Taylor & Francis
Taylor & Francis Group

Table of Content

Epistaxis and Its Associated Factors Among Precollege Students in Southern Ethiopia

Gemechu Ameya, Gelila Biresaw, Hayat Mohammed, Abebayehu Chebud, Melese Meskele, Mohammed Hussein & Muktar Endris

ORIGINAL RESEARCH

Epistaxis and Its Associated Factors Among Precollege Students in Southern Ethiopia

Gemechu Ameya [ID][1]
Gelila Biresaw[2]
Hayat Mohammed[3]
Abebayehu Chebud[4]
Melese Meskele[5]
Mohammed Hussein[6]
Muktar Endris[7]

[1]Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Kotebe Metropolitan University, Addis Ababa, Ethiopia; [2]Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia; [3]Department of Medical Laboratory Science, College of Medicine and Health Science, Jimma University, Jimma, Ethiopia; [4]Melka Oda Primary Hospital, West Guji, Melka Sodo, Ethiopia; [5]Worabe Comprehensive Specialized Hospital, Worabe, Ethiopia; [6]Dessie Selam General Hospital, Dessie, Ethiopia; [7]Galikoma Health Center, Afar Region, Galikoma, Ethiopia

Background: Epistaxis is one of the most common otorhinolaryngological emergencies affecting the majority of the population in their lifetime, with some of them requiring serious medical attention. This study aimed to assess the prevalence and associated factors of epistaxis among pre-college students in Wolaita Sodo, Ethiopia.
Methods: An institution-based cross-sectional study was conducted. Data were collected using a pre-tested interviewer administered questionnaire. The study participants were selected by systematic random sampling technique. A logistic regression analysis was employed to assess the presence and strength of association factors with epistaxis. An adjusted odds ratio with 95% confidence interval was used to determine the presence and strength of the association at 0.05 level of significance.
Results: Of 387 participants, 57.1% of them were male, and the mean age of all participant was 18.05±1.401 SD years. The overall epistaxis prevalence was 108 (27.9%). Blood group O, which accounted for about 43.4% was more prevalent. Blood group O (AOR=3.96, 95% CI=1.5–10.4), participants who drink coffee daily (AOR=2.75, 95% CI=1.0–7.4), and participants who took a bath frequently with both hot and cold-water (AOR=4.55, 95% CI=1.1–18.6) were significantly associated with epistaxis.
Conclusion: The type of blood group, interval of coffee drinking, and type of bathing were significantly associated with epistaxis. Working on the identified associated factor and increased awareness about epistaxis for the students with effective first aid training is mandatory.
Keywords: epistaxis, blood group, southern Ethiopia, pre-college students

Introduction

Epistaxis is a bleeding from the nose due to rupture of tiny, distended vessels in the mucous membrane of any area of the nose.[1] There are a variety of causes associated with epistaxis, which are mainly categorized into an idiopathic and with symptoms of an underlying disease.[2] The majority of epistaxis are anterior bleeds type, which are responsible for about 90–95% of the cases.[3,4] In this type the most common site of bleeding is the anteroinferior aspect of the nasal septum in the anterior nasal cavity plexus vessels.[4] In posterior bleeding it is difficult to find the bleeding point and it is highly intense in many cases.[5] Posterior epistaxis is usually rare, it accounts for only 5–10% of cases.[6]

Although the majority of epistaxis cases are idiopathic,[7,8] some causes have been identified so far in different studies. Trauma,[7–9] hematological disorders,[2–4,8–11] anatomic deformities,[2,4,9] inflammatory reactions,[8,9] organ failure,[3,4,6,8,12] intranasal tumors,[2,3] cardiovascular diseases,[6,7,9] blood dyscrasias,[4,13] low humidity,[3,7,9,12] even

Correspondence: Gemechu Ameya
Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Kotebe Metropolitan University, P.O. Box: 3268, Addis Ababa, Ethiopia
Tel +251-91-783-7681
Email gemechuameya@gmail.com

vigorous nose blowing,[1,9] and nose picking[2,9,14] have been identified associated factors with epistaxis. In adults most cases are associated with medications such as non-steroidal anti-inflammatory drugs (NSAID)[5,6,9,10,12,15] and anti-coagulants such as heparin and warfarin.[4,6,8,11]

The most common inherited bleeding disorders associated with epistaxis are hemophilia A, hemophilia B, and von Willebrand diseases.[9,11] Epistaxis is the most common symptom in approximately 60% of the patients with von Willebrand's disease.[5] Superficial bleeding like epistaxis is usually associated with platelet defects or vascular disorders.[11] Epistaxis is a common bleeding event that may be a symptom of coagulopathy.[16,17] It is also more prevalent in a person with O-blood group. This blood type is associated with a lower expression of von Willebrand factor compared with non O-blood groups.[18] This blood group also has a longer bleeding time compared to other blood groups, which confers the relative bleeding tendency.[19] Therefore, this study helps to describe the extent of epistaxis and to predict the most likely factors associated with epistaxis.

There is limited study and data related to the associated factors of the case in the given area need to be identified. To the best of our knowledge, there is no study done in our setting to identify either the prevalence or associated factors on epistaxis. The true prevalence of epistaxis is hard to investigate because of the self-limiting characteristics of the disease. An episode of self-limiting epistaxis does not get reported.[12] This makes the problem mostly ignored, and results in limited awareness in society on emergency first aid management of the disease.[25] It is unclear how much the level of epistaxis is and which factors locally are associated to the case. Basically the distribution of epistaxis is common among young adults.[5,7] If no efforts are put to determine the factors locally influencing epistaxis, it will be difficult to manage and treat epistaxis. The aim of this study was to assess the prevalence and associated factors of epistaxis among pre-college students in southern Ethiopia.

Methods
Study Design and Settings
An institutional-based cross-sectional study was conducted. The study was conducted in Wolaita Sodo town, the administrative center of the Wolaita Zone of the Southern Nations, Nationalities, and Peoples Region, Ethiopia. The lowest altitude of the town is 1,600 and the highest is 2,222 meters above sea level. And the

mean annual temperature is 20°C. In this city there are four pre-college (preparatory high schools); three of them private schools and the other is a public school. The estimated number of pre-college students was about 3,718.

Study Population
Students attending a preparatory high school in Wolaita Sodo town were used as a source of population. Selected students attending in a preparatory high school who fulfilled the inclusion criteria and were willing to participate in the study were the study population. Students who were on anti-thrombotic drugs and students who feel sick or had discomfort were excluded from the study.

Sample Size Determination
Sample size was calculated based on a single population proportion formula using the following assumptions. Due to the lack of a similar study in the study area, *P*-value=0.5 was used with 0.05 degree of freedom. After adding 10% potential non-response, the sample size became 422.

Sampling Method
The number and list of students in each school was obtained from the school director office of each preparatory high school. Based on the obtained information the study population was proportionally allocated to each preparatory high school under the study. For each high school, the allocated number of sample size of students was further allocated proportionally to each batch. The study subject was selected by systematic random sampling method. The first study subject was determined by lottery method.

Data Collection Tool and Procedure
Data was collected using a pre-tested and semi-structured interviewer administered questionnaire that is developed based on a previous study.[3,7,14,18–26] The questionnaire was first prepared in English and translated to local language (Amharic) for the data collection. The question was back-translated to English to check its consistency. The data collection tool contains questions related to socio-demographic characteristics of participants, epistaxis status, health-related factors, behavioral factors, and diet habits of the participants. The data were collected by five data collectors after receiving training.

Specimen Collection and Processing
The capillary blood from the finger was collected by cleaning the skin of the area around the fingertip with 70% isopropyl

alcohol in a circular fashion beginning at the site and moving out ward. And the blood was collected by piercing the fingertip with a sterile lancet. Drops of blood were placed onto microscopic slides and a thin blood film wass made on the first slide for blood morphology examination and the blood grouping was done on the other slides using anti-A, anti-B, and anti-D reagents. The prepared thin blood film was fixed on the slide using methanol alcohol. Slides were stained using Giemsa stain in batch in the laboratory. Finally slides were examined under a microscope.

Data Quality Control

Data quality was ensured from data collection up to final laboratory results by following the prepared standard operating procedure. The questionnaire was prepared in English then it was translated to Amharic for data collection and back-translated to English to check for consistency and completeness. Cross-checking of completeness of questionnaires was done during and after data collection. To ensure the validity and reliability of data collection, a pretest was done in Boditi secondary and preparatory high school in 5% of the sample size. Based on the finding of pre-test, necessary correction and modification were done.

Data Analysis and Interpretation

Data was first checked manually for completeness and then was coded and entered into Epi-Data version 3.1 statistical software and cleaned thoroughly before being transported to SPSS version 21 for further analysis. Descriptive statistics such as mean, frequencies, and percentages were used to describe and summarize the data. Binary logistic regression was used to determine the association between outcome variable and independent variables. Variables with a P-value$\leq$0.25 in univariable binary logistic regression analysis were candidates for multivariable analysis and factors with $P<0.05$ in the final model were considered as statistically significant. The degree of association between dependent and independent variables were assessed using an adjusted odds ratio at 95% CI.

Ethical Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. Ethical clearance to conduct the study was obtained from the institutional ethical review of the college of medicine and health science, Arba Minch University. The institutional ethical review board was organized from seven committee members from different departments. Further permission was obtained from the zone education office and school directors before starting data collection. We guaranteed confidentiality by excluding names or any other personal identifiers from data-collection sheets and reports. The identifier for each eligible subject was replaced by a code, and no master code exists that allows the research data to be linked with the identifiers. Participants were informed about the aim of the study, the advantages of the study, and their rights even to stop in the middle of the procedure. Written and oral consent were taken from each participant before data collection. The study participants with critical results were attached to a clinical setting and all female participants with the Rh-negative blood group received information about what precautions they should take when they reach child-bearing age. All students with the history of epistaxis got training on emergency management of nose bleeding.

Results

Socio-Demographic Characteristics of Study Participants

A total of 387 students participated in the study, with a response rate of 91.7%. The mean age of participants'

Table 1 Socio-Demographic Characteristics of the Study Participants (n=387)

Variables Category		Frequency	Percent (%)
Age	15–17	133	34.4
	18–20	233	60.2
	>20	21	5.4
Sex	Male	221	57.1
	Female	166	42.9
Level of education	Grade 11th	211	54.5
	Grade 12th	176	45.5
Participants' Fathers Job	Daily laborer	52	13.4
	Governmental employee	187	48.3
	Merchant	94	24.3
	Private worker	45	11.6
	Farmer	9	
Participants' Mothers Job	Housewife	165	42.6
	Governmental employee	121	31.3
	Merchant	76	19.6
	Private worker	23	5.9
	Farmer	2	
Family Income	≤1,000 birr	12	3.1
	1,001–3,000 birr	110	28.4
	>3,000	265	68.5
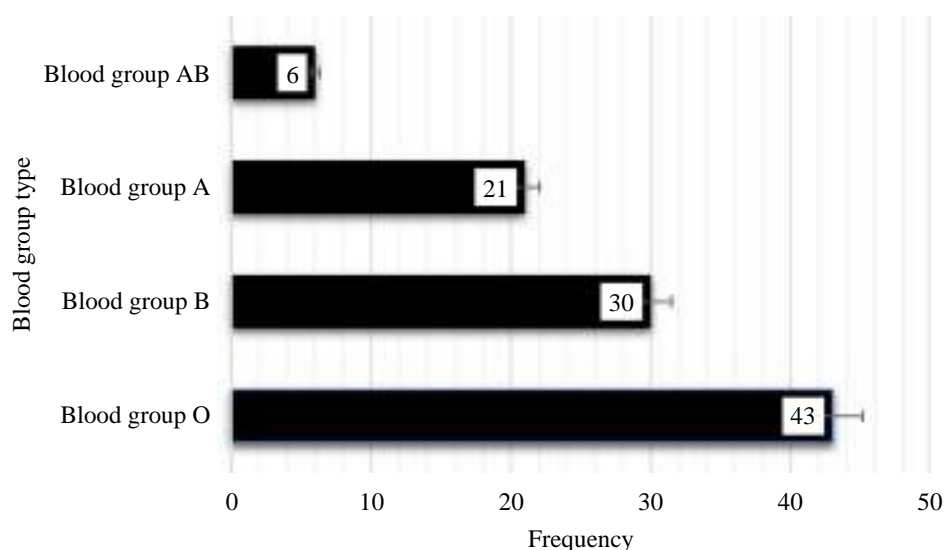
Figure 1 Blood group distribution of the study participants (n=387).

was 18.05±1.401 SD years. More than half (221, 57.1%) of the respondents were male. Regarding the job of the respondent's parent, 187 (48.3%) fathers were governmental employees and 165 (42.6%) mothers were housewifes. The majority of study participants (375, 96.9%) family income was above 1,000 birr per month (Table 1).

Blood Group Distribution, Health, and Behavioral-Related Characteristics of the Study Participants

The majority of study participants (43.4%) have blood type O, followed by blood type B, while AB was the least prevalent type (Figure 1). The majority (345, 89.1%) of participants had normocytic normochromic RBC appearance. Among 108 epistaxis cases, 37 (34.1%) of them had a family history of epistaxis and nine (2.3%) participants had heart disease. None of the study participants were aware of any coagulation disorder (Table 2).

A few (2.6%) of study participants had a history of alcohol consumption and, from those participants, six of them took once a week. Almost all (99.5%) participants had no history of cigarette smoking. Slightly higher than half of the study participant took a bath once a week, while nearly 40% of then took one 3-times per week. About one third of the participant used only cold water for bathing, and almost the same proportion use both warm and cold water (Table 2).

Habit of Diet of the Study Participant

More than half (61.8%) of the participants had no habit of eating "datta" (spice mainly prepared from chili pepper, ginger, and garlic). Half of the participants that had a habit of eating "datta" ate only once a day with different foods. From all participants, 186 (48.1%) of them had a history of drinking coffee. Of these, about one third of them drunk only

Table 2 Blood Group Distribution and Health-Related Characteristics of the Study Participants

Variables	Category	Frequency	Percent (%)
RBC Morphology	Normocytic Normochromic	345	89.1
	Normocytic Hypochromic	27	7.0
	Other	15	3.9
Heart Disease	Yes	9	2.3
	No	378	97.7
Alcohol	Yes	10	2.6
	No	377	97.4
Cigarette Smoking	Yes	2	
	No	385	99.5
Frequency of Bathing	Once a week	199	51.4
	Two times a week	30	7.8
	Three times a week	153	39.5
	Every day	5	
Type of bathing	Cold shower only	135	34.9
	Hot shower only	128	33.1
	Both hot and cold shower	124	32.0

once per week. One hundred and thirty-four (34.6%) of the participants reported the habit of drinking ginger tea and the majority of them drinking it 2–3 times in a week. Nearly half of the participants consumed vegetable as a usual food and 94 (39.8%) of them ate vegetables 2–3 times in a week. Slightly more than half of the participants had the habit of eating fruit and 81 (40.3%) of them ate it only once in a week (Table 3).

Prevalence of Epistaxis
The overall epistaxis level in this study was 108 (27.9%) (95% CI=23–32%). From all of the epistaxis participants, slightly more than half had their last episode within 3–6 months and 42.6% of them reported their number of episodes was between 2–5 periods within a year. The same

Table 3 Habit of Diet of the Study Participants (n=387)

Variables	Category	Frequency	Percent (%)
Datta eating	Yes No	148 239	38.2 61.8
Datta eating frequency	Daily Once a week Sometimes	105 40 3	71.0 27.0 2.0
Coffee drinking	Yes No	186 201	48.1 51.9
Frequency of coffee drinking	Daily Once a week 2–3 times a week Sometimes	62 67 56 1	33.3 36.0 30.1 0.6
Ginger tea	Yes No	134 253	34.6 65.4
Frequency of ginger tea	Daily Once a week 2–3 times a week Sometimes	14 12 97 11	10.9 9.0 72.4 8.2
Types of food consumed	Vegetables Meat Cereals Vegetables and cereals Cereals and meat	180 51 89 52 15	46.5 13.2 23.0 13.4 3.9
Fruit Consumption	Yes No	201 186	51.9 48.1
Frequency of fruit consumption	Daily Once a week 2–3 times a week Sometimes	50 81 68 2	24.9 40.3 33.8 1.0

proportion of epistaxis participants reported their bleeding time was between 7–10 minutes. About 30% of them responded that their cause of bleeding was stress only (Table 4).

Factors Associated with Epistaxis
Factors associated with epistaxis were assessed by binary logistic regression. In univariable analysis, sex, blood type, type and interval of bathing, frequency of "Datta" eating, frequency of coffee drinking, habit of drinking ginger tea, and fruit consumption had P-values less than 0.25 and the variables were selected for multivariable analysis. In multivariable analysis, the blood type of the students, coffee drinking, and type of bathing remained significantly and independently associated with epistaxis. Those participants with blood group O were about 4-times more likely to be affected by epistaxis than participants with non-O blood type (AOR=3.96, 95% CI=1.5–10.4). Epistaxis was 2.6-times higher in participants who drink coffee daily than those

Table 4 Epistaxis-Related Characteristics of the Study Participants (n=108)

Variables	Category	Frequency	Percent (%)
Last episode of epistaxis	Before 1 year 6–12 months 3–6 months <3 months	4 36 57 11	3.7 33.3 52.8 10.2
Number of episodes within a year	Once 2–5 6–7 8–10 >10	4 46 39 14 5	3.7 42.6 36.1 13.0 4.6
Nasal bleeding time	2–3 min 4–6 min 7–10 min >10 min	42 19 46 1	38.9 17.6 42.6 0.9
Cause of nasal bleeding	Trauma only Nose picking Dry weather only Stress only Hot bath Both dry weather and hot bath Both dry weather and stress Both trauma and stress	8 14 27 32 1 3 11 12	7.4 13.0 25.0 29.6 0.9 2.8 10.2 11.1

Table 5 Factors Associated with Epistaxis Among the Participants (n=387)

Variables	Epistaxis	None Epistaxis	COR (95% CI)	AOR (95% CI)	P-value
Sex					
Male	77	144	2.33 (1.4–3.8)	2.09 (0.7–5.8)	0.158
Female	31	135	Ref.	Ref.	
Blood group					
Non-O-blood group	43	176	Ref	Ref.	
O-blood group	65	103	2.58 (1.6–4.1)	3.96 (1.5–10.4)	0.005*
Interval of bathing					
Once per week	55	144	1.13 (0.7–1.8)	0.59 (0.2–1.8)	0.364
Twice per week	13	17	2.26 (1.0–5.1)	0.79 (0.2–3.8)	0.766
More than twice per week	40	118	Ref.	Ref.	
Type of water for Bathing					
Cold water	24	111	Ref.	Ref.	
Hot water	40	88	2.10 (1.2–3.7)	1.98 (0.6–6.4)	
Both hot and cold water	44	80	2.54 (1.4–4.5)	4.55 (1.1–18.6)	0.035*
Datta eating interval					
Daily	54	51	2.74 (1.3–5.9)	1.13 (0.3–4.7)	0.868
No daily	12	31	Ref.	Ref.	
Coffee drinking habit					
Yes	71	115	2.74 (1.7–4.4)	2.6 (0.9–7.3)	0.064
No	37	164	Ref.	Ref.	
Coffee drinking frequency					
Daily	32	30	2.33 (1.2–4.3)	2.75 (1.0 −7.4)	0.044*
Not Daily	39	85	Ref.	Ref.	
Drinking Ginger tea					
Yes	52	82	2.23 (1.4–3.5)	1.5 (0.5–4.3)	0.416
No	56	197	Ref.	Ref.	

Note: *Significant association.

participant who do not drink daily (AOR=2.75, 95% CI=1.0–7.4). Participants who took baths by both hot and cold-water were 4.6-times more likely to develop epistaxis than those who took cold showers (AOR=4.55, 95% CI:=1.1–18.6) (Table 5).

Discussion

The findings from this study showed that 27.9% (95% CI=23–32%) of the study participants had epistaxis. This finding is in line with the study conducted in Dar es Salaam, Tanzania (23.4%).[7] In this study the level of epistaxis is higher compared to the findings of studies conducted in India (7.5%) and Nigeria (0.5%).[3,28] This discrepancy could be because the study finding only represents the result from a single hospital and may be due to inadequate samples in the Nigerian study. Many studies[3,12,15,27] conducted on epistaxis are hospital-based, but epistaxis cases

are mostly self-limiting,[12] and only a few seeks medical attention;[6,7] so that the number of cases that will be presented in a health institution are rare.

Contrary with the above findings, the current study showed that the level of epistaxis is lower than the studies conducted in Saudi Arabia and India, where the level of epistaxis were 49% and 45%, respectively.[12,15] This might be attributable to the fact that the findings of a study conducted in Saudi Arabia were due to an incorporated large number of participants that were found in the kingdom of Saudi Arabia. In the case of India the discrepancy may be due to the different study design, which is retrospective, and the data collected for a prolonged time.

Out of the 108 participants with epistaxis, 60.2% of them were known to have blood type O, which was also significantly associated with the case. This indicates the prevalence of epistaxis is higher in blood type O than in the

participants with other blood groups. Similar findings were observed in studies done in Nepal and India.[18,27] The possible reason for the observed high association may be that the O blood group is known to be associated with a lower expression of von Willebrand factor which plays an important role in clotting compared with non-O blood groups. Bleeding time is recorded to be slightly longer in blood group O.[19]

Type of bathing was also found to be significantly associated with epistaxis. In the current study participants who took a shower frequently by both hot and cold-water were more likely to developepistaxis. This may be due to the fluctuation in body temperature that affects the blood pressure of the participants. A study conducted in China showed the hourly temperature had a significant lag effect on blood pressure.[28] Blood pressure changes with temperature based on different temperature and variation in blood pressure may induce epistaxis. Some other studies[3,7,17,26,29] show significant relations between epistaxis and hypertension. Unlike in our study, ingesting hot beverages and taking a hot bath were also highlighted as a contributing factor for epistaxis.[30] The variation observed may be due to the study season and the difference in environmental temperature.

In the current study, the interval of coffee drinking habit was associated with epistaxis. Study participants who drink coffee daily were more likely to develop epistaxis than their counterparts. The findings of another study showed that caffeine in energy drinks has a nose bleeding effect.[31,32] One of the most common causes of nose bleeds is dryness in the nasal passages, which can occur as a result of caffeine. This may be because caffeine dries out the body by pulling moisture from the mucous membranes of the nasal passages.

Conclusion

This study revealed that the level of epistaxis among preparatory students in Wolaita Sodo is relatively high. Blood type O, drinking coffee daily and bathing with warm and cold water were significantly associated with epistaxis. Working on the identified associated factor is important to reduce the effect of the cases. It is better to increase awareness about epistaxis for the students and it is also better to give first aid training for the school community. Therefore, the health office and health extension workers need to better strengthen and maintain the local information dissemination network on epistaxis and its right time of commencement community-based information education and communication on epistaxis.

Disclosure

The authors report no conflicts of interest for this work.

References

1. Sandra M, Brooks-Brunn J. *Brunner & Suddarth's Textbook of Medical-Surgical Nursing*. 10th ed. Philadelphia: Lippincott Williams & Wilkins; 2004.
2. Reyre A, Michel J, Santini L, et al. Epistaxis: the role of arterial embolization. *Diagn Interv Imaging*. 2015;96(7–8):757–774.
3. Moran N, Das D. Epistaxis—incidence, etiology, and management: A hospital-based study. *Clin Rhinology*. 2016;9(1):18–20.
4. Viehweg TL, Roberson JB, Hudson JW. Epistaxis: diagnosis and treatment. *J Oral Maxillofacial Surg*. 2006;64(3):511–518.
5. Marajo M, Secchi D, Lucia M, Indolfo P, Rabesquine MM. Epistaxis. *Prevailing Factors Treat*. 2009;13(4):381–385.
6. Villwock JA, Jones K. Recent trends in epistaxis management in the United States 2008–2010. *JAMA Otolaryngol*. 2013;139 (12):1279–1284.
7. Japhet MG, Phillipo LC. Etiological profile and treatment outcome of epistaxis at a tertiary care hospital in Northwestern Tanzania: a prospective review of 104 cases. *BMC Ear Nose Throat Disord*. 2011;11:8.
8. Pope LER, Hobbs CGL. Epistaxis: an update on current management. *Postgrad Med J*. 2005;81(955):309–314.
9. Fatakia A, Winters R, Amedee R. Epistaxis: a Common Problem. *Ochsner J*. 2010;10(3):176–178.
10. Fathy H, Ismail R, Mahmoud N, Abdel WM. Recurrent epistaxis in children: when should we suspect coagulopathy? *Egypt J Otolaryngology*. 2014;30(2):106–111. doi:10.4103/1012-5574.1 33207
11. Salih AA, Majeed EA. Relation between Epistaxis and hematological – disorders. *Arch Med Health Sci*. 2012;18(3):143–149.
12. Alhaddad MS, Almulhim K, Mubarak IAS, Alotaibi N, Hussain MAS, Alyahya KA. Prevalence of epistaxis in saudi population. *Int J Sci Study*. 2017;5(9):96–99.
13. Soto-Galindo GA, Treviño González JL. Epistaxis diagnosis and treatment update: a review. *Ann Otolaryngol Rhinol*. 2017;4(4):1176.
14. Michael R, Meera C, Derek L, et al. *Epistaxis as a Common Presenting Symptom of Glanzmann's Thrombasthenia, a Rare Qualitative Platelet Disorder: Illustrative Case Examples*. Hindawi Publishing Corporation; 2017.
15. Sharma K, Kumar S, Islam T, Krishnatreya M. A retrospective study on etiology and management of epistaxis in elderly patients. *Arch Med Health Sci*. 2015;3(2):234–238. doi:10.4103/2321-4848.171911
16. Kemal O, Müderris T, Sevil E, Kutlar G. Relation of mean platelet volume and red blood cell distribution width with epistaxis. *Laryngoscope*. 2015;125(4):788–790. doi:10.1002/lary.24990
17. Karabulut AE, Çevik Y, Emektar E, Çorbacioğlu Ş, Dağar S, Yardim O. Analysis of mean platelet volume and red blood cell distribution width in recurrent epistaxis. *Turkish J Em Med*. 2018;18(2):67–70. doi:10.1016/j.tjem.2018.02.001
18. Adhikari P, Pramanik T. Relation of blood group with epistaxis. *Int Arch Otorhinolaryngology*. 2008;12(4):513–516.

19. Adhikari P, Pramanik T, Pokharel R, Khanal S. Relationship between blood group and epistaxis among Nepalese. *Nepal Med College J.* 2008;10(4):264–265.

20. Simmen DB, Jones NS, Cummings CW, Flint PW, Harker LA. Epistaxis. *Otolaryngology Head Neck Surg.* 2005;5:682–693.

21. Ando Y, Iimura J, Arai S, et al. Risk factors for recurrent epistaxis: importance of initial treatment. *Auris Nasus Larynx.* 2014;41 (1):41–45.

22. Varshney S, Saxena RK. Epistaxis: a retrospective clinical study. *Indian J Otolaryngology Head Neck Surg.* 2005;57:2.

23. Pallin DJ, Chng YM, McKay MP. Epidemiology of epistaxis in U.S. emergency departments, 1992 to 2001. *Ann Emerg Med.* 2005;46:77–81.

24. Sauter TC, Hegazy K, Hautz WE. Epistaxis in anticoagulated patients: fewer hospital admissions and shorter hospital stays on rivaroxaban compared to phenprocoumon. In: *Clinical Otolaryngology.* 2017:1–6.

25. Alshehri F, Alluwaim F, Alyahya K. Teachers' awareness regarding emergency management of epistaxis inside the school; alahssa, Saudi Arabia. *Open J Prev Med.* 2018;8:44–55.

26. Acar B, Yavuz B, Yıldız E, Ozkan S, Ayturk M, Sen O. A possible cause of epistaxis: increased masked hypertension prevalence in patients with epistaxis. *Braz J Otorhinolaryngol.* 2017;83(1):45–49.

27. Mirdha M, Jena SK. Distribution of blood group and its relation to bleeding time and clotting time. *Int J Med Sci Public Health.* 2016;5 (12):2566–2569.

28. Xu D, Zhang Y, Wang B, et al. Acute effects of temperature exposure on blood pressure: an hourly level panel study. *Environ Int.* 2019;124:493–500.

29. Kodiya AM, Labaran AS, Musa E, Mohammed GM, Ahmad E. Epistaxis in Kaduna, Nigeria: A review of 101 cases. *Afr Health Sci.* 2012;12(4):479–482.

30. Ahmed HS, Abdullah MA, Ahmed AA, Manal IBM, Marzouki HZ. Epistaxis: what do people know and what do they do? *Global j Ortholrygology.* 2018;13:2.

31. Nowak D, Jasionowski A. Analysis of the consumption of caffeinated energy drinks among Polish adolescents Int. *J Environ Res Public Health.* 2015;12:7910–7921.

32. Kapner AD Ephedra and energy drinks on college campuses. the higher education center for alcohol and other drug abuse and violence prevention. 2004. Available from: http://files.eric.ed.gov/fulltext/ED537639.pdf. Accessed on 5 March 2020.

Recombinant FVIII Products (Turoctocog Alfa and Turoctocog Alfa Pegol) Stable Up to 40°C

Mariasanta Napolitano, Arne Agerlin Olsen, Anne Mette Nøhr & Hermann Eichler

ORIGINAL RESEARCH

Recombinant FVIII Products (Turoctocog Alfa and Turoctocog Alfa Pegol) Stable Up to 40°C

Mariasanta Napolitano [1]
Arne Agerlin Olsen[2]
Anne Mette Nøhr[2]
Hermann Eichler[3]

[1]Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties (PROMISE), University of Palermo Reference Regional Center for Thrombosis and Hemostasis, Hematology Unit, Palermo, Italy; [2]Novo Nordisk A/S, Biopharm Manufacturing Development, Gentofte, Denmark; [3]Saarland University and Saarland University Hospital, Institute of Clinical Haemostaseology and Transfusion Medicine, Homburg (Saar), Germany

Purpose: The stability under high-temperature conditions of factor VIII (FVIII) concentrates for replacement therapy is of critical importance to patients, particularly those who reside in, or travel to, regions with high ambient temperatures. Concerns about product stability may limit or prevent access to treatment for patients and may limit their ability to live a close-to-normal life. This study evaluated the effect of hot and humid storage conditions on the long-term stability of the recombinant FVIII products, turoctocog alfa and turoctocog alfa pegol.

Methods: Turoctocog alfa samples were assessed for stability at 30°C for 9 months or 40°C for 3 months following storage at 5°C for 21 or 27 months, respectively, while turoctocog alfa pegol samples were assessed at 30°C for 12 months or 40°C for 3 months following storage at 5°C for 18 or 27 months, respectively. In addition, turoctocog alfa and turoctocog alfa pegol dry powders were evaluated for stability at 5°C/ambient humidity (AH) for 30 months, 30°C/75% relative humidity (RH) for 12 months and 40°C/75% RH for 6 months. Both studies utilized a range of product strengths. Key stability assessments included oxidized forms, potency, water content and high molecular weight protein (HMWP).

Results: Both turoctocog alfa and turoctocog alfa pegol remained stable following storage at 40°C/75% RH for 3 months, and at single temperatures (5°C/AH, 30 and 40°C/75% RH), without any major increase in HMWP or any impairment of potency or water content.

Conclusion: Turoctocog alfa and turoctocog alfa pegol offer stability at 40°C for up to 3 months without jeopardizing the quality of each product. These stability characteristics may offer patients flexibility with product storage and daily use.

Keywords: factor VIII, hemophilia A, storage flexibility, temperature stability, turoctocog alfa, turoctocog alfa pegol

Plain Language Summary

For patients with hemophilia A and their caregivers, it is crucial that factor VIII (FVIII) products used for replacement therapy can be stored under intense heat for long time periods. Concerns about whether their FVIII products are affected by intense heat may prevent patients from accessing treatment and living a normal life when they are in hot and humid regions. This study therefore evaluated the effect of temperatures up to 40°C and humid storage conditions on the long-term stability of the FVIII products: turoctocog alfa and turoctocog alfa pegol.

Turoctocog alfa samples were assessed for stability at 30°C for 9 months or 40°C for 3 months following storage at 5°C for 21 or 27 months, respectively, while turoctocog alfa pegol samples were assessed at 30°C for 12 months or 40°C for 3 months following storage at 5°C for 18 or 27 months, respectively. In addition, turoctocog alfa and turoctocog alfa pegol were evaluated for stability at 5°C/ambient humidity (AH) for 30 months, 30°C/75% relative humidity (RH) for 12 months and 40°C/75% RH for 6 months.

Correspondence: Anne Mette Nøhr
Biopharm Manufacturing Development, Novo Nordisk A/S, Nybrovej 80, Gentofte 2820, Denmark
Tel +45 3075 1619
Email amnq@novonordisk.com

We found both FVIII products remained stable following storage at 40°C/75% RH for 3 months, and at single temperatures (5°C/AH, 30 and 40°C/75% RH), without any major increase in protein products or impairment of potency or water content.

Turoctocog alfa and turoctocog alfa pegol offer stability at 40°C for up to 3 months without jeopardizing the quality of each product. These stability characteristics offer patients flexibility with product storage and daily use.

Introduction

In patients with hemophilia A, it is recommended to treat bleeds with factor VIII (FVIII) within 2 hours,[1,2] which requires access to replacement factor at all times. Furthermore, FVIII prophylaxis—the gold standard of care for severe hemophilia A patients—requires patients or their caregivers to administer regular intravenous injections of FVIII (usually every other day or 3 times a week with standard half-life FVIII concentrates[2] and once or twice weekly with extended half-life [EHL] FVIII products).[3]

Manufacturers recommend storing both standard and EHL FVIII products in a refrigerator (or below 25°C) and reconstituting them at room temperature. However, some patients are unaware that most FVIII products remain stable for a limited time at higher temperatures;[4–15]

therefore, their adherence to (and satisfaction with) therapy may be adversely affected by the perceived limitations of FVIII product storage conditions, particularly when patients are traveling.[15–17] With reference to product-related factors for hemophilia treatment, patients have reported a preference for storage-flexible products that allow reduced injection times, greater patient adherence, less waste, greater ease of handling, and fewer restrictions in daily activities such as travel and sports.[15]

The structural stability of recombinant therapeutic products can affect biological activity, clearance, and immunogenicity, with increasing instability occurring over long-term storage.[18] While the World Health Organization recommendations are that the stability of therapeutic products should be assessed based on climatic zones with a maximum temperature of 30°C and 75% relative humidity (RH; Figure 1), they also recognize that storage conditions often exceed these temperatures.[19] It is thus crucial to also assess the stability of FVIII products at temperatures above 30°C.

Turoctocog alfa (NovoEight®, Novo Nordisk A/S, Bagsværd, Denmark) and turoctocog alfa pegol (an extended half-life glycoPEGylation of turoctocog alfa, Esperoct®, Novo Nordisk A/S, Bagsværd, Denmark) are recombinant FVIII (rFVIII) products developed to prevent and treat bleeding

ICH Stability Zone I   ICH Stability Zone II   ICH Stability Zone III   ICH Stability Zone IV
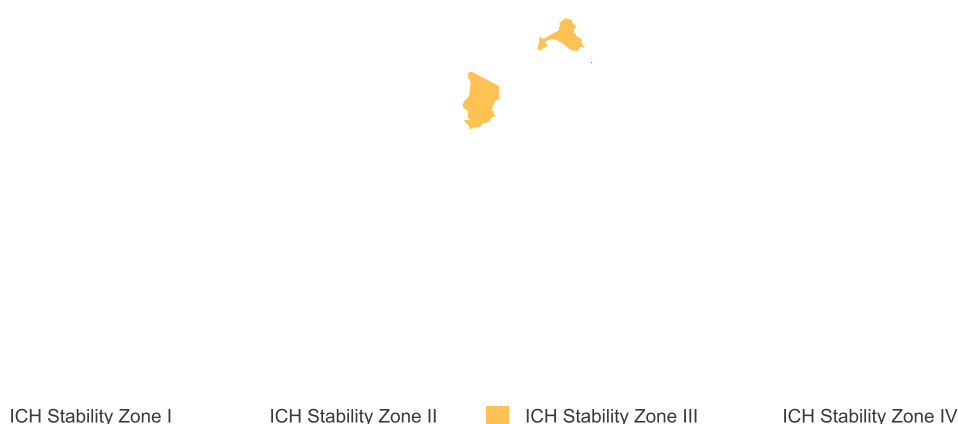
Figure 1 ICH quality guidelines for pharmaceutical stability storage. (https://q1scientific.com/ich-quality-guidelines/)
Notes: Long-term testing conditions for: Zone I, 21°C/45% RH (temperate); Zone II, 25°C/60% RH (subtropical and Mediterranean); Zone III, 30°C/35% RH (hot/dry); Zone IV, 30°C/75% RH (hot/very humid). Data from WHO.[19]
Abbreviations: ICH, International Council on Harmonisation; RH, relative humidity; WHO, World Health Organization.

episodes in patients with hemophilia A.[20–26] The activated forms of both products have the same primary structure as native activated FVIII.[27] The production steps for turoctocog alfa are the same as for turoctocog alfa pegol prior to PEGylation;[27–29] importantly, PEGylation of turoctocog alfa does not affect product stability.[27] Both products are supplied as a dry powder in single-dose vials of 250, 500, 1000, 1500, 2000, and 3000 IU (turoctocog alfa)[7,8] and 500, 1000, 1500, 2000, and 3000 IU (turoctocog alfa pegol).[13,14] The current approved storage and shelf life of the dry powder for both products is storage at 5°C for up to 30 months, during which time the products may be stored at room temperature (≤30°C) for ≤12 months or up to 40°C for ≤3 months.[7,8,13,14]

Patients may live in, or travel to, countries with a hot and humid climate. To allow these patients to use turoctocog alfa and turoctocog alfa pegol safely, we evaluated the effect of high-temperature, high-humidity storage conditions on the long-term stability of both products. In the first study, turoctocog alfa samples were assessed at 30°C for 9 months or 40°C for 3 months following storage at 5°C for 21 or 27 months, respectively, while turoctocog alfa pegol samples were assessed at 30°C for 12 months or 40°C for 3 months following storage at 5°C for 18 or 27 months, respectively. The second study assessed the dry powders of turoctocog alfa and turoctocog alfa pegol when stored at 5°C/ambient humidity (AH) for 30 months, 30°C/75% RH for 12 months, and 40°C/75% RH for 6 months (Figure 2).

Patients and Methods
Product and Sample Preparation
Commercially available turoctocog alfa and turoctocog alfa pegol in dry-powder batches were each evaluated at

their lowest and two highest strengths: 250, 2000, and 3000 IU for turoctocog alfa, and 500, 2000, and 3000 IU for turoctocog alfa pegol (Figure 2).

Long-Term Stability Study
Turoctocog Alfa
The long-term stability of turoctocog alfa dry-powder batches was assessed during single temperature storage at 40°C (±2°C) and 75% RH (±5% RH) for 3 months, and at 30°C (±2°C) and 75% RH (±5% RH) for 9 months. A reference sample for each dose was maintained at 5°C (±3°C) in darkness and AH for 3 months. Prior to the start of this study, the turoctocog alfa samples had been stored at 5°C (±3°C) in darkness and AH for 21 and 27 months (Figure 2).

Turoctocog Alfa Pegol
Dry-powder batches of turoctocog alfa pegol were assessed for long-term stability at 40°C (±2°C) in darkness and 75% RH (±5% RH) for 3 months, and at 30°C (±2°C) and 75% RH (±5% RH) for 12 months. Prior to the start of this study, the dry products had been stored at 5°C (±2°C) in darkness and AH for 18 and 27 months for consecutive temperature storage (Figure 2).

Stability Assessments and Assay Methods
All studies were performed according to current International Conference on Harmonisation (ICH) bracketing design guidelines.[30] The key stability parameters assessed for both products were those from the drug product specifications that are susceptible to change during storage and/or those that could potentially influence product quality: namely, oxidized forms, potency, water content, and high-molecular-weight protein (HMWP). For
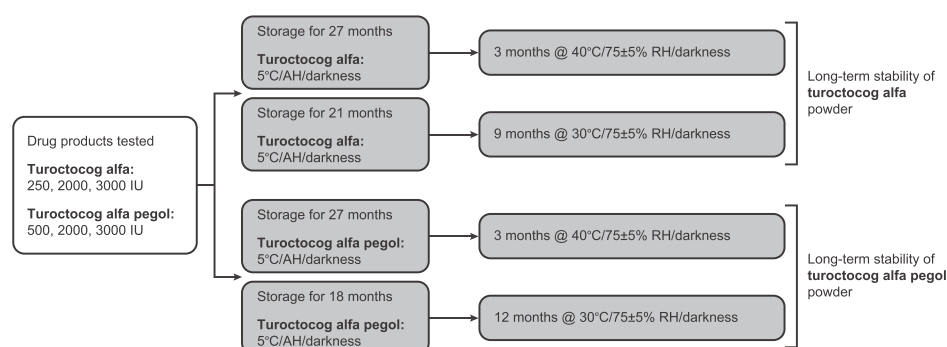
Figure 2 Study design.
Notes: The stability of turoctocog alfa dry powder product was assessed after storage at 5°C for 27 months followed by 40°C for 3 months (reference samples maintained at 5°C for 3 months) and after storage at 30°C for 9 months following storage at 5°C for 21 months. The stability of turoctocog alfa pegol dry powder product was also assessed after storage at 5°C for 27 months, followed by 40°C for 3 months and after storage at 30°C for 12 months following storage at 5°C for 18 months.
Abbreviations: AH, ambient humidity; IU, international units; RH, relative humidity.

oxidized forms, potency, water content, and HMWP, the results from all assays were evaluated against predetermined acceptance limits and calculated using appropriate statistical methods.[30] Potency was expressed as % target. The specific ranges for each assay are noted within the appropriate sections below.

Oxidized Forms

Oxidized forms within the reconstituted turoctocog alfa and turoctocog alfa pegol products were assessed using reverse-phase-high performance liquid chromatography (RP-HPLC). Analysis was performed on an HPLC system equipped with processing software and a 4.0 x 250-mm, C_4 5 µm, 300 Å column (Novo Nordisk Pharmatech A/S, Køge, Denmark). The column temperature was set at 40°C for turoctocog alfa and 40°C ± 2°C for turoctocog alfa pegol, with a detection wavelength of 215 nm. A gradient of 35–100% eluent B (0.09% [turoctocog alfa] or 0.14% [turoctocog alfa pegol] trifluoroacetic acid [TFA] in 80% acetonitrile in purified water) and 65–0% eluent A (0.1% [turoctocog alfa] or 0.15% [turoctocog alfa pegol] TFA in purified water) was applied over a 40-min period with a flow rate of 1 mL/min. The composition of 100% eluent B was then maintained for 5 min, before being changed back to the initial conditions over 1 min, followed by column equilibration for 14 min, resulting in a total run time of 60 min. Oxidized forms were calculated as the percentage area on the resulting chromatograms. RP-HPLC chromatograms showed all components were present in the drug substances and therefore in the drug products (chromatograms not shown).

Potency

The potency of turoctocog alfa and turoctocog alfa pegol was investigated using the chromogenic assay (Chromogenix Coamatic® Factor VIII) and the SynthASil (HemosIL, Instrumentation Laboratory) activated partial thromboplastin time reagent.[27]

Turoctocog alfa and turoctocog alfa pegol product samples, calibrator, and blank samples were analyzed in triplicate on the ACL® Elite Pro analyzer. Absorbance readings for product, reference samples, and calibrator were used to calculate potency using slope–ratio analysis or parallel line regression (chromogenic assays). Potency was expressed as % target (European Pharmacopoeia acceptance limits are 80–125% of target).[31]

Water Content

The water content of turoctocog alfa and turoctocog alfa pegol samples was evaluated using near-infrared (NIR) spectroscopy for the high-temperature stability samples only. NIR spectroscopy was conducted using a Fourier transform-NIR Spectrometer (MPA, Bruker, Billerica, MA, USA) equipped with an integrating sphere (or equivalent). Both products were scanned in the frequency ranges of 7502.1–6098.1 and 5450.1–4597.7 nm at a resolution of 8 cm^{-1}, with an average of 32 scans being recorded for each spectrum. The spectrometer was equipped with OPUS software (Bruker) (or equivalent) and data were analyzed using a partial least squares fit method. Turoctocog alfa and turoctocog alfa pegol samples were analyzed without any pretreatment; however, NIR spectroscopy data were pretreated using vector normalization and a first derivative to enhance spectral information and correct interferences from the analyzed material that might otherwise induce baseline drift and changes in maximum absorbance.[32] Karl–Fischer coulometry was used as the reference method for determining the calibration function and to analyze samples in cases where NIR results were not accepted (for example, due to persistent outliers).[31]

High Molecular Weight Protein

Size exclusion-HPLC (SE-HPLC) was used to determine the presence of protein aggregates in the turoctocog alfa and turoctocog alfa pegol samples. Prior to SE-HPLC analysis, the samples were reconstituted in either 1.0 mL (250-IU samples [turoctocog alfa] and 500-IU samples [turoctocog alfa pegol]) or 4.3 mL (2000- and 3000-IU samples, both products) of 0.9% sodium chloride solution. SE-HPLC measurements for turoctocog alfa were performed using an HPLC system equipped with a Shodex PROTEIN KW-803, 8 × 300-mm column (Shodex), or equivalent. For turoctocog alfa pegol, SE-HPLC measurements were performed using a Sepax Technologies SRT SEC-500, 7.8 × 300-mm, 5-µm, 500 Å column (Sepax Technologies, Newark, DE, USA). An elution flow rate of 0.4 mL/min was employed for turoctocog alfa, using a column temperature of 30°C and excitation and emission detection wavelengths of 285 and 335 nm, respectively. For turoctocog alfa pegol, an elution flow rate of 0.3 mL/min was used, with a column temperature of 23°C, an excitation detection wavelength of 285 nm, and an emission detection wavelength of 345 nm. The eluent buffer consisted of 10-mM TRIS, 10-mM $CaCl_2$, 300-mM NaCl,

and 5% 2-propanol at pH 7.0 (turoctocog alfa) or 10-M BIS-TRIS propanol, 10-mM calcium acetate hydrate, 0.5-M sodium chloride, and 10% isopropanol at a pH adjusted to 6.8 with acetic acid (turoctocog alfa pegol). The injection load volume was within the validated range of the method (0.95–7.8 μg). Run times for products reconstituted in 4.3- and 1.0-mL 0.9% sodium chloride solutions were ≥70 and ≥80 min, respectively, for turoctocog alfa, and 60 min for turoctocog alfa pegol. HMWP content was determined by calculating the area percentage of the HMWP peak on the resulting chromatogram.

Results

Oxidized Forms

Oxidized forms, which are inactive forms of rFVIII that can reduce potency,[33] are the stability-limiting factor. Oxidized forms increased as a function of time and temperature following an Arrhenius correlation for both products (Figure 3A). Results for turoctocog alfa samples stored at consecutive temperatures of 30°C/75% RH for 9 months (Figure 3B) or 40°C/75% RH for 3 months (Figure 3C), following storage at 5°C for 21 or 27 months, respectively, showed that levels of oxidized forms were within the predetermined acceptance limits for all batches. Similar results were seen for turoctocog alfa pegol samples stored at consecutive temperatures of 30°C/75% RH for 12 months (Figure 3G) or 40°C/75% RH for 3 months (Figure 3H), following storage at 5°C for 18 or 27 months, respectively. There was a small increase in the occurrence of oxidized forms of both turoctocog alfa and turoctocog alfa pegol following storage at single temperatures of 5°C, 30°C/75% RH, and 40°C/75% RH for 30, 12, and 6 months, respectively (Figure 3D–F and Figure 3I–K, respectively). For both products, oxidized forms were close to 5% after storage at 30°C/75% RH for 12 months (Figures 3E and J) and approaching 7% after storage at 40°C/75% RH for 6 months (Figures 3F and K).

Potency

As potency is used as the basis for dosage calculations and treatment strategies,[34] it is important to determine whether potency is affected by hot and humid storage conditions. Changes in potency were within the limits for all turoctocog alfa batches when stored at consecutive temperature conditions for 9 months (Figure 4A) or 3 months (Figure 4B), respectively. Potency was also within the limits for all turoctocog alfa pegol batches when stored at consecutive

temperature conditions for 12 months (Figure 4F) or 3 months (Figure 4G), respectively. These limits are also in line with the European Pharmacopoeia acceptance criteria of –20% to +125% from target.[31] The results of the potency test of dry turoctocog alfa and turoctocog alfa pegol samples stored at single temperatures for 30, 12, and 6 months, respectively, are shown in Figure 4C–E and Figure 4H–J, respectively. Potency was maintained within the limits for all turoctocog alfa and turoctocog alfa pegol sample strengths tested.

Water Content

Excessive water content can increase degradation and reduce the stability of a therapeutic product.[35] When turoctocog alfa batches were stored at consecutive temperature conditions for 9 months (Figure 5A) or 3 months (Figure 5B), respectively, water content for all batches was within the range of 1–2%, corresponding to the observed ranges in stability studies. Likewise, water content was also within this upper range for all turoctocog alfa pegol batches when stored at consecutive temperature conditions for 12 months (Figure 5F) or 3 months (Figure 5G), respectively. There was a small increase in the water content with increasing temperature for both turoctocog alfa and turoctocog alfa pegol samples following storage at single temperatures for 30, 12, and 6 months, respectively (Figure 5C–E and Figure 5H–J, respectively). Water content was approximately 1% after storage at 30°C/75% RH for 12 months (Figure 5D and I) and <2% after storage at 40°C/75% RH for 6 months (Figure 5E and J).

High Molecular Weight Protein

Protein product aggregates, including HMWP, are described as potent inducers of immune responses to therapeutic protein products.[36] When turoctocog alfa batches were stored at consecutive temperature conditions for 9 months (Figure 6A) or 3 months (Figure 6B), respectively, HMWP for all batches was within the range of 2–4%. Similarly, HMWP was also within this upper range for all turoctocog alfa pegol batches when stored at consecutive temperature conditions for 12 months (Figure 6F) or 3 months (Figure 6G), respectively. Small increases in HMWP were observed for both turoctocog alfa and turoctocog alfa pegol samples following storage at single temperatures for 30, 12, and 6 months, respectively (Figure 6C–E and Figure 6H–J, respectively); for both products, HMWP levels were ≤3% after storage at 30°C/
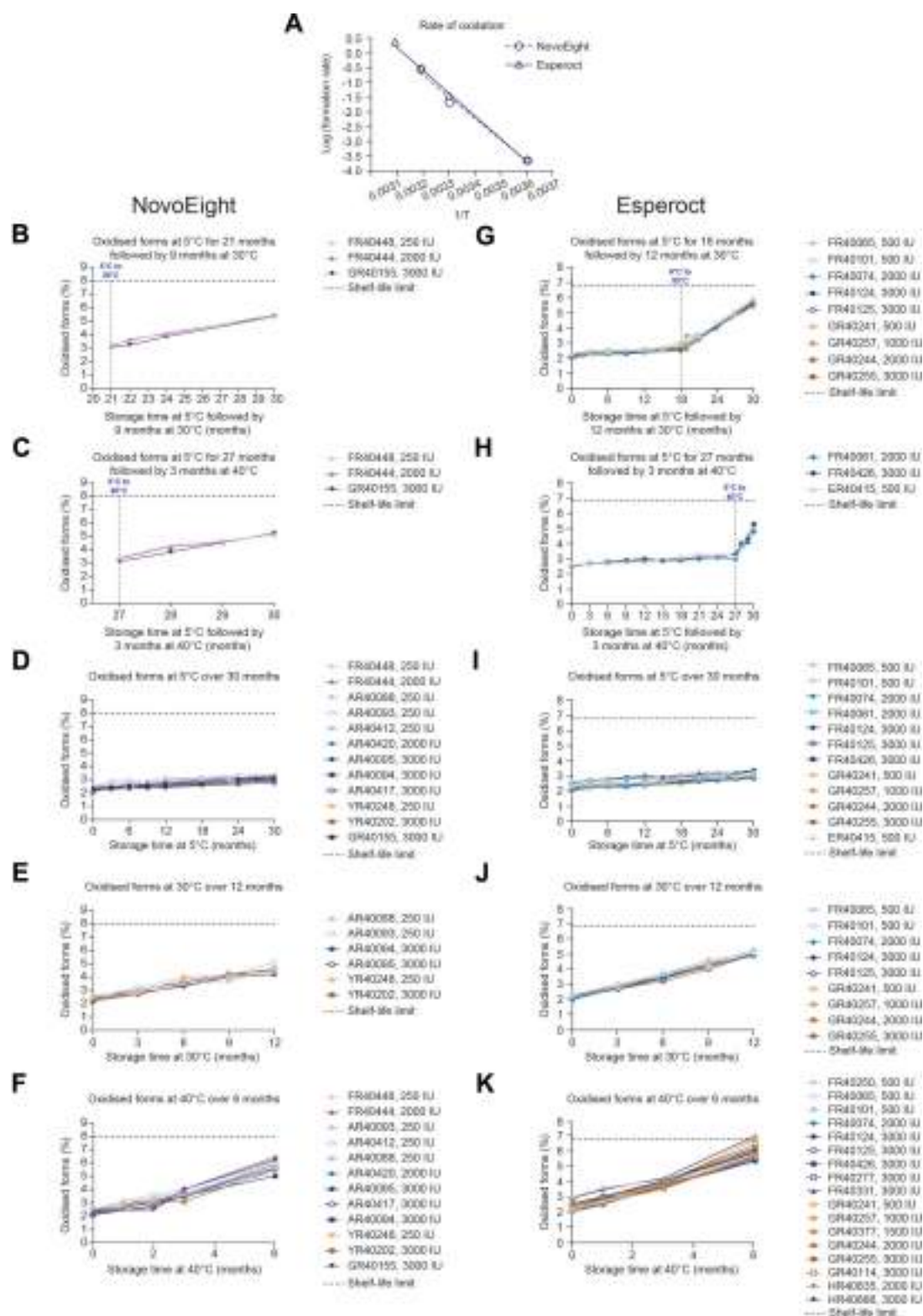
Figure 3 Oxidized forms of turoctocog alfa dry powder samples as the rate of oxidation (Arrhenius correlation) (**A**), when stored at 5°C for 21 months followed by storage at 30°C for 9 months (**B**), 5°C for 27 months followed by storage at 40°C for 3 months (**C**), and when stored at 5°C for 30 months (**D**), 30°C for 12 months (**E**), and 40°C for 6 months (**F**). Oxidized forms of turoctocog alfa pegol dry powder samples when stored at 5°C for 18 months followed by storage at 30°C for 12 months (**G**), 5°C for 27 months followed by storage at 40°C for 3 months (**H**), and when stored at 5°C for 30 months (**I**), 30°C for 12 months (**J**), and 40°C for 6 months (**K**). Shelf-life limits for both products are shown by the horizontal dotted lines.
Abbreviations: IU, international units; T, temperature (in degrees Kelvin).
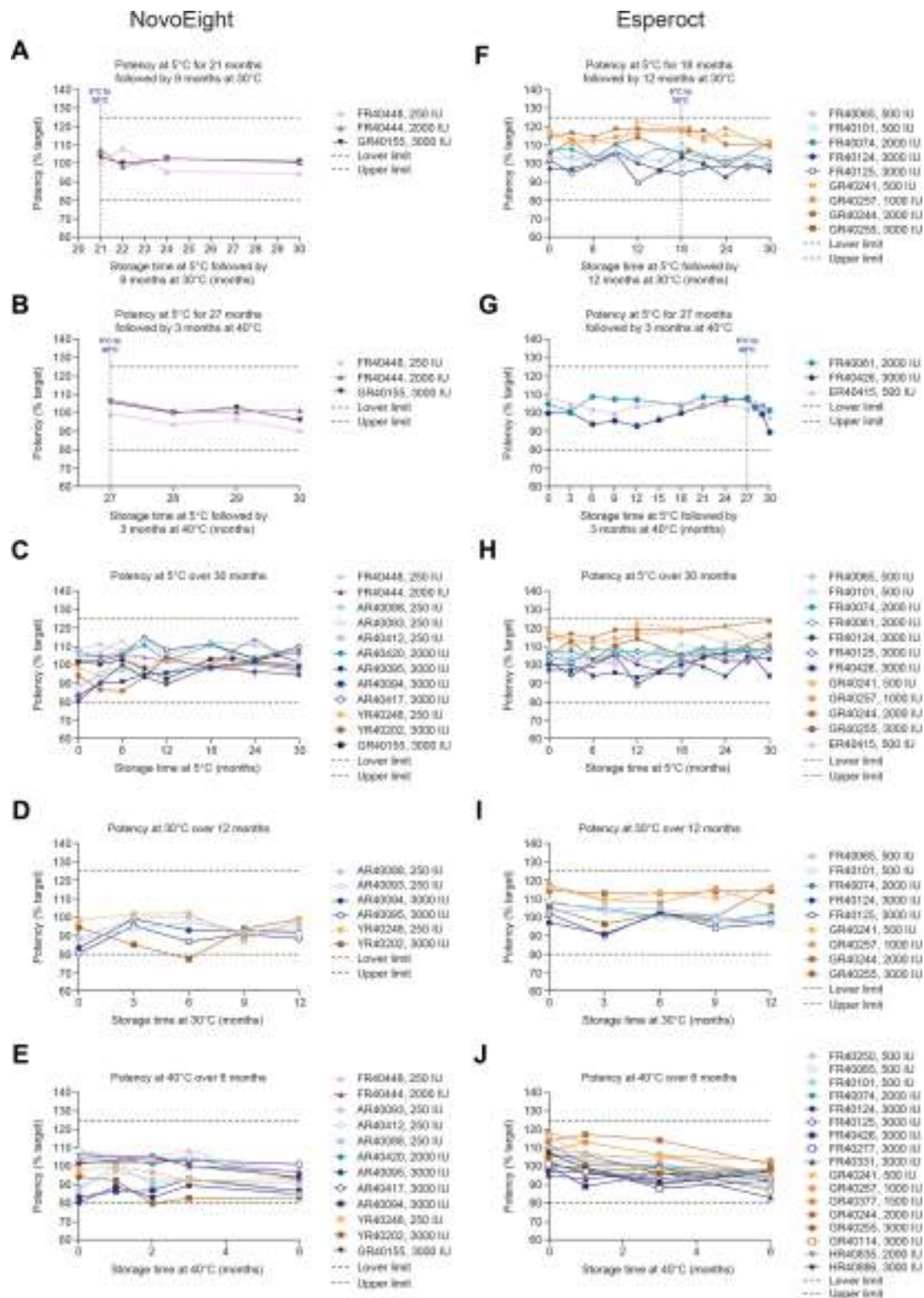
Figure 4 Potency of turoctocog alfa dry powder samples when stored at 5°C for 21 months followed by storage at 30°C for 9 months (**A**), 5°C for 27 months followed by storage at 40°C for 3 months (**B**), 5°C for 30 months (**C**), 30°C for 12 months (**D**), and 40°C for 6 months (**E**). Potency of turoctocog alfa pegol dry powder samples when stored at 5°C for 18 months followed by storage at 30°C for 12 months (**F**), 5°C for 27 months followed by storage at 40°C for 3 months (**G**), 5°C for 30 months (**H**), 30°C for 12 months (**I**), and 40°C for 6 months (**J**). Upper and lower shelf-life limits for both products are shown by the horizontal dotted lines.
Abbreviation: IU, international units.
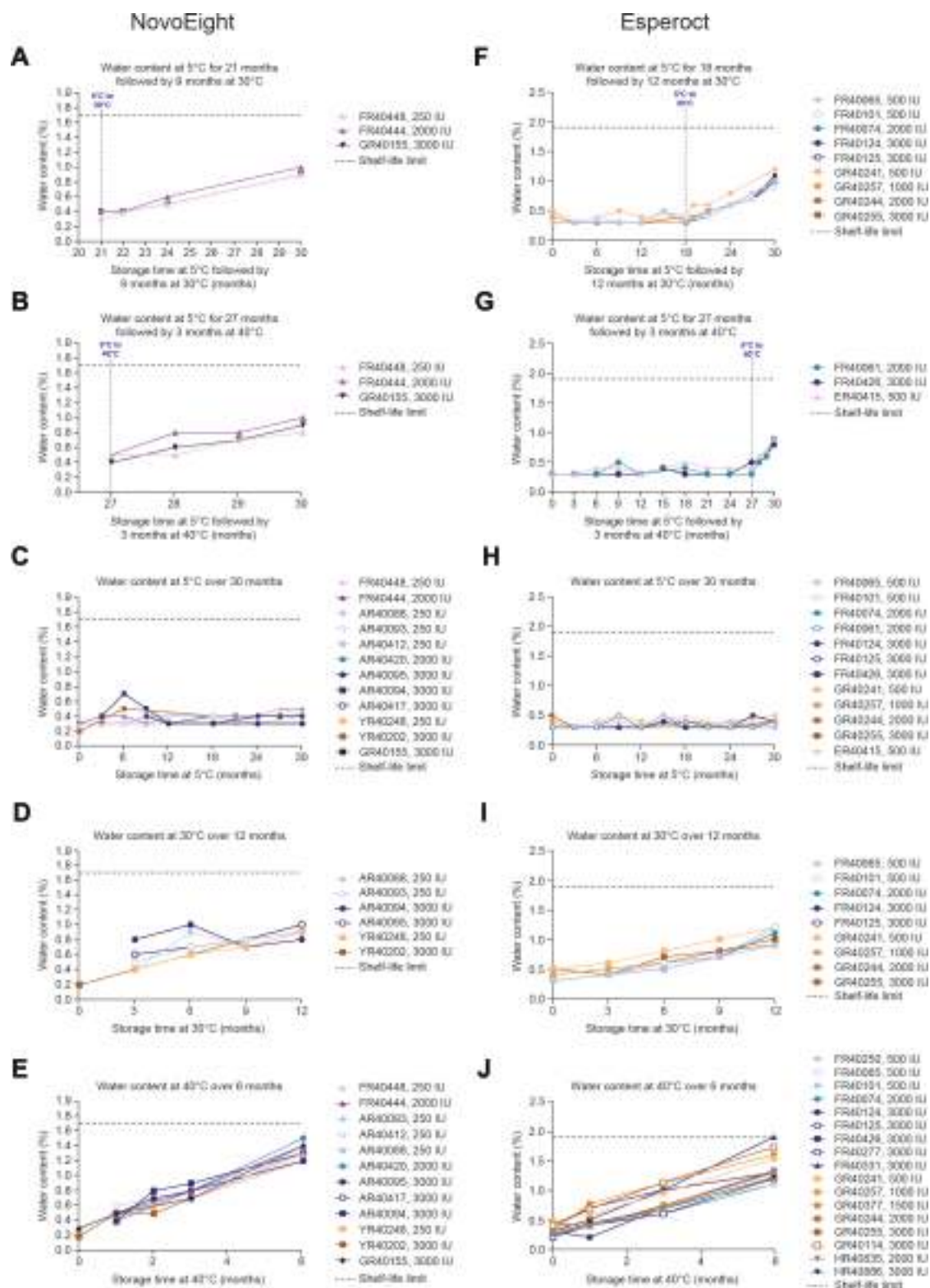
Figure 5 Water content of turoctocog alfa dry powder samples when stored at 5°C for 21 months followed by storage at 30°C for 9 months (**A**), 5°C for 27 months followed by storage at 40°C for 3 months (**B**), 5°C for 30 months (**C**), 30°C for 12 months (**D**), and 40°C for 6 months (**E**). Water content of turoctocog alfa pegol dry powder samples when stored at 5°C for 18 months followed by storage at 30°C for 12 months (**F**), 5°C for 27 months followed by storage at 40°C for 3 months (**G**), 5°C for 30 months (**H**), 30°C for 12 months (**I**), and 40°C for 6 months (**J**). Shelf-life limits for both products are shown by the horizontal dotted lines.
Abbreviation: IU, international units.

16

Journal of Blood Medicine 2021:12
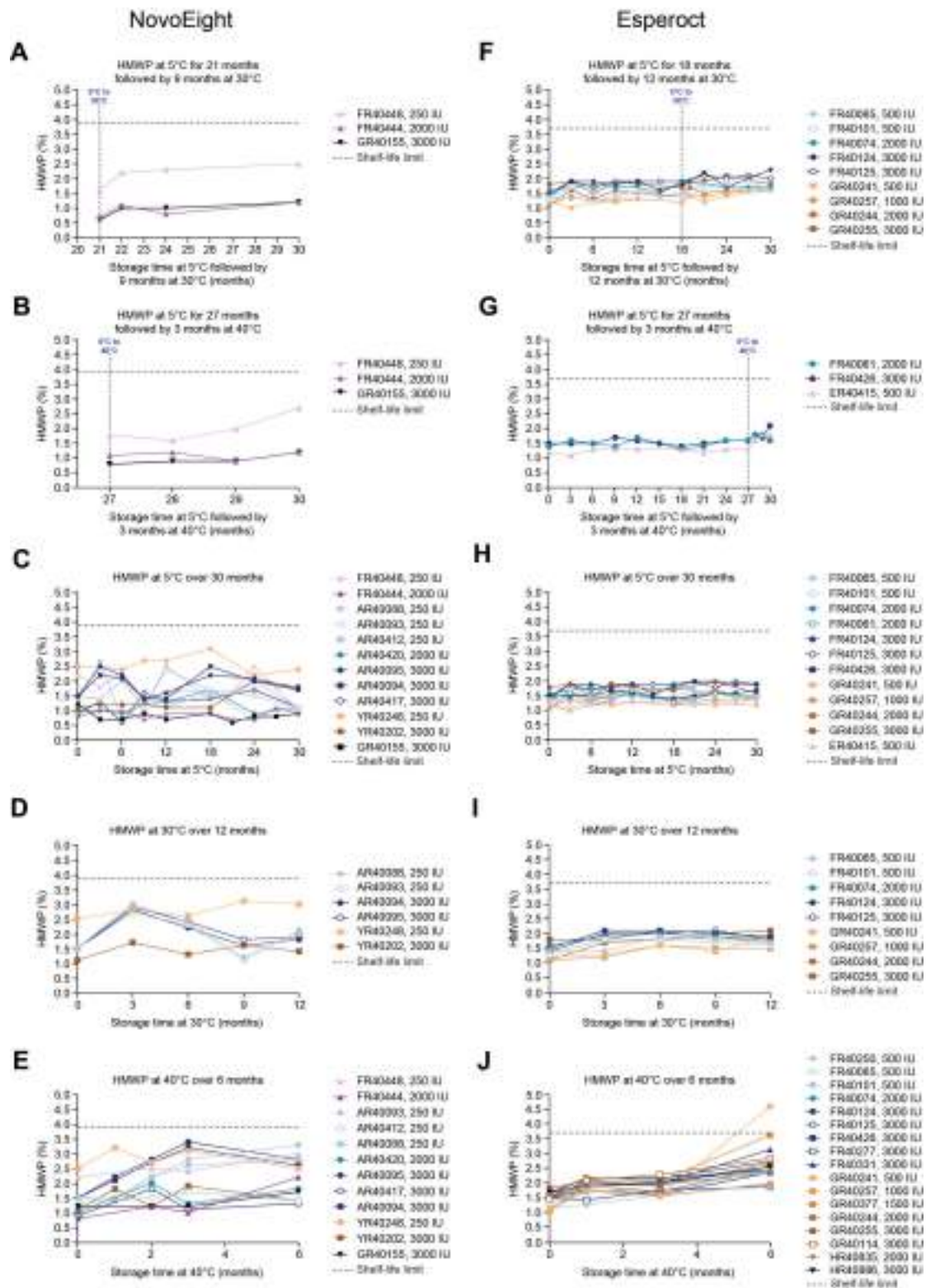
Figure 6 HMWP of turoctocog alfa dry powder samples when stored at 5°C for 21 months followed by storage at 30°C for 9 months (**A**), 5°C for 27 months followed by storage at 40°C for 3 months (**B**), 5°C for 30 months (**C**), 30°C for 12 months (**D**), and 40°C for 6 months (**E**). HMWP of turoctocog alfa pegol dry powder samples when stored at 5°C for 18 months followed by storage at 30°C for 12 months (**F**), 5°C for 27 months followed by storage at 40°C for 3 months (**G**), 5°C for 30 months (**H**), 30°C for 12 months (**I**), and 40°C for 6 months (**J**). Shelf-life limits for both products are shown by the horizontal dotted lines.
Abbreviations: HMWP, high molecular weight protein; IU, international units.

75% RH for 12 months (Figure 6D and I) and, for all but one batch (turoctocog alfa pegol at 500 IU), <4% after storage at 40°C/75% RH for 6 months (Figure 6E and J).

It was noted that there was some variability in HMWP for both turoctocog alfa and turoctocog alfa pegol due to analytical issues encountered at low concentrations. HMWP is oligomeric forms of the monomer of turoctocog alfa and turoctocog alfa pegol, eluting ahead of the monomer in SE-HPLC.

Discussion

The ICH guidelines state that well-defined stability studies should be developed to confirm product stability during the intended storage period.[30] We assessed key stability parameters for both turoctocog alfa and turoctocog alfa pegol; these parameters are susceptible to change during storage and/or could potentially influence product quality, namely oxidized forms, potency, water content, and HMWP. There were no major increases in oxidized forms or HMWP and no impairment in potency or water content for either product; most results were within each product's range. Although FVIII activity (potency) and protein aggregates (HMWP) were largely unaffected, a small increase in oxidized forms was observed after storage at 30°C/75% RH for 12 months and after storage at 40°C/75% RH for 6 months. However, the changes in oxidized forms for both turoctocog alfa and turoctocog alfa pegol at high temperatures up to 40°C did not affect the quality of either product. Oxidized forms are inactive forms of rFVIII and a correlation between potency and oxidized forms can be expected.[33] For example, oxidation of FVIII has been reported to correlate with the oxidation of free Cys and disulfide bonds, leading to structural change and loss of potency.[33]

Interestingly, turoctocog alfa can be subjected to variable storage conditions, including cycling between 5°C and ≤40°C, and subsequent storage for 3 months up to 40°C, without loss of stability.[37] As of yet, no temperature cycling data are available for turoctocog alfa pegol. However, among commercially available EHL-rFVIII products, turoctocog alfa pegol is the only one that has shown stability at 40°C.[13] Thus, the current stability data should reassure patients who are receiving turoctocog alfa and considering switching to turoctocog alfa pegol. Furthermore, as the current study shows that both products can be safely stored at temperatures up to 40°C/75% RH for 3 months without compromising product potency, patients can also be reassured that the dose on the vial

label is unaffected—and therefore dose adjustments are not required—when either product is stored outside the refrigerator for several months. Patients should note the date when turoctocog alfa and turoctocog alfa pegol are taken out of the fridge so they can ensure that the duration of room-temperature storage does not exceed 3 months.

Home treatment of hemophilia is necessary and recommended: the World Federation of Hemophilia recommends acute bleeds should be treated as quickly as possible, preferably within 2 hrs.[2] Patients/caregivers of those with hemophilia A have reported preferences for FVIII products that can be stored at higher temperatures for long periods of time, both overall and when travelling.[15,16] However, in one small study, only 13% of patients and caregivers were aware of their product's storage conditions,[38] while another study showed that 80% of patients/caregivers who stored FVIII in the refrigerator did not know that their product may be safely stored at room temperature for a limited number of months.[15] Therefore, it is important for patients to know if there is a meaningful, clinical difference between an FVIII product kept outside of the fridge for 48 hrs (for example) compared with one kept in the fridge for 3 months. Our study shows that both turoctocog alfa and turoctocog alfa pegol remained stable when stored at a relatively high ambient room temperature and humidity (40°C/75% RH) for up to 3 months following an extended storage period at 5°C. Two other rFVIII products (Advate® and Recombinate® [Takeda, Lexington, MA]) are thermostable at 30°C–40°C;[39,40] however, at the time of writing and to the best of our knowledge, turoctocog alfa and turoctocog alfa pegol are the only FVIII and EHL FVIII products, respectively, licensed for storage at temperatures up to 40°C.

These data are of clinical importance, as they demonstrate that the stability of both products may offer flexibility and greater convenience in product storage and daily use. Benefits of increased storage flexibility and portability include the ability to keep more FVIII product at home, the ability to travel more easily, reduced daily disruption[15,16] and, potentially, lead a more active lifestyle. The resulting improvement in convenience may also contribute to better treatment outcomes and quality of life by facilitating early treatment of bleeds[15,17] and improving adherence to the recommended prophylaxis schedules. These benefits would be particularly relevant for patients who live in, or who travel to, countries where daytime temperatures are consistently or transiently above 30°C, and even up to 40°C. It is therefore crucial for hemophilia care providers to clearly explain to patients and/or

caregivers that FVIII products such as turoctocog alfa and turoctocog alfa pegol can be kept at room temperature for extended periods (assuming no higher than 40°C) while remaining clinically effective to use.

Conclusion

Turoctocog alfa and turoctocog alfa pegol offer stability at 40°C/75% RH for up to 3 months without jeopardizing the quality of either product. These stability characteristics may be beneficial to patients by allowing flexibility in product storage and daily use.

Data Sharing Statement

Novo Nordisk's policy on data sharing may be found at https://www.novonordisk-trials.com/how-access-clinical-trial-datasets.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

MN has acted as a consultant for Amgen, Bayer, BIOFVIIIx and Novo Nordisk, and received speaker fees from Baxalta, Bayer, CSL Behring, Kedrion, Novo Nordisk, Sobi, Takeda, and Octapharma; AAO and AMN are employees of Novo Nordisk A/S. The authors report no other conflicts of interest in this work.

References

1. Pipe S. Antihemophilic factor (recombinant) plasma/albumin-free method for the management and prevention of bleeding episodes in patients with hemophilia A. *Biologics*. 2009;3:117–125. doi:10.2147/btt.2009.2872
2. Srivastava A, Brewer A, Mauser-Bunschoten E, et al. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1–47.
3. Mancuso ME, Santagostino E. Outcome of clinical trials with new extended half-life FVIII/IX concentrates. *J Clin Med*. 2017;6(4):39. doi:10.3390/jcm6040039
4. Advate [prescribing information]. Baxter Healthcare Corporation; 2011. Available from: https://www.fda.gov/downloads/BloodBloodProducts/ucm059095.pdf. Accessed September 18, 2018.
5. Kogenate [summary of product characteristics]. Bayer HealthCare Manufacturing SRL; 2018. Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000275/human_med_000879.jsp&mid=WC0b01ac058001d124. Accessed September 28, 2018.
6. NUWIQ [prescribing information]. Octapharma AB; 2015. Available from: https://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/UCM461328.pdf. Accessed September 28, 2018.
7. NovoEight® [summary of product characteristics]. Novo Nordisk A/S; 2018. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002719/WC500157553.pdf. Accessed August 23, 2018.
8. NovoEight®. [Highlights of prescribing information]. Available from: https://www.novo-pi.com/novoeight.pdf. Accessed August 10, 2018.
9. Jivi [summary of product characteristics]. Bayer AG; 2019. Available from: https://www.ema.europa.eu/en/documents/product-information/jivi-epar-product-information_en.pdf. Accessed November 14, 2019.
10. ELOCTATE [summary of product characteristics]. Swedish Orphan Biovitrum AB; 2019. Available from: https://www.ema.europa.eu/en/documents/product-information/elocta-epar-product-information_en.pdf. Accessed November 14, 2019.
11. ADYNOVATE [package insert]. Baxalta US Inc; 2019. Available from: https://www.fda.gov/files/vaccines,%20blood%20&%20biologics/published/Package-Insert—ADYNOVATE.pdf. Accessed November 14, 2019.
12. AFSTYLA [summary of product characteristics]. CSL Behring GmBH; 2019. Available from: https://www.ema.europa.eu/en/documents/product-information/afstyla-epar-product-information_en.pdf. Accessed November 14, 2019.
13. ESPEROCT. [Summary of product characteristics]. Available from: https://www.ema.europa.eu/en/documents/product-information/esperoct-epar-product-information_en.pdf. Accessed 14 November 2019.
14. ESPEROCT [package insert]. Novo Nordisk A/S; 2019. Available from: https://www.fda.gov/media/120351/download. Accessed November 14, 2019.
15. Tischer B, Marino R, Napolitano M. Patient preferences in the treatment of hemophilia A: impact of storage conditions on product choice. *Patient Prefer Adherence*. 2018;12:431–441. doi:10.2147/PPA.S151812
16. DiBenedetti D, Coles T, Sharma T, Pericleous L, Kulkarni R. Assessing patients' and caregivers' perspectives on stability of factor VIII products for haemophilia A: a web-based study in the United States and Canada. *Haemophilia*. 2014;20(4):e296–e303. doi:10.1111/hae.12459
17. Saxena K. Barriers and perceived limitations to early treatment of hemophilia. *J Blood Med*. 2013;4:49–56. doi:10.2147/JBM.S43734
18. Shannon E, Daffy J, Jones H, Paulson A, Vicik SM. Etanercept (Enbrel) alternative storage at ambient temperature. *Clin Pharmacol*. 2017;9:87–99. doi:10.2147/CPAA.S131832

19. World Health Organization. WHO Expert Committee on Specifications for Pharmaceutical Preparations. WHO Technical Report Series 953; forty-third report; 2009. Available from: http://apps.who.int/iris/bitstream/handle/10665/44553/WHO_TRS_953_eng.pdf?sequence=1. Accessed November 1, 2018.

20. Kulkarni R, Karim F, Glamocanin S, et al. Results from a large multinational clinical trial (guardian3) using prophylactic treatment with turoctocog alfa in paediatric patients with severe haemophilia A: safety, efficacy and pharmacokinetics. *Haemophilia*. 2013;19 (5):698–705. doi:10.1111/hae.12165

21. Santagostino E, Lentz SR, Misgav M, et al. Safety and efficacy of turoctocog alfa (NovoEight®) during surgery in patients with haemophilia A: results from the multinational guardian clinical trials. *Haemophilia*. 2015;21(1):34–40. doi:10.1111/hae.12518

22. Lentz S, Misgav M, Ozelo M, et al. Results from a large multinational clinical trial (guardian1) using prophylactic treatment with turoctocog alfa in adolescent and adult patients with severe haemophilia A: safety and efficacy. *Haemophilia*. 2013;19(5):691–697. doi:10.1111/hae.12159

23. Curry N, Albayrak C, Escobar M, et al. Once-weekly prophylaxis with glycoPEGylated recombinant factor VIII (N8-GP) in severe haemophilia A: safety and efficacy results from pathfinder 2 (randomized Phase III trial). *Haemophilia*. 2019;25(3):373–381. doi:10.1111/hae.13712

24. Giangrande P, Andreeva T, Chowdary P, et al. Clinical evaluation of glycoPEGylated recombinant FVIII: efficacy and safety in severe haemophilia A. *Thromb Haemost*. 2017;117(2):252–261. doi:10.1160/TH16-06-0444

25. Hampton K, Chowdary P, Dunkley S, et al. First report on the safety and efficacy of an extended half-life glycoPEGylated recombinant FVIII for major surgery in severe haemophilia A. *Haemophilia*. 2017;23(5):689–696. doi:10.1111/hae.13246

26. Meunier S, Alamelu J, Ehrenforth S, et al. Safety and efficacy of a glycoPEGylated rFVIII (turoctocog alpha pegol, N8-GP) in paediatric patients with severe haemophilia A. *Thromb Haemost*. 2017;117 (9):1705–1713. doi:10.1160/TH17-03-0166

27. Stennicke HR, Kjalke M, Karpf DM, et al. A novel B-domain O-glycoPEGylated FVIII (N8-GP) demonstrates full efficacy and prolonged effect in hemophilic mice models. *Blood*. 2013;121 (11):2108–2116. doi:10.1182/blood-2012-01-407494

28. Ellgaard TW, Bindslev L, Kamstrup S. Evaluation of the virus clearance capacity and robustness of the manufacturing process for the recombinant factor VIII protein, turoctocog alfa. *Protein Expr Purif*. 2017;129:94–100. doi:10.1016/j.pep.2016.09.002

29. Agerso H, Stennicke HR, Pelzer H, et al. Pharmacokinetics and pharmacodynamics of turoctocog alfa and N8-GP in haemophilia A dogs. *Haemophilia*. 2012;18(6):941–947. doi:10.1111/j.1365-2516.2012.02896.x

30. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline: quality of Biotechnological Products: stability Testing of Biotechnological/Biological Products Q5C. Current Step 5 version. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-topic-q-5-c-quality-biotechnological-products-stability-testing-biotechnological/biological-products_en.pdf. Accessed May 19, 2020.

31. European Pharmacopoeia. 10th Edition. Available from: https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-10th-edition. Accessed May 19, 2020.

32. Eldin A, Shalaby A. Comparison of FT-NIR transmission and HPLC to assay montelukast in its pharmaceutical tablets. *Am J Analytical Chem*. 2011;2(8):885–891. doi:10.4236/ajac.2011.28102.

33. Wang W, Wang YJ, Kelner DN. Coagulation factor VIII: structure and stability. *Int J Pharm*. 2003;259(1–2):1–15. doi:10.1016/S0378-5173(03)00227-8

34. Butenas S, Parhami-Seren B, Gissel MT, Gomperts ED, Fass DN, Mann KG. Potency and mass of factor VIII in FVIII products. *Haemophilia*. 2009;15(1):63–72. doi:10.1111/j.1365-2516.2008.01826.x

35. Yoshioka S, Aso Y. Correlations between molecular mobility and chemical stability during storage of amorphous pharmaceuticals. *J Pharm Sci*. 2007;96(5):960–981. doi:10.1002/jps.20926

36. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS J*. 2006;8(3):E501–507. doi:10.1208/aapsj080359

37. Napolitano M, Nohr AM. The effect of fluctuating temperature on the stability of turoctocog alfa for hemophilia A. *Drugs R D*. 2019;19 (4):381–390. doi:10.1007/s40268-019-00290-3

38. Novais T, Duclos A, Varin R, Lopez I, Chamouard V. Treatment-related knowledge and skills of patients with haemophilia and their informal caregivers. *Int J Clin Pharm*. 2016;38(1):61–69. doi:10.1007/s11096-015-0207-z

39. Parti R, Ardosa J, Yang L, Mankarious M. In vitro stability of recombinant human factor VIII (Recombinate). *Haemophilia*. 2000;6(5):513–522. doi:10.1046/j.1365-2516.2000.00410.x

40. Parti R, Schoppmann A, Lee H, Yang L. Stability of lyophilized and reconstituted plasma/albumin-free recombinant human factor VIII (ADVATE rAHF-PFM). *Haemophilia*. 2005;11(5):492–496. doi:10.1111/j.1365-2516.2005.01128.x

Post-Transplant Maintenance Therapy for Patients with Acute Myeloid Leukemia: Current Approaches and the Need for More Trials

Rita Assi, Nohad Masri, Iman Abou Dalle, Jean El-Cheikh & Ali Bazarbachi

Open Access Full Text Article

REVIEW

Post-Transplant Maintenance Therapy for Patients with Acute Myeloid Leukemia: Current Approaches and the Need for More Trials

Rita Assi[1]
Nohad Masri[1]
Iman Abou Dalle[2]
Jean El-Cheikh [ID][2]
Ali Bazarbachi[2]

[1]Division of Hematology-Oncology, Lebanese American University and Lebanese American University Medical Center-Rizk Hospital, Beirut, Lebanon; [2]Division of Hematology-Oncology, American University of Beirut Medical Center, Beirut, Lebanon

Abstract: Relapse rates following allogeneic stem cell transplantation for acute myeloid leukemia remain unacceptably high and a major cause of death. Maintenance therapies post-transplant administered either to patients with impending relapse or at high risk of relapse could present a strategy to improve survival and overall outcomes. With the increasing use of molecular and genomic characterization of the disease, more novel therapies became available as maintenance strategies. These options were, however, hindered by excessive toxicities, mostly hematologic, especially with the use of myeloablative conditioning regimens. Several key questions have also emerged including the efficacy of these therapies, the duration of maintenance, as well as the potential modulation of the graft and the immune microenvironment. These issues are further complicated by the paucity of well-designed prospective randomized clinical trials evaluating these agents. Future directions in this field should include better risk stratification and patient selection based on assays of minimal residual disease, as well as the incorporation of novel targets and pathways of leukemogenesis. In this article, we highlight the current evidence behind the use of post-transplant maintenance therapy, the optimal patient and disease selection, as well as the challenges faced by these strategies in an area that remains quite controversial. We will focus on therapies targeting leukemia stem cells that directly or indirectly modulate the allografted immune microenvironment and augment the graft-versus-leukemia impact.

Keywords: AML, maintenance, relapse, target, MRD

Background

Acute myeloid leukemia (AML) remains the most common acute leukemia in adults with an incidence of 3–4 per 100,000 person per year. AML is a genetically and phenotypically heterogeneous and biologically dynamic spectrum of diseases.[1] Indeed, the clinical outcomes are largely determined by the patient's characteristics such as age, performance status and comoridities, as well as the leukemia features including the subtype (de novo versus secondary) and most importantly the genomic profile.[2] The recent advances in defining the molecular landscape of AML and its role in leukemogenesis have paved the way for the development and adaptation of novel targeted agents.

Following induction chemotherapy, patients achieving a morphologic leukemia-free state (complete remission (CR)) are mandated to receive a form of consolidation therapy aimed at the residual leukemic stem cells (LSCs) to prevent relapse and improve overall survival (OS).[3] A risk-adapted approach for relatively young or fit

Correspondence: Ali Bazarbachi
Department of Internal Medicine, American University of Beirut, Medical Center, P.O. Box 113-6044, Beirut, Lebanon
Email bazarbac@aub.edu.lb

AML patients in first CR (CR1) involves the assessment of this risk of relapse, leading to either chemotherapy continuation or allogeneic stem cell transplantation (ASCT), taking into account the presence of comorbidities, the donor type as well as the genetic characteristics of the disease.[4] In addition to pre-treatment risk stratification, the estimation of the leukemic burden while on therapy has recently emerged as a strong, independent and dynamic tool for individualizing post-induction treatment approaches. Either polymerase chain reaction (PCR), multiparameter flow cytometry (MFC) or the novel next-generation sequencing (NGS) can evaluate this measurable residual disease (MRD)[5–7]

Allogeneic Stem Cell Transplantation: Rationale and Nuances

Up to the current date, ASCT in first CR remains the most powerful antileukemic post-remission therapy. ASCT is generally recommended upfront for properly selected patients with high-risk cytogenetic features, those with intermediate and adverse-risk molecular findings, and patients with secondary AML. Patients with induction failure, post-induction residual disease and following salvage therapy are also referred for ASCT. In addition to potentially life-threatening complications of ASCT such as graft-versus-host disease (GVHD) and opportunistic infections, survival benefits recorded with ASCT are crippled by unacceptably high disease relapse rates,[8–10] hence the need for strategies to maintain remission and prevent relapses post-ASCT. Such interventions aim at reinforcing the graft-versus-leukemia (GVL) effect and/or eradicating persistent MRD, especially with the increasing availability of more sensitive techniques to detect any residual disease. Nevertheless, these maintenance therapies may represent over-treatment for patients with intermediate-risk disease, further subjecting them to long-term toxicities and disturbed quality of life (QoL), thereby reinforcing the need for a better selection of patients as well as strict and continuous MRD monitoring.

The transplantation field has tremendously evolved over the last two decades with refinements of indications as well as improvement in the safety profile of conditioning regimens and supportive care strategies. Nonetheless, risk factors for increasing mortality after relapse in an allografted patient still include, among others, a shorter time to recurrence and occurrence of GVHD prior to relapse[11] with significant improvement of overall survival (OS) for young patients relapsing in recent years (Bazarbachi et al, 2020).[12] Furthermore, a deeper understanding of factors facilitating disease relapse, such as molecular profile and role of MRD, has enabled more high-risk patients to receive post-transplant therapies to treat and even prevent relapses. Indeed, pharmacological intervention and manipulation of the disease kinetics in the early post-transplant phase could potentially collaborate with other strategies to improve overall outcomes,[13] possibly through up-regulation of tumor-associated antigens (TAA),[14] expansion of regulatory T-cells,[15] or acceleration of T-cell reconstitution.[16] With the availability of a wide array of novel and less toxic agents such as epigenetic modifiers, tyrosine kinase inhibitors (TKIs), BCL2 inhibitors and immune checkpoint inhibitors (ICPIs) among others, an intriguing strategy would be to preemptively use such molecules in an attempt to prevent relapses post-ASCT in specific subsets of high-risk patients. Nevertheless, we currently only have few randomized trials that offered a survival advantage for maintenance therapy in AML.

Selection of Optimal Candidates

Conducting either retrospective studies or prospective randomized trials to construct therapeutic strategies aiming at reducing post-ASCT relapse rates has been historically hampered by the depth of remission achieved as well as the intrinsic biologic apparatus of the disease. Cytogenetic abnormalities of AML knowingly dictate both the general outcomes of standard therapies and those following ASCT.[17] In view of the granular advances in the field of myeloid malignancies, considering specific subsets of AML patients for post-ASCT maintenance should therefore depend on the molecular and genomic characteristics of the disease itself at diagnosis.[18] Indeed, the presence of actionable or targetable mutations such as *FLT3*-ITD and *IDH*1/2 is a valuable opportunity to incorporate the approved corresponding inhibitors in the post-ASCT maintenance strategies. Novel molecular and MRD diagnostics are therefore of utmost importance to determine those who would benefit the most from personalized therapy options. As such, MRD status in the pre-transplant phase and more importantly detection of MRD early post-ASCT are crucial factors to implement therapy as they largely impact the likelihood and pace of disease relapse.[19,20]

In this setting, other variables including the donor source, intensity of conditioning regimen and GVHD

prophylaxis protocols (T-cell depletion and post-ASCT cyclophosphamide) might influence the risk of disease relapse.[21] While the implementation of reduced-intensity conditioning (RIC) has allowed more patients to receive ASCT,[22] it could potentially increase the rate of post-transplant relapse, as demonstrated by the large prospective randomized Phase III trial conducted by the Bone Marrow Transplant Clinical Trials Network.[23] Well-designed trials are eagerly needed to appropriately answer these challenging situations.

Challenges to Conduct Clinical Trials

In the presence of few prospective randomized trials, the decision to initiate post-ASCT maintenance therapy remains ambivalent in many situations. Early-phase studies assessing novel agents in the relapsed setting often exclude patients with prior history of ASCT given the plethora of complications they might experience, therefore resorting to agents previously approved for different indications or settings. This dilemma largely provides a protective blanket to access these drugs on an off-label indication, which could impede recruitment for prospective studies. Additionally, most currently ongoing maintenance trials using hypomethylating agents (HMA), targeted therapies and other molecules still demand rigorous eligibility criteria, thereby interfering with enrollment rate.

Optimal Timing and Duration of Maintenance Initiation

Starting maintenance therapy in the early post-ASCT phase should take into account the concomitant use of immunosuppressive drugs and their potential heightened hematological and organ toxicities, the risk of opportunistic infections and GVHD, as well as the possible drug–drug interactions (such as with calcineurin inhibitors), even when the acute toxicities of ASCT have seemingly resolved. An optimal maintenance approach is therefore difficult to be intercalated within the conditioning regimen itself and is reserved for a post-ASCT phase, mostly started between days 30 and 100 following transplantation. In this setting, pre- and post-ASCT MRD status could be valuable in planning and timing maintenance therapy. For those patients with impending signs of relapse by MRD testing or falling donor chimerism, a preemptive maintenance therapy could be started early post-ASCT, before overt morphological relapse.

Finally, the optimal duration of maintenance therapy has not been established for most cases, thereby affecting the QoL of these patients.

Available Maintenance Options: The Current Evidence
Targeting Epigenetics Pathways of Leukemia Cells
Hypomethylating Agents

The use of HMAs such as azacitidine and decitabine remains the most commonly adopted non-targeted strategy for the prevention of post-ASCT relapse owing in part to their acceptable safety profile.[24] The mechanism of action of HMAs post-ASCT is unclear, but they appear to silence tumor suppressor genes through epigenetic modification. At the preclinical level, these agents could also induce a GVL effect through stimulation of CD8+ T-cell responses to overexpressed tumor-associated antigens (TAAs) such as MAGE antigens.[25] This activity has led to the investigation of HMAs in a series of small trials, especially with the advancing field of MRD detection by sensitive techniques.

For example, AML patients with imminent relapse due to decreasing CD34 chimerism received pre-emptive azacitidine that delayed disease progression according to two studies.[26,27] The concurrent administration of donor lymphocyte infusion (DLI) did not, however, improve response rates or OS[27] and the majority of patients eventually experienced overt disease relapse.[26] In another study, azacitidine was also given sequentially with DLI and showed a low relapse rate and encouraging OS despite the presence of acute and chronic GVHD.[28]

In a Phase I dose-finding trial, azacitidine as monotherapy was given between on day +42 post-ASCT to 45 patients with AML (82%) and MDS, for up to four cycles at different dose levels 8, 16, 24, 32, and 40 mg/m^2.[29] Interestingly, two-thirds of AML patients were not in CR at the time of transplant. The recommended dose of azacitidine was reported to be 32 mg/m2 for 5 days in 30-day cycles because of dose-limiting but reversible thrombocytopenia. At 1-year follow-up, the median disease-free survival (DFS) was 58% for all enrolled patients and the 1-year OS rate was 77%. In another phase I/II study of 27 AML patients who received a RIC regimen followed by ASCT later showed that the subcutaneous administration of up to 10 cycles of azacitidine at 36 mg/m^2 for 5 days in 28-day cycles beginning at day 42 post-ASCT resulted in

the expansion of circulating regulatory T-cells with subsequent GVL response and no significant GVHD.[15] In a retrospective study of 18 allografted patients (13 AML and 5 MDS), including 50% of patients with a high or very high disease risk index, low-dose azacitidine started at a median of 60 days post-transplant was well tolerated and resulted in one-year disease-free survival (DFS) and OS of 63% and 70%, respectively.[30] A subsequent randomized phase III trial comparing azacitidine at 32 mg/m2 subcutaneously for 5 days in up to 12, 28-day cycles to no intervention in 87 patients with AML, myelodysplastic syndromes (MDS) or chronic myelomonocytic leukemia in remission was terminated early because of slow accrual.[31] At a median follow-up of 4.6 years in the azacitidine arm, available data suggest no significant effect of the HMA on relapse-free survival (RFS), except for a non-statistically significant trend for improvement in those who received at least 9 cycles of therapy.

The importance of MRD-adapted therapy is highlighted in the ongoing Phase II study (RELAZA2) whereby preemptive treatment with at least 6 cycles of azacitidine (75 mg/m^2 × 7 days) and for up to 18 additional months was evaluated.[32] The study enrolled patients in CR but with detectable MRD either after conventional chemotherapy or following ASCT. This preemptive MRD risk-adapted strategy was found to prevent or significantly delay disease relapse in 58% of patients who remained in CR after 6 months (95% CI: 44–72; p < 0.001). These results are encouraging and warrant further follow-up.

More recently, an oral azacitidine formulation CC-486 with extended dosing to prolong activity of azacitidine with sustained DNA hypomethylation showed promising results as maintenance therapy in a randomized trial following induction chemotherapy for AML.[33] CC-486 was then evaluated in a phase I/II trial of 30 patients (26 with AML and 4 MDS) who had undergone ASCT, given at 200–300 mg orally for 7 days or 150–200 mg orally for 14 days in up to 12, 28-day cycles.[34] The study resulted in 1-year RFS rates of 54% with the 7-day protocol and 72% with the 14-day regimen in the 28 evaluable patients, leading to estimated 1-year survival rates of 86% and 81%, respectively. The most common grade 3–4 treatment-related toxicities were gastrointestinal and hematologic toxicities, and two patients experienced severe chronic GVHD. A randomized, phase III trial evaluating CC-486 at the 200 mg 14-day dosing regimen as maintenance therapy post-ASCT for high-risk MDS and intermediate- or high-risk AML is currently enrolling.

On the other hand, a small study of decitabine administered at 5–15 mg/m2 intravenously for 5 days starting 50–100 days post ASCT for up to 8, 6-week cycles also exhibited favorable results with 2-year OS of 56% and cumulative incidence of relapse reaching 28%.[35] However, the majority (75%) of patients experienced grade 3–4 hematologic toxicities during therapy. While decitabine did not increase the rate of chronic GVHD, there was a trend for increased FOXP3 expression and T-reg cells in the lymphocyte environment in a correlative study that was not statistically meaningful.

Interpreting the results of these studies remains challenging and controversial, as they are small and mostly uncontrolled. As such, the optimal timing of HMA initiation post-ASCT and dosing need to be explored further to establish efficacy at preventing relapses and avoid unnecessary toxicities, especially in patients who can be cured with ASCT alone. In patients with detectable MRD or mixed chimerism, pre-emptive treatment with HMA could potentially delay or even prevent relapses in AML and MDS patients.[36]

More recently, there has been a growing interest in evaluating HMA as partners to novel promising agents such as the BCL2 inhibitor venetoclax, ICPs, FLT3 inhibitors, as well as isocitrate dehydrogenase (IDH) inhibitors and studies are ongoing (Table 1).

Histone Deacetylase Inhibitors (HDACi)

The class I/II HDACi have presented as potential promising agents in AML/MDS owing to large induction effects on cell-cycle arrest and differentiation, as well as pro-apoptotic effects on myeloid cells through epigenetic modifications of histones.[37] HDACi have also exhibited some antileukemic and immunomodulatory roles through the control of cytokine secretion. This is further evidenced by the panobinostat activity, a potent oral inhibitor of class 1, 2, and 4 deacetylases, in the PANOBEST trial.[38] This study enrolled 42 patients with high-risk AML or MDS who had received ASCT and panobinostat was started at a median of 98 days (60–150) post-ASCT. Two-thirds of these patients were transplanted in active disease. While only 22 (54%) of the 42 patients completed 1 year of therapy because of adverse events, the cumulative incidence at relapse remained 21% at 2 years, resulting in 2-year OS and DFS rates of 88% and 74%. More importantly, panobinostat was found to inhibit the suppressive function of T-regs when used at low doses and enhance their function at higher doses,[39] thereby playing a possible

Table 1 Some of the Ongoing Trials Evaluating Various Targets for Post-Allogeneic Stem Cell Transplantation Strategies

Target	Drug(s)	Clinical Phase	Endpoints	Status	Identifier
FLT3					
	Crenolanib	II	PFS; DFS; OS; GVHD; 100-day TRM	Recruiting	NCT02400255
	Gilteritinib	III	RFS, OS, GVHD, EFS	Active, not recruiting	NCT02997202
TP53					
	APR-246 + Azacytidine	II	Safety, tolerability, 1-year RFS	Recruiting	NCT03931291
Hh					
	Glasdegib	III	DFS	Not yet recruiting	NCT04168502
DNMT					
	Low-dose azacytidine	II	OS; relapse rate; safety	Recruiting	NCT01995578
	Azacytidine+ valproic acid	II	OS; time to relapse	Recruiting	NCT02124174
	Oral azacytidine (AMADEUS)	III	RFS, OS, NRM, GVHD	Recruiting	NCT04173533
	SGI-110+ DLI	II	DFS, OS	Not yet recruiting	NCT03454984
BCL-2					
	Venetoclax+ azacytidine	I	MTD; OS, RFS; GVHD	Recruiting	NCT03613532
	Venetoclax+ azacytidine	II	RFS, OS, GVHD	Recruiting	NCT04128501
	Venetoclax+ azacytidine (VIALE-T)	III	RFS, OS, GVHD, MRD	Recruiting	NCT04161885
HDAC					
	Panobinostat	III	OS, DFS, EFS, NRM, GVHD	Recruiting	NCT04326764
IDH2					
	Enasidenib	I	Safety, tolerability, GVHD, RFS	Recruiting	NCT03728335
	Enasidenib	I	MTD, DLT, GVHD	Recruiting	NCT03515512
	Enasidenib	II	OS, RFS, Safety and tolerability	Recruiting	NCT04522895
IL-15					
	N-803	II	Relapse rate, GVHD, MRD, OS	Recruiting	NCT02989844

Abbreviations: PFS, progression-free survival; OS, overall survival; RFS, relapse-free survival; EFS, event-free survival; GVHD, graft-versus-host disease; MTD, maximum tolerated dose; DLT, dose-limiting toxicity; DLI, donor lymphocyte infusion; MRD, minimal residual disease; NRM, non-relapse mortality; Hh, hedgehog pathway; DNMT, DNA methyltransferase; HDAC, histone deacetylase.

role in reducing GVHD. As these results are intriguing, a randomized multicenter phase III trial is currently comparing panobinostat 20 mg orally three times weekly every second week to the standard of care as maintenance post-ASCT. Vorinostat, another HDACi, is also being combined with low-dose azacytidine for post-ASCT in a currently ongoing phase I dose-escalation clinical trial.

Targeting Oncogenic Pathways of Leukemia Stem Cells
FLT3 Inhibitors

Treatment of *FLT3*-ITD mutated AML remains challenging due to significant relapse rates and short remissions with available therapies despite the common historical use of ASCT in first CR.[40] Nevertheless, *FLT3*-mutated AML is a heterogeneous disease that entails diversity in the type of *FLT3* mutations and their insertion site, the *FLT3*-ITD

allelic burden, and the presence of concurrent mutations; observations that further complicated the decision to proceed to ASCT in the first CR when feasible.[41–43] This controversy is evidenced by the European LeukemiaNet guidelines suggesting, with some controversy, that ASCT should not be offered to patients with low-mutant allelic ratio.[44–46] EBMT guidelines allowed ASCT in this setting and recommended it for all patients with *FLT3*-mutated AML (Bazarbachi et al, 2020).[47]

As such, the use of multi-kinase inhibitors of various generations has led to improved outcomes and achievement of deeper responses in *FLT3*-mutated AML. These TKIs, together with the incorporation of MRD assessment, have enabled the installation of post-transplant therapeutic strategies,[48] as the 1-year OS of patients who relapse post-ASCT drops to less than 20%.[11] (Bazarbachi et al, 2020).[12]

The enthusiasm of using FLT3 TKIs stems not only from their direct cytotoxic properties but also involve an

immunomodulatory effect synergizing with allografted T-cells. Several murine models have shown that sorafenib enhances the production of interleukin-15 (IL-15) production by leukemic cells, thereby promoting GVL effect.[16] The same experiment showed that sorafenib reduced the activating transcription factor (ATF4) expression in leukemic cells, a negative regulator of IRF-7 interferon regulatory factor-7 (IRF-7) activation, which further enhances IL-15 transcription when activated. The exact mechanisms of FLT3 TKIs immunogenicity remain to be elucidated.

One of the earliest and most promising post-transplant maintenance approaches has been the administration of FLT3 inhibitors, limited to date to *FLT3*-ITD mutated AML patients. Despite multiple retrospective and prospective randomized trials evaluating the efficacy and safety of the use of FLT3 inhibitors as post-transplant maintenance, there is still a debate on the best agent to be used (off-label use of sorafenib versus potent second-generation FLT3 inhibitors), dosing and time of initiation. A consensus by the EBMT Acute Leukemia Working Party recommended the use of sorafenib 400 mg twice daily in the post-transplant setting in the absence of active GVHD based on available data (Bazarbachi et al, 2020).[47] Previous retrospective studies have demonstrated a lower risk of disease relapse following ASCT in patients with *FLT3* ITD mutated AML who received post-transplant sorafenib maintenance (Antar, et al, 2014).[49–53]

In a phase I study involving 22 patients with *FLT3*-ITD AML receiving sorafenib maintenance post-ASCT, PFS at 1 year was 85% and OS was 95%.[54] Encouraging results were subsequently reported in other small trials of sorafenib maintenance compared to historical controls, showing markedly lower relapse rates, improved RFS and relatively tolerable toxicities, while not significantly affecting the rates of GVHD.[51–53,55–57] This is further supported by two registry studies from the European Society for Blood and Marrow Transplantation (EBMT) showing that post-transplant maintenance with sorafenib improved OS and leukemia-free survival (LFS) of allografted patients with *FLT3*-ITD positive AML (Bazarbachi et al, 2019)[58] and that sorafenib combined with DLI clearly improved OS and LFS of relapsed *FLT3*-ITD positive AML patients following ASCT. (Bazarbachi et al, 2019)[59]

In a prospective phase II controlled randomized trial (SORMAIN) of 83 patients with *FLT3*-ITD mutated AML, the administration of sorafenib for up to 24 months resulted in superior outcomes for patients in CR and no grade ≥2 GVHD compared to placebo. After a long median follow-up of 42 months, the 2-year RFS was 85% in the sorafenib group compared with 53% in the placebo group (HR=0.39, p=0.01), in addition to an OS benefit for the sorafenib group (HR=0.447; p=0.03).[60] Further follow-up showed that many patients will experience disease relapse when sorafenib is stopped at 24 months, suggesting a longer exposure to sorafenib might be needed to prevent late relapses. While SORMAIN trial constitutes the first placebo-controlled evidence that post-HSCT maintenance therapy could reduce the risk of relapse and death, this study enrolled patients who underwent transplantation in the first hematological CR, as well as those in the second or subsequent CR. Finally, the Chinese open-label, large randomized phase III trial assigned patients to receive sorafenib maintenance (n=100) or control (n=102) post-ASCT (Xuan et al 2020).[61] At a median follow-up of 21.3 months, the 1-year cumulative incidence of relapse was 7.0% (95% CI 3.1–13.1) in the sorafenib group and 24.5% (16.6–33.2) in the control group (hazard ratio 0.25, 95% CI 0.11–0.57; p=0.0010), with no treatment-related deaths and acceptable GVHD rates. Based on these available data, sorafenib is recommended by many authorities as a maintenance strategy to reduce post-ASCT relapses for *FLT3*-ITD-mutated AML (Bazarbachi et al, 2020).[47]

More recent data from the RATIFY trial that led to the US Food and Drug Administration (FDA) approval of midostaurin in 2017, proposed that the outcomes of patients who received this agent prior to ASCT were particularly encouraging.[62] In a phase II trial of midostaurin received as post-consolidation or post-ASCT maintenance, the 1-year relapse rate was encouragingly low at 9.2%.[63] In this German-Austrian AML Study Group 16–10, most patients discontinued midostaurin earlier than planned because of toxicities. This remains in line with prior reports on the drug's complex pharmacokinetic profile and drug–drug interactions that warrant close observation and dose adjustments to reduce toxicity.[64,65]

RADIUS is another phase II randomized study that accrued 60 patients with *FLT3*-ITD AML with stable engraftment post-ASCT to receive or not midostaurin for twelve 4-week cycles.[66] Unsurprisingly, the median RFS was not reached for either arm as the trial was not powered to detect any statistical difference (p=0.34) between subgroups.

The prospective cooperative group international phase III randomized trial (BMT-CTN 1506; NCT02997202) is seeking to confirm the impact of post-transplant gilteritinib

maintenance therapy versus placebo in patients with *FLT3*-mutated AML and has completed accrual at 346 patients. Gilteritinib is an effective and tolerable FLT3 inhibitor, with potent activity against both *FLT3*-ITD and *FLT3*-TKD mutations, particularly the kinase domain mutations at residue D835 and the gatekeeper mutation at residue F691.[67] Gilteritinib was recently approved for use in the relapsed/refractory setting[68] and was chosen for evaluation as post-ASCT maintenance owing to its safety profile and potent inhibition of FLT3 in vivo. Unfortunately, the use of placebo as control arm in this trial will not allow to answer the important question of whether Gilteritinib offers an additional benefit over sorafenib in that setting.

Quizartinib (AC220), a highly potent selective FLT3-ITD inhibitor was also studied in one small phase I trial where only 1 of 13 patients relapsed under therapy at the last follow-up.[69] Furthermore, toxicities were manageable and GVHD rate was not increased. However, increasing reports about resistance through point-mutant forms have been emerging, hence limiting single-agent use.[70]

Crenolanib, like gilteritinib, is another potent oral type 1 FLT3 TKI with extended activity against FLT3-ITD and resistance-conferring FLT3-D835 TKD mutants.[71] It is also under evaluation as a post-ASCT maintenance in a phase II trial (NCT02400255), in a cohort of patients transplanted in CR and in another group allografted with the residual disease with ≤10% bone marrow blasts. Crenolanib is started between days 45 to 90 after ASCT and for up to 2 years. It is important to note that phase II/III trials of post-ASCT maintenance involving the novel FLT3 TKIs do not use a first-generation inhibitor control, making it difficult to establish their superior efficacy in this setting.

Some unanswered questions remain regarding the use of FLT3 TKIs as maintenance post-ASCT. *FLT3*-ITD mutations, unlike *BCR-ABL1* fusions,[72] are not founding mutations but rather an important final step and one of many mutations found in leukemogenesis.[73,74] These include *WT1, IDH1, DNMT3A,* as well as *NUP98/NSD1* fusions, which are currently known to affect outcomes and response to therapy. Furthermore, FLT3 measuring assays are not cross-validated within trials along with considerable variability in the FLT3-ITD cut-off used (0.5 in the ELN recommendations, 0.7 in the RATIFY study) for treatment, as well as the dynamic changes that happen to this ratio over time. Until standardization of definitions, the indication of ASCT remains itself controversial in patients with low (<0.5) allelic ratio *FLT3*-ITD who have

a concomitant *NPM1* mutation and achieve MRD negative status on therapy (Bazarbachi et al, 2020).[47]

IDH1/IDH2 Inhibitors
Ivosidenib and enasidenib have been recently approved for the treatment of *IDH1* and *IDH2*-mutated AML, respectively.[75,76] Owing to the natural history of this subtype of AML and the relative safety of these agents, they could present as a promising option for maintenance therapy post-ASCT. Some trials (NCT03515512, NCT03564821) are currently evaluating the significance of these mutations and their role in post-ASCT relapses, as well as the safety of the corresponding targeted agents in this setting.

BCL2 Inhibitors
Venetoclax is a BCL2 inhibitor that competitively binds to the BH3 domain of BCL2, an anti-apoptotic protein, releases BH3-only proteins and induces apoptosis of hematologic malignant cells.[77] Venetoclax has been evaluated and is currently approved in combination with low-dose cytarabine and azacitidine or decitabine.[78,79] These studies have included only a few patients who relapsed after ASCT and still achieved CR with the combination. Two prospective trials investigating the efficacy of venetoclax in combination with azacitidine at improving RFS are currently enrolling AML patients for maintenance or preemptive therapy post-ASCT.

Hedgehog (Hh) Pathway Signaling Inhibitors
Anomalous hedgehog (Hh) pathway signaling is involved in the survival and proliferation of leukemia stem cells,[80] especially those resistant to chemotherapy.[81] Glasdegib, an oral small Hh inhibitor, has been recently FDA approved in combination with low-dose cytarabine for the treatment of AML patients not eligible for intensive therapy, after showing OS benefit.[82] Based on these findings, glasdegib is currently being evaluated in a phase II study for post-ASCT maintenance for AML patients at high-risk of relapse (NCT01841333).

Agents Targeting Mutated P53
AML and MDS with abnormal 17p or mutated *p53* are known to portend dismal outcomes with the highest risk of relapse even in the post-ASCT phase.[83] APR-246 is an agent that targets *p53* mutation in an attempt to restore its function and showed up to 80% CR rate in an early trial of patients with myeloid malignancies.[84] Based on this concept, a phase II trial studying the combination of azacitidine and APR-246 is currently enrolling allografted patients with MDS and

AML and mutated p53 (NCT03931291) with a primary endpoint being 1-year RFS.

Targeting Leukemic Surface Receptors
Monoclonal Antibodies

The use of antibody-drug conjugates (ADC) could achieve target specificity through inhibition of certain surface markers, such as CD33, expressed on the majority of myeloblasts. Gemtuzumab ozogamicin (GO) is a MoAb against CD33 conjugated to the toxin calicheamicin. In a small study of 10 relatively young patients allografted for high-risk AML, GO was administered with azacitidine as maintenance post-ASCT.[85] After a median number of 1.5 cycles only complicated by reversible hematological toxicities, 40% of patients relapsed.

Another newer generation anti-CD33 ADC Vadastuximab talirine (SGN33a) conjugated to a pyrrolobenzodiazepine dimer was studied as maintenance in the post-ASCT setting (NCT02326584), but the phase I/II trial was terminated early because of neutropenia and thrombocytopenia.

Immune-Mediated Therapies

Maintenance therapy with immune checkpoint inhibitors, such as nivolumab, is being investigated in clinical trials for patients with high-risk AML in remission post-consolidation, who are not candidates for ASCT.[86] For instance, using this selective immune modulation for post-ASCT maintenance may provide similar benefits and merits investigation owing to their inherent activity in AML. Nonetheless, issues related to acute GVHD are likely to emerge, as seen with previous studies of lenalidomide in this setting,[87] thereby limiting the wide adoption of these agents.[88–90]

Other agents on the outlook in this setting include anti-chemokine (C-X-C motif) receptor 4 (CXCR4) as well as CAR T-cell therapy.

Our Personalized Approach for Post-ASCT Maintenance

AML has increasingly presented itself as a poster child for personalized treatment approaches. ASCT by itself should not be regarded as an ultimate definitive therapy for all patients and with established poor outcomes for post-ASCT relapses, preventing one remains more beneficial than treating it. Nonetheless, we still have no simple algorithm or strategy to address post-ASCT relapses or

maintenance approaches. As delineated above, most available information is derived from phase II trials of HMAs and FTL3-ITD TKIs and few randomized data. Recent development of targeted agents made their use in the post-transplant setting more exciting taking into consideration the potential risks on GVHD and immune reconstitution post-ASCT. Furthermore, better MRD assessments facilitated the optimal selection of high-risk candidates who would benefit from such strategies.

Any treatment decision should therefore involve the patient's performance status, the pre-transplant disease course, the presence of actionable mutations, and the use of concurrent immunosuppressive medications as well as GVHD. Prognostication of high-risk AML patients has been recently refined, especially with the introduction of various MRD assays. These include MFC[5,91] and NGS-MRD monitoring, both shown to be predictive for post-transplant relapse and survival.[92,93]

In our clinical practice, we utilize patient and disease characteristics coupled with pre- and post-transplant MRD assays as metrics to counsel patients about their risk of relapse. Awaiting further validation, we believe these are useful parameters, especially when conjugated to risk-stratified maintenance approaches. Nonetheless, we recommend the use of off-label FLT3-TKIs such as sorafenib because of our favorable experience and the accumulating data with this regard, which led to the EBMT recommendations (Bazarbachi et al, 2020).[47] HMAs still represent a cornerstone maneuver to upregulate neoantigens and modulate immune responses post-ASCT when used alone or in various upcoming combinations (HMA+ DLI or venetoclax, etc.). One would, however, ask if pre-transplant therapy matters in this setting and whether responding favorably or not to azacitidine as initial therapy could affect the outcomes of post-ASCT maintenance. Novel agents such as ADCs and BCL2-inhibitors may provide a favorable approach despite little knowledge about the effect of these molecules on the graft and their potential toxicities. Immune stimulation with agents such as ICPs currently remains investigational awaiting well-designed clinical trials. Additionally, we must continue to explore the genetic profiling of AML and its ramifications.

Future Challenges and Directions

Disease relapse remains a paramount endpoint to treating physicians and patients, far beyond the use of survival endpoints alone based on small single-center trials. With the recent surge of therapeutic opportunities, the priority should

be to tailor randomized trials with refined conditioning regimens to post-transplant strategies while routinely incorporating MRD and genomic assays. This will require a solid partnership between the transplant community, academia and the pharmaceutical institutions for innovative and well-integrated approaches. A model trial in this setting also needs to assess the activity of a certain approach and its effect on GVHD. There is a steadily increasing number of novel agents, mostly of oral bioavailability, which could be preferred for maintenance therapy owing to their activity, dosing schedules, as well as minimal hematological toxicities. Other areas of interest include the use of MoAbs, ICP inhibitors and possibly products of cellular engineering (vaccines, modified chimeric antigen receptor T-cells, etc.). As a reflection of toxicities, we strongly support the integration of quality-of-life (QoL) metrics and patient-reported outcomes as informative endpoints in the design of these prospective randomized trials.

Disclosure

The authors report no conflicts of interest in this work.

References

1. The Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368:2059–2207. doi:10.1056/NEJMoa1301689
2. Döhner H, Estey E, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*. 2010;115(3):453–474.
3. Burnett A, Russell N, Hills R, et al. A randomized comparison of daunorubicin 90 mg/m2 vs 60 mg/m2 in AML induction: results from the UK NCRI AML17 trial in 1206 patients. *Blood*. 2015;125 (25):3878–3885. doi:10.1182/blood-2015-01-623447
4. Marcucci G, Haferlach T, Döhner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol*. 2011;29(5):475–486. doi:10.1200/JCO.2010.30.2554
5. Terwijn M, van Putten W, Kelder A, van der Velden V, Brooimans R, Pabst T. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/ SAKK AML 42A study. *J Clin Oncol*. 2013;31(31):3889–3897. doi:10.1200/JCO.2012.45.9628
6. Ivey A, Hills R, Simpson MA, et al. Assessment of minimal residual disease in standard- risk AML. *N Engl J Med*. 2016;374:422–433. doi:10.1056/NEJMoa1507471
7. Freeman S, Hills R, Virgo P, et al. Measurable residual disease at induction redefines partial response in acute myeloid leukemia and stratifies outcomes in patients at standard risk without NPM1 mutations. *J Clin Oncol*. 2018;36(15):1486–1497. doi:10.1200/JCO.2017.76.3425
8. Wingard J, Majhail N, Brazauskas R, et al. Long-term survival and late deaths after allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2011;29(16):2230–2239. doi:10.1200/JCO.2010.33.7212
9. Bejanyan N, Weisdorf D, Logan B, et al. Survival of patients with acute myeloid leukemia relapsing after allogeneic hematopoietic cell transplantation: a center for International Blood and Marrow Transplant Research Study. *Biol Blood Marrow Transplant*. 2015;21 (3):454–459. doi:10.1016/j.bbmt.2014.11.007

10. Ganzel C, Sun Z, Cripe L, et al. Very poor long-term survival in past and more recent studies for relapsed AML patients: the ECOG-ACRIN experience. *Am J Hematol*. 2018;93:1074–1081. doi:10.1002/ajh.25162
11. Thanarajasingam G, Kim H, Cutler C, et al. Outcome and prognostic factors for patients who relapse after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2013;19 (12):1713–1718. doi:10.1016/j.bbmt.2013.09.011
12. Bazarbachi A, Schmid C, Labopin M, et al. Evaluation of trends and prognosis over time in patients with AML relapsing after allogeneic hematopoietic cell transplant reveals improved survival for young patients in recent years. *Clin Cancer Res*. 2020b;3134.
13. Craddock C. Pharmacological methods to reduce disease recurrence. *Hematology Am Soc Hematol Educ Program*. 2013;2013:63–69. doi:10.1182/asheducation-2013.1.63
14. Choi J, Ritchey J, Prior J, et al. In vivo administration of hypomethylating agents mitigate graft-versus host disease without sacrificing graft-versus-leukemia. *Blood*. 2010;116(1):129–139. doi:10.1182/ blood-2009-12-257253
15. Goodyear O, Dennis M, Jilani N, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood*. 2012;119 (14):3361–3369. doi:10.1182/blood-2011-09-377044
16. Mathew N, Baumgartner F, Braun L, et al. Sorafenib promotes graft-versus-leukemia activity in mice and humans through IL-15 production in FLT3-ITD-mutant leukemia cells. *Nat Med*. 2018;24 (3):282–291. doi:10.1038/nm.4484
17. Armand P, Kim H, Zhang M, et al. Classifying cytogenetics in patients with acute myelogenous leukemia in complete remission undergoing allogeneic transplantation: a Center for International Blood and Marrow Transplant Research study. *Biol Blood Marrow Transplant*. 2012;18(2):280–288. doi:10.1016/j. bbmt.2011.07.024
18. Schlenk R, Döhner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358:1909–1918. doi:10.1056/NEJMoa074306
19. Walter R, Buckley S, Pagel J, et al. Significance of minimal residual disease before myeloablative allogeneic hematopoietic cell transplantation for AML in first and second complete remission. *Blood*. 2013;122(10):1813–1821. doi:10.1182/blood-2013-06-506725
20. Festuccia M, Deeg H, Gooley T, et al. Minimal identifiable disease and the role of conditioning intensity in hematopoietic cell transplantation for myelodysplastic syndrome and acute myelogenous leukemia evolving from myelodysplastic syndrome. *Biol Blood Marrow Transplant*. 2016;22(7):1227–1233. doi:10.1016/j.bbmt.2016.03.029
21. Milano F, Gooley T, Wood B, et al. Cord blood transplant in patients with minimal residual disease. *N Engl J Med*. 2016;375:944–953. doi:10.1056/NEJMoa1602074
22. D'Souza A, Lee S, Zhu X, Pasquini M. Current use and trends in hematopoietic cell transplantation in the United States. *Biol Blood Marrow Transplant*. 2017;23(9):1417–1421. doi:10.1016/j.bbmt.2017.05.035
23. Scott B, Pasquini M, Logan B, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35 (11):1154–1161. doi:10.1200/JCO.2016.70.7091
24. Cruijsen M, Hobo W, van der Velden W, et al. Addition of 10-day decitabine to fludarabine/total body irradiation conditioning is feasible and induces tumor-associated antigen specific T cell responses. *Biol Blood Marrow Transplant*. 2016;22(6):1000–1008. doi:10.1016/j.bbmt.2016.02.003
25. Sánchez-Abarca L, Gutierrez-Cosio S, Santamaría C, et al. Immunomodulatory effect of 5-azacytidine (5-azaC): potential role in the transplantation setting. *Blood*. 2010;115(1):107–121. doi:10.1182/blood-2009-03-210393
26. Platzbecker U, Wermke M, Radke J, et al. Azacitidine for treatment of imminent relapse in MDS or AML patients after allogeneic HSCT: results of the RELAZA trial. *Leukemia*. 2012;26(3):381–389. doi:10.1038/leu.2011.234

27. Craddock C, Jilani N, Siddique S, et al. Tolerability and clinical activity of posttransplantation Azacitidine in patients allografted for acute myeloid leukemia treated on the RICAZA trial. *Biol Blood Marrow Transplant.* 2016;22(2):385–390. doi:10.1016/j.bbmt.2015.09.004

28. Guillaume T, Florent Malard F, Leonardo Magro L, et al. Prospective phase II study of prophylactic low-dose azacitidine and donor lymphocyte infusions following allogeneic hematopoietic stem cell transplantation for high-risk acute myeloid leukemia and myelodysplastic syndrome. *Bone Marrow Transplant.* 2019;54(11):1815–1826. doi:10.1038/s41409-019-0536-y

29. de Lima M, Giralt S, Thall P, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome. *Cancer.* 2010;116(23):5420–5431. doi:10.1002/cncr.25500

30. El-Cheikh J, Massoud R, Fares E, et al. Low-dose 5-azacytidine as preventive therapy for relapse of AML and MDS following allogeneic HCT. *Bone Marrow Transplant.* 2017;52(6):918–921. doi:10.1038/bmt.2017.31

31. Oran B, de Lima M, Garcia-Manero G, et al. Maintenance with 5-azacytidine for acute myeloid leukemia and myelodysplastic syndrome patients. *Blood.* 2018;132(Supplement 1):971. doi:10.1182/blood-2018-99-111582

32. Platzbecker U, Middeke J, Sockel K, et al. Minimal-residual disease guided treatment with azacitidine in MDS/AML patients at imminent risk of relapse: results of the prospective RELAZA2 trial. *Blood.* 2017;130(Supplement 1):565.

33. Wei AH, Döhner H, Pocock C, et al. The QUAZAR AML-001 maintenance trial: results of a phase III international, randomized, double-blind, placebo-controlled study of CC-486 (oral formulation of azacitidine) in patients with acute myeloid leukemia (AML) in first remission. *Blood.* 2019;134(suppl2):LBA–3. doi:10.1182/blood-2019-132405

34. de Lima M, Oran B, Champlin R, et al. CC-486 maintenance after stem cell transplantation in patients with acute myeloid leukemia or myelodysplastic syndromes. *Biol Blood Marrow Transplant.* 2018;24 (10):2017–2024. doi:10.1016/j.bbmt.2018.06.016

35. Pusic I, Choi J, Fiala M, et al. Maintenance therapy with decitabine after allogeneic stem cell transplantation for acute myelogenous leukemia and myelodysplastic syndrome. *Bone Marrow Transplant.* 2015;21(10):1761–1769. doi:10.1016/j.bbmt.2015.05.026

36. Platzbecker U, Middeke J, Sockel K, et al. Measurable residual disease-guided treatment with azacitidine to prevent haematological relapse in patients with myelodysplastic syndrome and acute myeloid leukaemia (RELAZA2): an open-label, multicentre, Phase 2 trial. *Lancet Oncol.* 2018;19(12):1668–1679. doi:10.1016/S1470-2045(18)30580-1

37. Stahl M, Gore S, Vey N, Prebet T. Lost in translation? Ten years of development of histone deacetylase inhibitors in acute myeloid leukemia and myelodysplastic syndromes. *Expert Opin Investig Drugs.* 2016;25(3):307–317. doi:10.1517/13543784.2016.1146251

38. Bug G, Burchert A, Wagner E-M, et al. Phase I/II study of the deacetylase inhibitor panobinostat after allogeneic stem cell transplantation in patients with high-risk MDS or AML (PANOBEST trial). *Leukemia.* 2017;31(11):2523–2525. doi:10.1038/leu.2017.242

39. Shen L, Pili R. Class I histone deacetylase inhibition is a novel mechanism to target regulatory T cells in immunotherapy. *Oncoimmunology.* 2012;1(6):948–950. doi:10.4161/onci.20306

40. Brunet S, Labopin M, Esteve J, et al. Impact of FLT3 internal tandem duplication on the outcome of related and unrelated hematopoietic transplantation for adult acute myeloid leukemia in first remission: a retrospective analysis. *J Clin Oncol.* 2012;30(7):735–741. doi:10.1200/JCO.2011.36.9868

41. Kayser S, Dohner K, Krauter J, et al. Impact of allogeneic transplantation from matched related and unrelated donors on clinical outcome in younger adult AML patients with FLT3 internal tandem duplications. *Blood.* 2010;116(21):909. doi:10.1182/blood.V116.21.909.909

42. Sengsayadeth S, Jagasia M, Engelhardt B, et al. Allo-SCT for high-risk AML-CR1 in the molecular era: impact of FLT3/ITD outweighs the conventional markers. *Bone Marrow Transplant.* 2012;47 (12):1535–1537. doi:10.1038/bmt.2012.88

43. Deol A, Sengsayadeth S, Ahn K, et al. Does FLT3 mutation impact survival after hematopoietic stem cell transplantation for acute myeloid leukemia? A Center for International Blood and Marrow Transplant Research (CIBMTR) analysis. *Cancer.* 2016;122 (19):3005–3014. doi:10.1002/cncr.30140

44. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood.* 2017;129(4):424–447.

45. Sakaguchi M, Yamaguchi H, Najima Y, et al. Prognostic impact of low allelic ratio FLT3-ITD and NPM1 mutation in acute myeloid leukemia. *Blood Adv.* 2018;2(20):2744–2754. doi:10.1182/bloodadvances.2018020305

46. Straube J, Ling V, Hill G, Lane S. The impact of age, NPM1mut, and FLT3ITD allelic ratio in patients with acute myeloid leukemia. *Blood.* 2018;131(10):1148–1153. doi:10.1182/blood-2017-09-807438

47. Bazarbachi A, Bug G, Baron F, et al. Clinical practice recommendation on hematopoietic stem cell transplantation for acute myeloid leukemia patients with FLT3-internal tandem duplication: a position statement from the Acute Leukemia Working Party of the European Society for Blood and Marrow. *Haematologica.* 2020a;105 (6):1507–1516. doi:10.3324/haematol.2019.243410

48. Schuurhuis G, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood.* 2018;131(12):1275–1291. doi:10.1182/blood-2017-09-801498

49. Antar, A., Kharfan-Dabaja, M, Mahfouz, R, & Bazarbachi, A. Sorafenib maintenance appears safe and improves clinical outcomes in FLT3-ITD acute myeloid leukemia after allogeneic hematopoietic cell transplantation. *Clin Lymphoma Myeloma Leuk.* 2015;15 (5):298–302.

50. Borthakur G, Kantarjian H, Farhad Ravandi F, et al. Phase I study of sorafenib in patients with refractory or relapsed acute leukemias. *Haematologica.* 2011;96(1):62–68. doi:10.3324/haematol.2010.030452

51. Brunner A, Li S, Fathi A, et al. Haematopoietic cell transplantation with and without sorafenib maintenance for patients with FLT3-ITD acute myeloid leukaemia in first complete remission. *Br J Haematol.* 2016;175(3):496–504. doi:10.1111/bjh.14260

52. Battipaglia G, Ruggeri A, Massoud R, et al. Efficacy and feasibility of sorafenib as a maintenance agent after allogeneic hematopoietic stem cell transplantation for Fms-like tyrosine kinase 3-mutated acute myeloid leukemia. *Cancer.* 2017;123(15):2867–2874. doi:10.1002/cncr.30680

53. Battipaglia G, Massoud R, Ahmed S, et al. Efficacy and feasibility of sorafenib as a maintenance agent after allogeneic hematopoietic stem cell transplantation for Fms-like tyrosine kinase 3 mutated acute myeloid leukemia: an update. *Clin Lymphoma Myeloma Leuk.* 2019;19(8):506–508. doi:10.1016/j.clml.2019.04.004

54. Chen Y, Li S, Lane A, et al. Phase I trial of maintenance sorafenib after allogeneic hematopoietic stem cell transplantation for fms like tyrosine kinase 3 internal tandem duplication acute myeloid leukemia. *Biol Blood Marrow Transplant.* 2014;20(12):2042–2048. doi:10.1016/j.bbmt.2014.09.007

55. Sammons S, Pratz K, Smith B, Karp J, Emadi A. Sorafenib is tolerable and improves clinical outcomes in patients with FLT3-ITD acute myeloid leukemia prior to stem cell transplant and after relapse post-transplant. *Am J Hematol.* 2014;89(9):936–938. doi:10.1002/ajh.23782

56. Antar A, Kharfan-Dabaja M, Mahfouz R, Bazarbachi A. Sorafenib maintenance appears safe and improves clinical outcomes in FLT3-ITD acute myeloid leukemia after allogeneic hematopoietic cell transplantation. *Clin Lymphoma Myeloma Leuk.* 2015;15 (5):298–302. doi:10.1016/j.clml.2014.12.005

57. Pratz K, Rudek M, Smith B, et al. Prospective study of peri-transplant use of sorafenib as remission maintenance for FLT3-ITD patients undergoing allogeneic transplantation. *Biol Blood Marrow Transplant*. 2020;26(2):300–306. doi:10.1016/j.bbmt.2019.09.023

58. Bazarbachi A, Myriam Labopin M, Battipaglia G, et al. Sorafenib improves survival of FLT3-mutated acute myeloid leukemia in relapse after allogeneic stem cell transplantation: a report of the EBMT Acute Leukemia Working Party. *Haematologica*. 2019;104 (9):e398–e401. doi:10.3324/haematol.2018.211615

59. Bazarbachi A, Lab M, Battipaglia G, et al. Allogeneic stem cell transplantation for FLT3-mutated acute myeloid leukemia: in vivo T-cell depletion and posttransplant sorafenib maintenance improve survival. A Retrospective Acute Leukemia Working Party-European Society for Blood and Marrow Transplant. *Clinical Hematology International*. 2019:58–74. doi:10.2991/chi.d.190310.001

60. Burchert A, Bug G, Fritz L, et al. Sorafenib maintenance after allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia with FLT3–internal tandem duplication mutation (SORMAIN). *J Clin Oncol*. 2020;38(26):2993–3002. doi:10.1200/JCO.19.03345

61. Xuan L, Wang Y, Huang F, et al. Sorafenib maintenance in patients with FLT3-ITD acute myeloid leukaemia undergoing allogeneic hae-matopoietic stem-cell transplantation: an open-label, multicentre, randomised phase 3 trial. *Lancet Oncol*. 2020 Sep;21(9):1201–1212.

62. Stone R, Mandrekar S, Sanford B, Laumann K, Geyer S, Bloomfield C. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Med*. 2017;377:454–464. doi:10.1056/NEJMoa1614359

63. Schlenk R, Weber D, Fiedler W, Salih H, Wulf G, Salwender H. Midostaurin added to chemotherapy and continued single-agent maintenance therapy in acute myeloid leukemia with FLT3-ITD. *Blood*. 2019;133(8):840–851. doi:10.1182/blood-2018-08-869453

64. Levis M, Brown P, Smith B, Stine A, Pham R, Stone R. Plasma inhibitory activity (PIA): a pharmacodynamic assay reveals insights into the basis for cytotoxic response to FLT3 inhibitors. *Blood*. 2006;108(10):3477–3483. doi:10.1182/blood-2006-04-015743

65. Strati P, Kantarjian H, Ravandi F, Nazha A, Borthakur G, Daver N. Phase I/II trial of the combination of midostaurin (PKC412) and 5-azacytidine for patients with acute myeloid leukemia and myelo-dysplastic syndrome. *Am J Hematol*. 2015;90(4):276–281. doi:10.1002/ajh.23924

66. Maziarz R, Patnaik M, Scott B, Mohan S, Deol A, Rowley S. A phase 2 randomized trial investigating standard of care 6 midos-taurin after allogeneic stem cell-transplant in FLT3-ITD–mutated AML. *Blood*. 2018;132(Supplement 1):662. doi:10.1182/blood-2018-99-113582

67. Smith C, Wang Q, Chin C, Salerno S, Damon L, Levis M. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. *Nature*. 2012;485(7397):260–263. doi:10.1038/nature11016

68. Perl A, Altman J, Cortes J, Smith C, Litzow M, Baer M. Selective inhibition of FLT3 by gilteritinib in relapsed or refractory acute myeloid leukaemia: A multicentre, first-in-human, open-label, Phase 1–2 study. *Lancet Oncol*. 2017;18(8):1061–1075. doi:10.1016/S1470-2045(17)30416-3

69. Sandmaier B, Khaled S, Betul Oran B, Gammon G, Trone D, Frankfurt O. Results of a phase 1 study of quizartinib (AC220) as maintenance therapy in subjects with acute myeloid leukemia in remission following allogeneic hematopoietic cell transplantation. *Blood*. 2014;124(21):428. doi:10.1182/blood.V124.21.428.428

70. Smith C, Paguirigan A, Jeschke G, Lin K, Massi E, Theodore Tarver T. Heterogeneous resistance to quizartinib in acute myeloid leukemia revealed by single-cell analysis. *Blood*. 2017;130(1):48–58. doi:10.1182/blood-2016-04-711820

71. Galanis A, Ma H, Rajkhowa T, Ramachandran A, Small D, Cortes J. Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. *Blood*. 2014;123(1):94–100. doi:10.1182/blood-2013-10-529313

72. Heisterkamp N, Jenster G, Hoeve J, Zovich D, Pattengale P, Groffen J. Acute leukaemia in bcr/abl transgenic mice. *Nature*. 1990;344(6263):251–253. doi:10.1038/344251a0

73. Li L, Piloto O, Nguyen H, Greenberg K, Takamiya K, Racke F. Knock-in of an internal tandem duplication mutation into murine FLT3 confers myeloproliferative disease in a mouse model. *Blood*. 2008;111(7):3849–3858. doi:10.1182/blood-2007-08-109942

74. Jan M, Snyder T, Corces-Zimmerman M, et al. Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci Transl Med*. 2012;4(149):149ra118. doi:10.1126/scitranslmed.3004315

75. Roboz G, DiNardo C, Stein E, de Botton S, Mims A, Prince G. Ivosidenib induces deep durable remissions in patients with newly diagnosed IDH1-mutant acute myeloid leukemia. *Blood*. 2020;135 (7):463–471. doi:10.1182/blood.2019002140

76. Stein E, DiNardo C, Pollyea D, Fathi A, Roboz G, Altman J. Enasidenib in mutant IDH2 relapsed for refractory AML. *Blood*. 2017;130(6):722–731. doi:10.1182/blood-2017-04-779405

77. Uchida A, Isobe Y, Asano J, Uemura Y, Hoshikawa M, Takagi M. Targeting BCL2 with venetoclax is a promising therapeutic strategy for "double-proteinexpression" lymphoma with MYC and BCL2 rearrangements. *Haematologica*. 2019;104(7):1417–1421. doi:10.3324/haematol.2018.204958

78. Pollyea D, Stevens B, Jones C, Winters A, Pei S, Minhajuddin M. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med*. 2018;24(12):1859–1866. doi:10.1038/s41591-018-0233-1

79. DiNardo C, Wei A. How I treat acute myeloid leukemia in the era of new drugs. *Blood*. 2020;135(2):85–96. doi:10.1182/blood.2019001239

80. Fukushima N, Minami Y, Kakiuchi S, Kuwatsuka Y, Hayakawa F, Jamieson C. Small-molecule Hedgehog inhibitor attenuates the leukemia-initiation potential of acute myeloid leu-kemia cells. *Cancer Sci*. 2016;107(10):1422–1429. doi:10.1111/cas.13019

81. Terao T, Minami Y. Targeting hedgehog (Hh) pathway for the acute myeloid leukemia treatment. *Cells*. 2019;8(4):312. doi:10.3390/cells8040312

82. Cortes J, Heidel F, Hellmann A, et al. Randomized comparison of low dose cytarabine with or without glasdegib in patients with newly diagnosed acute myeloid leukemia or high-risk myelodysplastic syndrome. *Leukemia*. 2019;33(2):379–389. doi:10.1038/s41375-018-0312-9

83. Middeke J, Fang M, Cornelissen J, Mohr B, Appelbaum F, Stadler M. Outcome of patients with abnl(17p) acute myeloid leukemia after allogeneic hematopoietic stem cell transplantation. *Blood*. 2014;123 (19):2960–2967. doi:10.1182/blood-2013-12-544957

84. Sallman D, DeZern A, Garcia-Manero G, Steensma D, Roboz G, Sekeres M. Phase 2 results of APR-246 and azacitidine (AZA) in patients with TP53 mutant myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukemia (AML). *Blood*. 2019;134 (Supplement_1):676. doi:10.1182/blood-2019-131055

85. Oshikawa G, Kakihana K, Saito M, Aoki J, Najima Y, Kobayashi T. Post-transplant maintenance therapy with azacitidine and gemtuzu-mab ozogamicin for high-risk acute myeloid leukemia. *Br J Haematol*. 2015;169(5):756–759. doi:10.1111/bjh.13248

86. Kadia T, Cortes J, Ghorab A, Ravandi F, Jabbour E, Guastad N. Nivolumab (Nivo) maintenance (maint) in high-risk (HR) acute mye-loid leukemia (AML) patients. *J Clin Oncol*. 2018;36 (15_suppl):7014. doi:10.1200/JCO.2018.36.15_suppl.7014

87. Sockel K, Bornhaeuser M, Mischak-Weissinger E, Trenschel R, Wermke M, Unzicker C. Lenalidomide maintenance after allogeneic HSCT seems to trigger acute graft-versus-host disease in patients with high-risk myelodysplastic syndromes or acute myeloid leukemia and del(5q): results of the LENAMAINT trial. *Haematologica*. 2012;97(9):e34–5. doi:10.3324/haematol.2012.067629

88. Bashey A, Medina B, Corringham S, Pasek M, Carrier E, Vrooman L. CTLA4 blockade with ipilimumab to treat relapse of malignancy after allogeneic hematopoietic cell transplantation. *Blood*. 2009;113(7):1581–1588. doi:10.1182/blood-2008-07-168468

89. Davids M, Kim H, Bachireddy P, Costello C, Liguori R, Savell A. Ipilimumab for patients with relapse after allogeneic transplantation. *N Engl J Med*. 2016;375(2):143–153. doi:10.1056/NEJMoa1601202

90. Albring J, Inselmann S, Sauer T, Schliemann C, Altvater B, Kailayangiri S. PD-1 checkpoint blockade in patients with relapsed AML after allogeneic stem cell transplantation. *Bone Marrow Transplant*. 2017;52(2):317–320. doi:10.1038/bmt.2016.274

91. Walter R, Gooley T, Wood B, Milano F, Fang M, Sorror M. Impact of pretransplantation minimal residual disease, as detected by multiparametric flow cytometry, on outcome of myeloablative hematopoietic cell transplantation for acute myeloid leukemia. *J Clin Oncol*. 2011;29(9):1190–1197. doi:10.1200/JCO.2010. 31.8121

92. Thol F, Gabdoulline R, Liebich A, Klement P, Schiller J, Kandziora C. Measurable residual disease monitoring by NGS before allogeneic hematopoietic cell transplantation in AML. *Blood*. 2018;132(16):1703–1713. doi:10.1182/blood-2018-02-829911

93. Kim T, Ho Moon J, Ahn J, Kim Y, Lee S, Ahn S. Next-generation sequencing-based posttransplant monitoring of acute myeloid leukemia identifies patients at high risk of relapse. *Blood*. 2018;132 (15):1604–1613. doi:10.1182/blood-2018-04-848028

Basic Coagulation Profiles and Platelet Parameters Among Adult Type 1 and Type 2 Diabetes Patients at Dessie Referral Hospital, Northeast Ethiopia: Comparative Cross-Sectional Study

Hussen Ebrahim, Fikir Asrie & Zegeye Getaneh

Published online: 27 Jan 2021.

Submit your article to this journal

Article views: 745

View related articles

View Crossmark data

Citing articles: 5 View citing articles

ORIGINAL RESEARCH

Basic Coagulation Profiles and Platelet Parameters Among Adult Type 1 and Type 2 Diabetes Patients at Dessie Referral Hospital, Northeast Ethiopia: Comparative Cross-Sectional Study

Hussen Ebrahim [iD] [1]
Fikir Asrie [2]
Zegeye Getaneh [iD] [2]

[1]Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Wollo University, Dessie, Ethiopia; [2]Department of Hematology & Immunohematology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia

Background: Diabetes is a heterogeneous group of metabolic disorders characterized by hyperglycemia. The disease is highly associated with micro-vascular and macro-vascular complications. Thus, the main aim of this study was to compare basic coagulation profiles and platelet parameters among type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and healthy controls.

Methods: A comparative cross-sectional study was conducted at Dessie Referral Hospital from February to April 2019. A total of 180 study participants consisting of (60 T1DM, 60 T2DM, and 60 healthy controls) were enrolled using a systematic random sampling technique. Basic coagulation profiles and platelet parameters were determined using the HUMACLOT JUNIOR coagulometer and DIRUI BF 6500 automated hematology analyzer respectively. Non-parametric Kruskal–Wallis test supplemented with Dunn-Bonferroni correction and Spearman rank-order correlation test were used to compare basic coagulation profiles and platelet parameters among the groups. The test result was expressed in median and interquartile range and presented in texts and tables. P-value < 0.05 was considered to be statistically significant.

Results: Prothrombin time (PT) and international normalization ratio (INR) were significantly reduced in T2DM as compared to T1DM and healthy controls (p <0.05). Platelet distribution width (PDW) and mean platelet volume (MPV) were significantly increased in both T1DM and T2DM as compared to healthy controls (p <0.05). Moreover, PT and INR were negatively correlated with fasting blood glucose (FBG) among T1DM and PT, INR and activated partial thromboplastin time (APTT) were negatively correlated with FBG among T2DM.

Conclusion: Basic coagulation profiles and platelet parameters were significantly different between diabetes and controls where PT and INR in T2DM were significantly reduced as compared to T1DM and controls. However, PDW and MPV were significantly elevated in both T1DM and T2DM as compared to controls. Moreover, FBG was significantly negatively correlated with PT and INR among T1DM and FBG was significantly negatively correlated with PT, INR, and APTT among T2DM. Therefore, T2DM may be related to increased risk of thrombosis indicated by reduced PT and INR and high PDW and MPV than T1DM and controls. Basic coagulation profiles and platelet parameters should be regularly tested for early diagnosis and proper management of diabetes-related thrombosis.

Keywords: diabetes mellitus, basic coagulation profiles, platelet parameters, Dessie, Ethiopia

Correspondence: Hussen Ebrahim
Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Wollo University, Dessie 1145, Ethiopia
Tel +251921332772
Email husshosam@gmail.com

Background

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by hyperglycemia due to defects in insulin secretion, insulin action, or both. The disease is highly associated with micro-vascular and macro-vascular complications.[1,2] Diabetes can be diagnosed using laboratory test if one or more of the following criteria are met: If fasting plasma glucose level ≥7.0 mmol/l (126 mg/dl) or two-hour plasma glucose level ≥11.1 mmol/l (200 mg/dl) following a 75g oral glucose load or a random glucose level > 11.1 mmol/l (200 mg/dl) or HbA1c ≥ 48 mmol/l (equivalent to 6.5%).[1] Moreover, both T1DM and T2DM may be related to multiple consequences as glucose levels are associated with many physiological processes including lipid metabolism, the regulation of inflammation, vasodilatation, basic cell growth, and replication in uncontrolled diabetes.[3]

Diabetes is a risk factor for cardiovascular disease (CVD). Besides, some antecedent environmental and genetic factors precede the development of both diabetes and CVD. Independent risk factors including obesity, hypertension, toxins, and other genetic factors may be the possible risk factor and significantly related to glucose intolerance, hyperinsulinemia, dyslipidemia, insulin resistance syndrome, inflammation, thrombophilia, dysglycemia, oxidative stress, inflammation, endothelial dysfunction, and the generation of procoagulant and atherogenic lipoproteins which might be the common possible risk factors for both DM and CVD.[4–6]

The incidence and prevalence of DM rise quickly and becoming one of the most prevalent and costly chronic diseases worldwide.[1] International Diabetes Federation (IDF) reported in 2017, the number of people with DM was 424.9 million with an overall prevalence of 8.8%. In the same year, IDF reported an estimated 15.5 million adults aged 20–79 years were living with DM in the Africa region representing a regional prevalence of 3.3%.[1] Accelerated micro and macrovascular complications occurred in both types of diabetes. Moreover, both T1DM and T2DM are considered to be the risk factors for increased cardiovascular disease and stroke in unmanaged and poor glycemic control conditions.[7,8] Acute complications such as diabetic ketoacidosis, and hyperosmolar hyperglycemic state (HHS) and cardiovascular disease, stroke, chronic kidney failure, foot ulcers, impotence, and damage to the eyes commonly manifested as chronic complications of diabetes.[9]

Diabetes is significantly associated with metabolic and vascular disturbances.[10] An atherothrombotic disease that can affect the coronary, cerebral circulation and peripheral arteries are accelerated among T1DM and T2DM patient.[11,12] Atherothrombosis is the leading cause of morbidity and mortality in diabetes as it increases the risk of coronary heart disease, stroke, and peripheral arterial disease by 2- to 4-folds.[12] Eighty percent of the patient with DM died due to thrombotic complications and 75% of these death being due to cardiovascular complications.[11–14] The pathophysiological mechanisms related to the diabetic pro-thrombotic state are endothelial dysfunction, platelet hyperactivation, and increased activation of pro-thrombotic coagulation factors coupled with decreased fibrinolysis. Hyperglycemia and insulin deficiency in T1DM and hyperglycemia, IR, dyslipidemia, low-grade inflammation, oxidative stress, and other metabolic disorder in T2DM are the main contributors to the occurrence of diabetic pro-thrombotic conditions.[15]

Hyperglycemia, IR, dyslipidemia, hypertension, and the presence of excess free fatty acids may cause endothelial dysfunction through multiple mechanisms.[11] In most cases, hyperglycemia can directly influence the vulnerability of vascular endothelium by changing its glycocalyx layers thereby this process enhances platelet-endothelial cell adhesion and release of coagulation factors and therefore, triggers occlusive thrombus formation.[16,17] Besides, hyperglycemia and IR increase the accumulation of reactive oxygen species and reactive nitrogen species through the process of oxidative stress that can change the structure and functional activity of vascular endothelium. In general, hyperglycemia and IR could cause the disturbance between vasodilators and vasoconstrictors which increases the risk of atherosclerosis and thrombus formation.[11,18]

Hyperglycemia and IR in diabetes can change and up-regulate the gene expression pathway involving the coagulation protein as it may increase the generation of oxidative stress. Therefore, this process increases the synthesis of pro-thrombotic coagulation factors such as fibrinogen, tissue factor (TF) and factor VII (FVII), plasminogen activator inhibitor-1 (PAI-1), and other pathological pro-inflammatory cytokines. Moreover, hyperglycemia can trigger the formation of glycated fibrinogen through the glycation process resulted in the formation of fibrin clot which is dense in structure and resistant to fibrinolysis. Therefore, this change may increase the risk of atherothrombosis and CVD.[11,12,18]

In diabetes, hyperglycemia, insulin resistance, insulin deficiency, cellular abnormalities, metabolic disorder, inflammation, and oxidative stress are involved in dysregulation of several signaling pathways stimulating platelet enhanced adhesion, activation, and aggregation.[15] It is found that hyperglycemia causes platelet to become large and hyperactivated. Therefore, larger platelets release more prothrombotic factors such as thromboxane A_2.[19] In addition, markers of fibrinolysis are abnormal in people with metabolic syndrome, and fibrinolytic dysfunction is markedly increased.[20]

Different studies involved coagulation profiles and platelet parameters among diabetic patient-reported contrasting findings. Some studies reported that normal APTT and PT,[21,22] and some other studies reported that significantly prolonged APTT and PT among patients with T2DM[23,24] and some other related studies also reported that shortened APTT and PT among T2DM patient.[25,26] Activated partial thromboplastin time is a screening test for intrinsic and common pathways of coagulation and PT is a screening test for extrinsic and common pathways of coagulation systems standardized by using INR to normalize for the variable responsiveness of thromboplastin reagents.[27] Platelet count and platelet indices are important parameters that can reflect the number, size, and activity of platelets.[28] Basic coagulation tests and platelet parameters serve as important biomarkers to assess the coagulation factors and platelet parameters to predict the progression of cardiovascular and thrombosis complications among diabetic patients.

Methods and Materials
Study Design, Period, and Area
A comparative cross-sectional study was conducted from February to April 2019 at Dessie Referral Hospital, Northeast Ethiopia. The hospital provides emergency, antiretroviral therapy services, chronic care, surgical, dental, medical, pediatric, gynecologic, obstetric, and other services for more than 4 million clients.

Study Participants
The study was conducted on a total of 180 study participants. Study participants who had a history of known inherited bleeding disorders, hypertension, chronic renal disease, chronic liver disease, any history of malignancy, infectious diseases (human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV)),

those with the habit of smoking, regular chat chewing and alcohol drinker were excluded from the study. Moreover, pregnant and lactating mothers, patients receiving anticoagulant and antiplatelet agent therapy, and oral contraceptive users were also excluded from the study.

Sample Size Determination and Sampling Techniques
The rule of thumb that has been recommended by van Voorhis and Morgan, has been applied to determine the sample size.[29] Thus, a total of 180 study participants (60 T1DM, 60 T2DM, and 60 age and gender-matched controls) were enrolled in the study. A systematic random sampling technique was employed to recruit diabetic cases from Dessie referral hospital chronic care clinics and controls from Dessie Blood Bank.

Data Collection and Laboratory Analysis
Socio-demographic characteristics and clinical information of study participants were collected using a structured questionnaire. Blood sample was collected from the study participants following standard operating procedures (SOPs) by qualified laboratory personnel after informed consent has been obtained. Following an aseptic vein puncture, about 5.7 mL of fasting blood sample was collected with a sterile disposable syringe then; about 2.7 mL of the collected blood sample was dispensed into the test tube containing 0.3 mL of 3.2% trisodium citrate. Platelet-poor plasma (PPP) was obtained by centrifuging at 1500 g for 15 minutes. APTT, PT, and INR were determined using the HUMAN CLOT JUNIOR coagulation analyzer (Wiesbaden, Germany). First, 50 microliters (μL) of test PPP were warmed at 37°C for 5 minutes. At the same time, the APTT reagent and calcium chloride buffer solution were simultaneously incubated then, 50 μL of APTT reagents were added to the warmed platelet-poor plasma and then incubated at 37°C for 3 minutes followed by the addition of 50 μL pre-warmed calcium chloride buffer solution. Therefore, the analyzer read the clotting time of APTT and displayed the result in seconds. The PT reagent which contains lyophilized thromboplastin of rabbit brain was reconstituted by mixing one vial of thromboplastin reagent and one vial calcium chloride buffer solution followed by the vial stand undistributed for 30 minutes at room temperature then, the mixture incubated at 37°C for 10 minutes. One hundred μL of the test PPP was added into the test cuvette followed by incubation at 37°C for 3 minutes. Subsequently, 200 μL of the pre-warmed PT

reagent rapidly added and the time is taken for clot formation (in seconds) was recorded and at the same time, INR was calculated and displayed from PT output. For platelet analysis, about 3 mL of whole blood was dispensed into EDTA test tube and properly mixed. Platelet parameters were determined using DIRUI BF 6500 automated hematology analyzer (DIRUI INDUSTRIAL CO. LTD., P.R., CHINA).

Data Quality Management

Blood sample was collected and processed according to the SOPs. Samples were properly mixed and homogenized by inverting 8–10 times and safety procedures and specimen handling procedures were strictly followed. The performance of automated instruments was maintained by daily running of two-level controls (Normal and pathological) for coagulometer and daily background checking for hematology analyzer was conducted. Daily cleaning of automated analyzers and other equipment before leaving the laboratory was conducted. Training was given and daily regular supervision was conducted for data collectors.

Data Management and Analysis

Data were coded, entered, and cleaned using Epi Data 3.1 version and then exported to statistical package for social sciences (SPSS) version 21.0 (IBM Corporation, Armonk, NY, USA). The Kolmogorov–Smirnov test was conducted to check the normality of continuous variables. Levene's tests were conducted to check the homogeneity of variance. Kruskal–Wallis test supplemented with Dun-Bonferroni correction was used to compare the median of continuous variables. Spearman rank-order correlation test was used to determine the correlation between fasting blood glucose (FBG), PT, INR, APTT, platelet count, PDW, and MPV. The results were summarized using median and interquartile range (IQR) and presented using tables and texts. P-value < 0.05 was considered as statistically significant.

Results

Socio-Demographic Characteristics of Study Participants

In this study, a total of 180 adult study participants were included. The study participants were categorized into three groups; T1DM, T2DM, and healthy blood donors as a control, and 60 adult individuals were involved in

each study group. The median age of the study participants were 30, 34.5, and 31.0 years for the respective T1DM, T2DM, and control groups. From the total study participants, 95 of them (52.8%) were males (Table 1).

Clinical Characteristics of Study Participants on Diabetes

A total of 120 DM study participants comprised of 38 (63.3%) of T1DM and 33 (55%) of T2DM were below 5 years based on the duration of treatment (Table 2).

Basic coagulation abnormality was high in T2DM in which 11.7% of T1DM and 16.7% of T2DM had shortened PT whereas 53.3% of T1DM and 63.3% of T2DM had shortened APTT (Table 3).

Table 1 Socio-Demographic Distribution of Study Participants at Dessie Referral Hospital, Northeast Ethiopia from February to April 2019 (N=180)

Variables	Category	T1DM; N=60 n (%)	T2DM; N=60 n (%)	Controls; N=60 n (%)
Age	18–24	18 (30.0)	13 (21.7)	17 (28.3)
	25–34	17 (28.3)	17 (28.3)	21 (35.0)
	35–44	15 (25.0)	14 (23.3)	13 (21.7)
	≥45	10 (16.7)	16 (26.7)	9 (15.0)
Gender	Male	35 (58.3)	29 (48.3)	31 (51.7)
	Female	25 (41.7)	31 (51.7)	29 (48.3)
Residence	Urban	37 (61.7)	38 (63.3)	49 (81.7)
	Rural	23 (38.3)	22 (37.7)	11 (18.3)
Religion	Orthodox	12 (20.0)	20 (33.3)	20 (33.3)
	Muslim	46 (76.7)	37 (61.7)	34 (56.7)
	Protestant	2 (3.3)	3 (5.0)	6 (10.0)
Educational level	Illiterate	11 (18.3)	16 (26.7)	4 (6.7)
	Primary school	22 (36.7)	14 (23.3)	4 (6.7)
	Secondary school	12 (20.0)	15 (25)	10 (16.7)
	Diploma	12 (20.0)	11 (18.3)	23 (38.2)
	Degree and above	3 (5.0)	4 (6.7)	19 (31.7)
Marital status	Single	22 (36.7)	14 (23.3)	24 (40.0)
	Married	36 (60.0)	42 (70.0)	33 (55.0)
	Divorced	2 (3.3)	4 (6.7)	3 (5.0)

Abbreviations: T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

Table 2 Clinical Characteristics of T1DM and T2DM Study Participants at Dessie Referral Hospital, Northeast Ethiopia from February to April 2019 (N=120)

Variables	Category	T1DM; N=60 n (%)	T2DM; N=60 n (%)
Medication Regimen	Insulin	60(100)	–
	Metformin	-	37 (61.6)
	Glibenclamide	-	7 (11.7)
	Metformin and Glibenclamide	-	12 (20.0)
	Insulin and Oral hypoglycaemic Agents	-	4 (6.7)
Diabetic Ketoacidosis	Yes	4 (6.7)	-
	No	56 (93.3)	60 (100)
HHS	Yes	1 (1.7)	1 (1.7)
	No	59 (98.3)	59 (98.3)
Foot ulcer	Yes	-	3 (5.0)
	No	60 (100)	57 (95.0)
Visual Disturbance	Yes	-	4 (6.7)
	No	60 (100)	56 (93.3)
Neuropathy	Yes	1 (1.7)	2 (3.3)
	No	59 (98.3)	58 (96.7)
Duration of Treatment	0–5 years	38 (63.3)	33 (55.0)
	6–10 years	17 (28.3)	7 (28.3)
	> 10 years	5 (8.3)	10 (16.7)
Duration of DM	0–5 years	38 (63.3)	33 (55.0)
	6–10 years	17 (28.3)	7 (28.3)
	> 10 years	5 (8.3)	10 (16.7)

Abbreviations: IQR, interquartile range; HHS, hyperglycemic hyperosmolar state; FBG, fasting blood glucose.

Comparison of Basic Coagulation Profiles and Platelet Parameters Among T1DM, T2DM, and Healthy Controls

The non-parametric Kruskal–Wallis test was used to compare the median value among the groups. The finding of the Kruskal–Wallis test for PT, INR, PDW, and MPV showed a statistically significant median (IQR) difference among T1DM, T2DM, and healthy controls (p <0.05) (Table 4).

Multiple Comparisons of Basic Coagulation Profiles and Platelet Parameters Among T1DM, T2DM, and Healthy Controls

Dunn-Bonferroni correction was conducted for multiple comparisons between the groups. In multiple pairwise comparisons with Dunn-Bonferroni correction, the median and IQR of PT and INR among T2DM were the statistically significant difference as compared to T1DM and healthy controls (p<0.05). The median and IQR of PDW and MPV in both T1DM and T2DM showed a statistically significant difference as compared to healthy controls (p <0.05) (Table 5).

Correlation of FBG with Basic Coagulation Profiles and Platelet Parameters Among T1DM and T2DM

In this study, Spearman rank order correlation showed that there were statistically significant negative correlation between FBG, PT and INR in T1DM (rho= - 0.260, p =0.045), rho = −0.273, p =0.035)) respectively and there were statistically significant negative correlation between FBG, PT, INR and APTT in T2DM (rho = −0.399, P=0.002), rho =0.392, p=0.002), rho=−0.303, p=0.019)) (Table 6).

Discussion

Atherothrombotic disease is the leading cause of morbidity and mortality in patients with diabetes which is usually associated with both metabolic and vascular abnormalities.[10] Diabetes considered to be the independent risk factor for the development of atherosclerosis. Therefore, atherosclerosis is the main cause of macrovascular complications,[1,24] and therefore, causes increased platelet activation, activation of coagulation factors, and hypo fibrinolysis significantly associated with an increased risk of cardiovascular disease.[1,24–26]

In this study, PT and INR were significantly reduced in T2DM patients as compared to T1DM and healthy controls. This result was similar to the finding reported in India and Nigeria[30,31] where PT was reduced among T2DM and in Gahanna[26] PT and INR was found significantly reduced among T2DM as compared to controls (p<0.05). The possible reason for this might be due to the presence of different factors associated with hyperglycemia, IR, dyslipidemia, low-grade inflammation, and oxidative stress possibly the common contributors for endothelial dysfunction, platelet hyperactivation, and increased activation of prothrombotic coagulation factors.[15,18]

On contrary, a study conducted in Egypt[10] and Sudan[32] showed that PT between T2DM and controls had no significant difference. The reason for this

Table 3 Basic Coagulation Profiles and Platelet Parameters of Study Participants at Dessie Referral Hospital, Northeast Ethiopia from February to April 2019 (N=180)

Variables	Category	T1DM n (%)	T2DM n (%)	Controls n (%)	Reference Range
PT (sec.)	Shortened Normal prolonged	7 (11.7) 32 (53.3) 21 (35)	10 (16.7) 40 (66.7) 10 (16.7)	2 (3.3) 32 (53.3) 26 (43.3)	14–16 sec
INR	shortened Normal prolonged	5 (8.3) 38 (63.3) 17 (28.3)	5 (8.3) 47 (78.3) 8 (13.3)	0 (0) 40 (66.7) 20 (33.3)	0.8–1.2
APTT (sec.)	shortened Normal prolonged	32 (53.3) 28 (46.7) 0	38 (63.3) 21 (35) 1 (1.7)	32 (53.3) 28 (46.7) 0	24–36 sec
Platelet count (×10³/ul)	Low Normal high	2 (3.3) 56 (93.3) 2 (3.3)	3 (5) 56 (93.3) 1 (1.7)	0 60 (100) 0	150–400×10³/ul
PDW (%)	Low Normal high	8 (13.3) 29 (48.3) 23 (38.3)	12 (20) 33 (55) 15 (25)	17 (28.3) 41(68.3) 2 (3.3)	15–18 fl
MPV (fl)	Low Normal high	0 59 (98.3) 1(1.7)	1 (1.7) 58 (96.7) 1 (1.7)	0 60 (100) 0	7–13 fl

Abbreviations: PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international normalization ratio; PDW, platelet distribution width; MPV, mean platelet volume; ul, microliter; fl, femtoliter.

variation might be due to sample size variation, variation in study design, and the variation in the study population. On the other hand, a study finding in Nigeria showed PT was significantly elevated in T2DM as compared to controls.[23] This variation might be due to the presence of elevated levels of in vitro inhibitor of coagulation such as D-dimer, thrombin-antithrombin complex, and prothrombin activation fragment 1+2 which are markers of coagulation activation that may cause prolonged PT.[23] In this study, PT and INR revealed a statistically significant difference between T1DM and T2DM individuals. This variation might be due to T2DM have elevated levels of free fatty acids, elevated insulin levels accompanied with insulin resistance, and elevated levels of PAI-1 but reduced in T1DM, which can affect the regulation of many physiological processes, including alterations in fibrin clot parameters, endothelial dysfunction, atherosclerotic plaque formation and cardiac lipotoxicity.[3]

Table 4 Comparison of Coagulation Profiles (PT, INR, and APTT) and Platelet Parameters (Platelet Count, PDW, and MPV) Among Study Groups at Dessie Referral Hospital, Northeast Ethiopia from February to April 2019 (Kruskal–Wallis Test)

Variables	T1DM N=60 Median [IQR]	T2DM N=60 Median [IQR]	Controls N=60 Median [IQR]	P-value
PT (sec.)	13.2 (2.9)	12.5 (2.9)	13.9 (1.7)	0.001*
INR	1.13 (0.25)	1.03 (0.25)	1.15 (0.14)	0.001*
APTT (sec.)	24.4 (5.3)	23.1 (4.0)	23.4 (5.1)	0.212
Platelet count (×10³/ul)	242.0 (109)	264.5 (109)	277.0 (75)	0.127
PDW (%)	17.1 (2.5)	17.0 (2.5)	15.6 (1.0)	0.004*
MPV (fl)	10.2 (1.5)	10.3 (1.6)	9.7 (1.2)	<0.001*
FBG (mg/dl)	200 (200)	239.5 (149)	88.5 (12)	

Notes: *Significant at p<0.05, p-value <0.05 considered to statistically significant.

Table 5 Post Hoc Test with Dunn-Bonferroni Correction for Multiple Comparisons Among Study Groups at Dessie Referral Hospital, Northeast Ethiopia from February to April 2019 (Pairwise Multiple Comparisons)

Variables	Pairwise Multiple Comparisons (Dunn-Bonferroni Correction)		
	T1DM vs T2DM	T1DM vs Controls	T2DM vs Controls
PT (sec.)	0.033*	0.910	0.001*
INR	0.025*	0.982	0.001*
PDW (%)	0.711	<0.001*	<0.001*
MPV (fl)	1.00	0.007*	0.026*
FBG (mg/dl)	0.338	<0.001*	<0.001*

Notes: N=120, *significant at p<0.05, P-value <0.05 considered statistically significant.F

Table 6 Correlation of FBG with Basic Coagulation Profiles and Platelet Parameters Among T1DM and T2DM at Dessie Referral Hospital, Northeast Ethiopia from February to April 2019 (Spearman Rank-Order Correlation Test)

Group of Study Participant	Variables	Correlation Coefficient (Spearman Rho)	p-value
T1DM	PT	− 0.260	0.045*
	INR	−0.273	0.035*
	APTT	−0.158	0.227
	Platelet count	−0.086	0.512
	PDW	0.112	0.395
	MPV	0.156	0.234
T2DM	PT	−0.399	0.002*
	INR	−0.392	0.002*
	APTT	−0.303	0.019*
	Platelet count	−0.161	0.218
	PDW	0.150	0.252
	MPV	0.015	0.910

Notes: N=120, *significant at p<0.05, P-value <0.05 considered statistically significant.

In this study, platelet parameters (PDW and MPV) showed that there was a statistically significant increase among T1DM and T2DM as compared to healthy controls (P<0.05). This finding was in agreement with the studies done in India and Ethiopia which reported an increased in PDW and MPV in T2DM than controls, respectively.[32,33] Similarly, other studies conducted in Turkey and Nigeria reported that MPV was significantly increased in T2DM as compared to controls.[34–36] Moreover, this study was in concurrent finding in Poland reported that increased MPV and PDW in T1DM as compared to controls,[37] and a similar finding was reported in Nigeria where high MPV in T1DM as compared to controls.[38] The reason might be since hyperglycemia causes non-enzymatic glycation of proteins on the surface of the platelet thereby reduces membrane fluidity and its elasticity and increases its reactivity through direct osmotic effects on platelets. This results in osmotic swelling of platelets and may cause an increased rate of platelet turnover.[39] On the other hand, discordance finding was reported in China found that no significant PDW difference between T2DM and controls.[40] The reason for this variation might be due to variation in sample size and variation in a population.

The present study finding showed that FBG in T1DM was statistically negatively correlated with PT and INR and FBG in T2DM was negatively correlated with PT, INR, and APTT. This finding was in agreement with a study done in Indonesia that revealed a negative correlation between FBG and PT among T2DM.[41] The reason might be since hyperglycemia exerted a procoagulant effect indicated by the elevation of thrombin-antithrombin complexes, soluble tissue factor, increased levels of thromboxane A_2, VWF, FVIII, tissue plasminogen activator (TPA), fibrinogen, and increased the level of PAI. The differential effects of hyperglycemia and IR suggested that patients with hyperglycemia due to IR are especially susceptible to thrombotic events by concurrent insulin-induced impairment of fibrinolysis and glucose-induced activation of coagulation.[11,16] On the other hand, Nnenna et al, and Fadairo et al, in Nigeria reported that a positive correlation existed between FBG and APTT, PT, and INR in DM patients.[23,42] The reason for this variation might be due to variation in sample size and coagulation analyzer.

The present study showed that no significant correlation between FBG and APTT, platelet count, PDW, and MPV in T1DM and no significant correlation between FBG and platelet count, PDW, and MPV in T2DM. A similar finding was reported in Iran which found that no statistically significant correlation between FBG and platelet count in DM patients.[43] The contrary finding was reported in China which found a significant positive correlation between FBG and platelet count, PDW, and MPV in T2DM patients,[40] and in Nigeria reported a significant positive correlation between FBG and platelet count in T1DM.[38] The reason for this variation might be due to variation in the population, variation sample size, and variation in automated hematology analyzer.

Conclusion

In this study, basic coagulation profiles and platelet parameters were significantly different between diabetes and controls where PT and INR in T2DM were significantly reduced as compared to T1DM and controls. However, PDW and MPV were significantly elevated in both T1DM and T2DM as compared to controls. Moreover,FBG was significantly negatively correlated with PT and INR among T1DM and FBG was significantly negatively correlated with PT, INR, and APTT among T2DM. Therefore, T2DM may be related to increased risk of thrombosis indicated by reduced PT and INR and high PDW and MPV than T1DM and controls. Basic coagulation profiles and platelet parameters should be regularly tested for early diagnosis and proper management of diabetes-related thrombosis. A longitudinal study should be conducted to establish the cause-effect relationship between DM, coagulation profiles, and platelet parameters. Further study will be required to determine the association between   diabetes-related complications, coagulation profiles, and platelet parameters.

Abbreviations

APTT, activated partial thromboplastin time; CVD, cardiovascular disorder; DM, diabetes mellitus; FBG, fasting blood glucose; FVII, factor VII; IDF, International Diabetes Federation; INR, international normalization ratio; IR, insulin resistance; MPV, mean platelet volume; PAI-1, plasminogen activator inhibitor-1; PDW, platelet distribution width; PT, prothrombin time; SOP, standard operating procedure; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TF, tissue factor.

Data Sharing Statement

The authors confirmed that all the data for this manuscript are available; if someone wants to request the data they can contact the corresponding author.

Ethics and Consent Statement

The study was approved by the School of Biomedical and Laboratory Sciences Research and Ethical Review Committee (SBMLS/21/23/11). A letter of support was secured from the zonal health office and a permission letter was obtained from the clinical director of the hospital. Written informed consent was taken from each study participant. Individuals who had coagulation abnormality were linked to the responsible clinician in Dessie referral hospital for proper treatment. This study was conducted in accordance with the declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no conflicts of interest.

References

1. International Diabetes Federation. Diabetes Atlas. sixth edition, 2013. Available from: http://www.diabetesatlas.org. Accessed November 10, 2018.
2. Gundogan K, Bayram F, Capak M, Tanriverdi F, Karaman A. Prevalence of metabolic syndrome in the Mediterranean region of Turkey: evaluation of hypertension, diabetes mellitus, obesity, and dyslipidemia. *Metab Syndr Relat Disord*. 2009;7:427–434. doi:10.1089/met.2008.0068
3. Sobczak, Amélie IS, Alan SJ. Coagulatory Defects in Type-1 and Type-2 Diabetes. *Int J Mol Sci*. 2019;20:6345. doi:10.3390/ijms20246345
4. Odegaard AO, Jacobs DR, Sanchez OA, Goff DC, Reiner AP, Gross MD. Oxidative stress, inflammation, endothelial dysfunction, and incidence of type 2 diabetes. *Cardiovasc Diabetol*. 2016;15 (1):51. doi:10.1186/s12933-016-0369-6
5. Soedamah-Muthu SS, Chaturvedi N, Toeller M, et al. Risk Factors for Coronary Heart Disease in Type 1 Diabetic Patients in Europe: the Eurodiab Prospective Complications Study. *Diab Care*. 2004;27 (2):530–537. doi:10.2337/diacare.27.2.530
6. Kendall DM, Harmel AP. The metabolic syndrome, type 2 diabetes, and cardiovascular disease: understanding the role of insulin resistance. *Am J Manag Care*. 2002;8(20 Suppl):S635–S653.
7. Yngen DM. Platelet Hyperactivity in Diabetes Mellitus. *Eur Cardiol Rev*. 2005;1(1):1–5. doi:10.15420/ECR.2005.1o
8. Wou C, Unwin N, Huang Y, Roglic G. Implications of the growing burden of diabetes for premature cardiovascular disease mortality and the attainment of the Sustainable Development Goal target. *Cardiovasc Diagn Ther*. 2019;9(2):140–149. doi:10.21037/cdt.2018.09.04
9. Use of glycated hemoglobin. (HbA1c) in the diagnosis of diabetes mellitus. *Diabetes Res Clin Pract*. 2011;93(3):299–309. doi:10.1016/j.diabres.2011.03.012
10. Fattah MA, Shaheen MH, Mahfouz MH. Disturbances of Haemostasis in Diabetes Mellitus. *Dis Markers*. 2004;19 (6):251–258. doi:10.1155/2004/797458

11. Grant PJ. Diabetes mellitus as a prothrombotic condition. *J Intern Med*. 2007;262(2):157–172. doi:10.1111/j.1365-2796.2007.01824.x

12. Carr ME. Diabetes mellitus A hypercoagulable states. *J Diabetes Complications*. 2001;15(11):44–54. doi:10.1016/S1056-8727(00)00132-X

13. Kim JH, Bae HY, Kim SY. Clinical Marker of Platelet Hyperreactivity in Diabetes Mellitus.Diabetes. *Metab J*. 2013;37 (6):423–428. doi:10.4093/dmj.2013.37.6.423

14. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diab Care*. 1993;16:434–444. doi:10.2337/diacare.16.2.434

15. Vazzana N, Ranalli P, Cuccurullo C, Davì G. Diabetes mellitus and thrombosis. *Thromb Res*. 2012;129(3):371–377. doi:10.1016/j.thromres.2011.11.052

16. Lemkes BA, Hermanides J, Devries JH, Holleman F, Meijers JCM, Hoekstra JBL. Hyperglycemia: a prothrombotic factor?: hyperglycemia and hypercoagulability. *J Thromb Haemost*. 2010;8 (8):1663–1669. doi:10.1111/j.1538-7836.2010.03910.x

17. Byberg L, Siegbahn A, Berglund L, McKeigue P, Reneland R, Lithell H. Plasminogen Activator Inhibitor-1 Activity Is Independently Related to Both Insulin Sensitivity and Serum Triglycerides. *Arterioscler Thromb Vasc Biol*. 1998;18(2):258–264. doi:10.1161/01.ATV.18.2.258

18. Alzahrani S, Ajjan R. Review article on coagulation and fibrinolysis in diabetes. *Diab Vasc Dis Res*. 2010;7(4):260–273. doi:10.1177/1479164110383723

19. Ulutas KT, Dokuyucu R, Sefil F, et al. Evaluation of mean platelet volume in patients with type 2 diabetes mellitus and blood glucose regulation: a marker for atherosclerosis? *Int J Clin Exp Med*. 2014;7 (4):955–961.

20. Sapkota B, Shrestha S, Poudel S. Association of activated partial thromboplastin time and fibrinogen level in patients with type II diabetes mellitus. *BMC Res Notes*. 2013;6:485. doi:10.1186/1756-0500-6-485

21. Erem C, Hacıhasanoğlu A, Ş Ç, et al. Coagulation and Fibrinolysis Parameters in Type 2 Diabetic Patients with and without Diabetic Vascular Complications. *Med Princ Pract*. 2005;14(1):22–30. doi:10.1159/000081919

22. Abdulla AM, Elmissbah TE, Hamid EM, A-ltom FO, Faisal M. Assessment of Coagulation Process in Diabetic Patients using Prothrombin Time and Activated Thromboplastin Time Tests. *Int J Multidiscip Curr Res*. 2017;4(3):84–88.

23. Fadairo JK, Atere AD, Odunayo T, Abiodun OP. Assessment of Some Coagulation Indices among Type II Diabetic Subjects in a Tertiary Facility in South West Region, Nigeria. *IOSR J Dent Med Sci*:. 2016;15(6):159–163.

24. Thukral S, Hussain S, Bhat S, Kaur N, Reddy A. Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) in Type 2 Diabetes Mellitus, a Case-Control Study. *Diab Mellit*. 2018;5(8):H5–H9.

25. Banini AE, Boyd LC, Hoffman M. Elevated prothrombin level and shortened clotting times in subjects with type 2 diabetes. *J Thromb Haemost*. 2007;5(3):638–639. doi:10.1111/j.1538-7836.2007.02366.x

26. Awuku YA, Adu P, Ampomah LTW, et al. High risk of coagulopathy among Type-2 Diabetes Mellitus clients at a municipal hospital in Ghana. *Ghana Med J*. 2017;51(3):101–107. doi:10.4314/gmj.v51i3.2

27. George LA, Lambert MP. *Coagulation Cascade and Fibrinolysis Pathway: Assessment In Nonmalignant Hematology*. Cham: Springer International Publisher; 2016:221–233.

28. Swaminathan A, Amitkumar K, Ganapathy S, Ayyavoo S. Evaluation of mean platelet volume and other platelet parameters in subjects with Type-2 diabetes mellitus. *Natl J Physiol Pharm Pharmacol*. 2017;7(1):51–54. doi:10.5455/njppp.2016.6.0719518072016

29. Wilson Van Voorhis CR, Morgan BL. Understanding Power and Rules of Thumb for Determining Sample Sizes. *Tutor Quant Methods Psychol*. 2007;3(2):43–50. doi:10.20982/tqmp.03.2.p043

30. Onyema Eledo B. Assessment of some haemostatic parameters among diabetes mellitus patients in bayelsa state: a case study at the federal medical centre, Yenagoa. *Eur J Clin Biomed Sci*. 2017;3 (5):91–96. doi:10.11648/j.ejcbs.20170305.12

31. Dhule S, Gawali S. Platelet aggregation and clotting time in type 2 diabetic males. *Natl J Physiol Pharm Pharmacol*. 2014;4 (2):121–123. doi:10.5455/njppp.2014.4.290920131

32. Pujani M, Gahlawat H, Agarwal C, Chauhan V, Singh K, Lukhmana S. Platelet parameters: can they serve as biomarkers of glycemic control or development of complications in the evaluation of type 2 diabetes mellitus? *Iraqi J Hematol*. 2018;7(2):72–78. doi:10.4103/ijh.ijh_8_18

33. Biadgo B, Melku M, Mekonnen Abebe S, Abebe M. Hematological indices and their correlation with fasting blood glucose level and anthropometric measurements in type 2 diabetes mellitus patients in Gondar, Northwest Ethiopia. *DiabetesMetabSyndrObes*. 2016;9:91–99. doi:10.2147/DMSO

34. Dindar S, Cinemre H, Sengul E, Annakkaya A. Mean Platelet Volume is Associated with Glycaemic Control and Retinopathy in Patients with Type 2 Diabetes Mellitus. *West Indian Med J*. 62 (6):519–523. doi: 10.7727/wimj.2012.284.

35. Akinsegun A, Olusola DA, Sarah J-O, et al. Mean platelet volume and platelet counts in type 2 Diabetes: mellitus on treatment and non-diabetic mellitus controls in Lagos, Nigeria. *Pan Afr Med J*. 2014;18:42. doi:10.11604/pamj.2014.18.42.3651

36. Citirik M, Beyazyildiz E, Simsek M, Beyazyildiz O, Haznedaroglu IC. MPV may reflect subclinical platelet activation in diabetic patients with and without diabetic retinopathy. *Eye*. 2015;29 (3):376–379. doi:10.1038/eye.2014.298

37. Malachowska B, Tomasik B, Szadkowska A, et al. Altered Platelets' morphological parameters in children with type 1 diabetes – a case-control study. *BMC Endocr Disord*. 2015;15(17):1–7. doi:10.1186/1472-6823-15-1

38. Isaac IZEO. Some Haematological Parameters in Patients with Type-1 Diabetes in Sokoto, North-Western Nigeria. *J Blood Lymph*. 2012;03(01):1–4. doi:10.4172/2165-7831.1000110

39. Keating FK, Sobel BE, Schneider DJ. Effects of increased concentrations of glucose on platelet reactivity in healthy subjects and patients with and without diabetes mellitus. *Am J Cardiol*. 2003;92 (11):1362–1365. doi:10.1016/j.amjcard.2003.08.033

40. Chen X, Fang L, Lin H, et al. The Relationship between Type 2 Diabetes and Platelet Indicators. *Iran J Public Health*. 2017;46 (6):1211–1216.

41. Indranila KS. Molecular aspect correlation between glycated hemoglobin (hba1c), prothrombin time (pt) and activated partial thromboplastin time (aptt) on type 2 diabetes mellitus (t2dm). *Indones J Clin Pathol Med Lab*. 2016;23(1):1–6.

42. Nnenna I, Obioma A. Evaluation of Haemostatic Parameters of Diabetic Patients Accessing Care in Some Selected Health Care Facilities in Port Harcourt Metropolis, Nigeria. *Sci Technol*. 2016;3(2):119–134.

43. Ziaee A, Ghorbani A, Kalbasi S, Hejrati A, Moradi S. Association of hematological indices with prediabetes: A cross-sectional study. *Electron Physician*. 2017;9(9):5206–5211. doi:10.19082/5206

Taylor & Francis

Low Blood Donation Practice of Health Sciences College Students in Northeast Ethiopia: A Cross-Sectional Study

Mekedes Dejene, Azeb Tefera, Abebe Dires, Sisay Gedamu, Yemiamrew Getachew & Sewunet Ademe

ORIGINAL RESEARCH

Low Blood Donation Practice of Health Sciences College Students in Northeast Ethiopia: A Cross-Sectional Study

This article was published in the following Dove Press journal:
Journal of Blood Medicine

Mekedes Dejene[1]
Azeb Tefera[1]
Abebe Dires [2]
Sisay Gedamu [2]
Yemiamrew Getachew[3]
Sewunet Ademe [2]

[1]Emergency Department, Dessie Referral Hospital, Dessie, Ethiopia; [2]Department of Nursing, School of Nursing and Midwifery, College of Medicine & Health Sciences, Wollo University, Dessie, Ethiopia; [3]Department of Community and Mental Health, School of Nursing and Midwifery, College of Medicine & Health Sciences, Wollo University, Dessie, Ethiopia

Introduction: Blood transfusion is a basic and an emergency intervention in health care facilities which has a great role in reducing significant morbidity and mortality. However, there is a major shortage of blood and blood products in developing countries including Ethiopia. This study aimed to assess practice of blood donation and associated factors among health science college students in Dessie town, northeast Ethiopia.
Methods: An institution-based cross-sectional study was conducted among health science college students from May to June 2019. A pre-tested and self-administered structured questionnaire was used for data collection. Multivariable logistic regression analysis model was applied to identify independent predictors of blood donation practice at the level of significance below 0.05.
Results: Overall, 12.4% (95% CI: 9.5–15.5) of participants had been donated blood at least once in their lifetime. However, 59.2% of participants have willingness to donate blood in the future. In this study, older age (≥25years) (AOR=2.30, 95% CI: 1.18–4.46), had family history of blood transfusion (AOR=3.55, 95% CI: 1.71–7.36), had knowledge (AOR=2.09, 95% CI: 1.04–4.17) and favorable attitude (AOR=2.41, 95% CI: 1.01–5.75) about blood donation were significantly associated with practice of donating blood.
Conclusion: In this study, blood donation practice of health sciences college students was found to be low. Age, family history of blood transfusion, knowledge and attitude towards blood donation were independent predictors of blood donation practice. Therefore, Red Cross societies, Dessie town health office, health science colleges and other stakeholders should enhance the awareness of college students regarding the importance of donating blood.
Keywords: blood donation, college students, northeast Ethiopia

Introduction

Blood transfusions help to save millions of lives every year and support complex medical and surgical procedures.[1] It is a basic and an emergency intervention in health care facilities which has a significant role in reducing morbidity and mortality. In addition, blood has an essential life-saving role in maternal and childcare interventions that could occur in human made and natural disasters. However, in many countries there is no adequate supply of blood available to fulfill the demand.[2]

There was a marked difference in the accessibility of blood between high and low income countries. The average annual collection of blood per blood center was

Correspondence: Abebe Dires
Email abede9907@gmail.com

30,000 units of blood in high-income countries as compared to 3,700 in low-income countries.[3] In principle, there are three types of blood donors. These are; replacement, paid, and voluntary donors.[4] The World Health Organization (WHO) is recommending all countries should obtain their blood supplies from voluntary donors by the year 2020. But the majority of blood donation practices are replacement type of donation.[5]

From the 92 million units of blood collected annually, almost half was collected in high-income countries.[3] Similarly, more than 34 million peoples were donated blood from 189 national societies.[6] Collected bloods could be discarded due to different reasons. The WHO's global database on blood safety indicated that, about 1.6 million units of collected blood was discarded annually due to the presence of infections like HIV, hepatitis virus and syphilis.[7]

In developing countries, the habit of blood donation was much lower than that of high income countries.[6] In sub-Saharan Africa, blood helps to reduce morbidity and mortality of young children and pregnant women in particular. However, many deaths could be prevented if national blood services and hospitals have adequate supplies of blood.[8] From 2011 to 2014, the overall blood collections were increased by 19% among 14 African countries, and in Ethiopia the services was increased by 73.8%.[9] The Ethiopian Red Cross Society (ERCS) is the first organization in establishing blood bank service in the country. After the federal ministry of health has taken over the service from ERCS in 2012, the rate of national blood collection was increased from 40,000 units of blood in 2011 to 88,000 in 2014 and the involvement of voluntary blood donors had been increased from 10% in 2011 to 70% in 2014.[10] According to WHO, countries' blood transfusion service was recommended to be based on voluntary blood donors by 2020.[11]

The global burden of injuries and death from road traffic accidents was increased, particularly in Africa.[12] The majority of deaths after traumatic injuries is as a result of hemorrhage.[2] In addition, hemorrhage has been identified as the leading cause of maternal mortality worldwide and in Ethiopia.[13–18] Generally, 50–80% of all blood transfusions were given to treat patients with severe anemia as a result of hemorrhage and trauma.[19] Thus, timely access to blood transfusion has a great role to reduce significant morbidity and mortality in health care facilities. Although the global need of blood supply has been increased progressively, there is still evidence of a shortage of blood and blood products in developing countries including Ethiopia. In low income countries, lack of knowledge and poor attitude about donating blood were the major factors affecting blood donation practice.[20] To what extent eligible segments of the population in Ethiopia have donated blood is vital to enhance community awareness interventions which are essential to meet the demand for blood. In Ethiopia, numerous studies have been conducted regarding blood donation at community level. However, there are few studies about the practice of blood donation among health science college students.

This study aimed to assess blood donation practices of health science college students and any associated factors in Dessie town, northeast Ethiopia.

Methods and Materials
Study Design, Period and Setting
An institution-based cross-sectional study was conducted among health science college students in Dessie town, northeast Ethiopia from May to June 2019. Dessie town is located 523 kilometers (KM) far from Bahir Dar (Regional city of Amhara) and 401 KM northeast of Addis Ababa (the capital city of Ethiopia). The town has one public referral hospital and three private hospitals. The referral hospital is designed to serve up to 5,000,000 populations. There is one blood bank in the town that works in conjunction with the Red Cross society. There are four health science colleges in the town, with a total of 3,560 students. Nursing, midwifery, public health officers and medical laboratory were the main programs executed in those colleges.

Population
The source populations were all health science college students founded in Dessie town and the study populations were randomly selected health science college students during data collection period.

Sample Size
A single population proportion formula [$n = (Z a/2)^2 P (1-P)/d^2$] was used to estimate the sample size by considering the following assumptions: Proportion of 50%, 95% confidence level, 5% margin of error, and 10% non-response rate.[21] Therefore, the final sample size was 423.

Sampling Technique and Procedure
From the four health science colleges found in Dessie town, the total number of students in each college was

Total number of health science colleges and students in Dessie town (n=3560)

| Dessie HSC= 1700 | Alkan HSC= 659 | Tropical HSC=590 | Dandiboru HSC= 631 |

Proportional allocation at college level

| 201 | 78 | 70 | 74 |

Proportional allocation based on year of study

1st yr= 54	1st yr = 16	1st yr = 18	1st yr = 21
2nd yr= 48	2nd yr = 27	2nd yr = 15	2nd yr = 17
3rd yr= 62	3rd yr = 20	3rd yr = 20	3rd yr = 16
4th yr= 37	4th yr = 15	4th yr = 17	4th yr = 20

Simple random sampling

Total sample size (n= 423)

Figure 1 Sampling procedures on the practice of blood donation among health science college students of Dessie town, northeast Ethiopia, 2019.
Abbreviation: HSC, health science college.

determined. Proportional allocation was made at the level of college and year of entry to select students. Finally, a simple random sampling technique was used to select study participants (Figure 1).

Data Collection Tool and Procedures

For data collection, self-administered structured question-naire was used which was adopted from previous studies.[21-24] Socio-demographic variables, questions assessing the knowledge, attitude and practice of students about blood donation were included in the questionnaire. In addition, participants' willingness to donate blood was also assessed (Supplementary material).

Data Quality Management

To maintain the quality of the data, four nurse data collectors and two public health officers as a supervisor were recruited and trained for two days regarding the objective of the study and other ethical issue. In addition, the data collection tool was pre-tested by assumption of 5% of the total sample size within one week prior to the actual data collection period in Wollo University health science students. Based on pre-test evaluation, the tool was modified and the necessary corrections were made. Finally, an organized and valuable tool was made ready and used for the actual data collection.

Operational Definitions

Practice of blood donation: Students who had donated blood at least once in his or her lifetime.

Knowledgeable about blood donation: Among knowledge assessing questions, students who score was greater than or equal to a mean value of 0.5 were considered knowledgeable. Whereas, those who scored less than the mean value 0.5 were not considered to be knowledgeable.

Favorable attitude towards blood donation: Those respondents who scored the mean value of 0.3 and above among attitude assessing questions.

Unfavorable attitude towards blood donation: Those respondents who had scored below the mean value of 0.3 answering attitude assessing questions.

Data Analysis

Data was entered using Epi-info version 7 and analyzed by SPSS (statistical package for social science) version 20. Data were reported using mean for continuous variables and proportions and tables were used to describe categorical variables. Bivariable and multivariable logistic analyses model were used with odds ratio and 95% confidence interval. In bivariable analysis, variables with p-value of 0.2 and below were entered into multivariable analysis model. In multivariable analysis model, variables with p-value < 0.05 were considered statistically significant.

Results
Socio-Demographic Characteristics of Study Participants

In this study, 412 students were participated with response rate of 97.4%. The mean age of participant was 19.5 years and 62.6% were in the age group of 18–24 years. Overall, 55.6% of the participants were male, 64.8% were from an urban area, 36.9% were students of public health, and 28.9% were second year students (Table 1).

Table I Socio-Demographic Characteristics of Respondents for Practice of Blood Donation Among Health Science College Students in Dessie Town, 2019 (n = 412)

Variables	Frequency	Percent (%)
Age category in completed year		
18–24	258	62.6
≥25	154	37.4
Sex		
Male	229	55.6
Female	183	44.4
Religion		
Orthodox	186	45.2
Muslim	127	30.8
Protestant	99	24.0
Ethnicity		
Amhara	357	86.7
Oromo	28	6.8
Tigray	19	4.6
Others [b]	8	1.9
Original Residence		
Urban	267	64.8
Rural	145	35.2
Type of department		
Nursing	80	19.4
Public Health Officer	152	36.9
Medical Laboratory	101	24.5
Midwifery	79	19.2
Student's study Year		
First	107	26.0
Second	119	28.9
Third	96	23.3
Forth	90	21.8

Note: [b]Afar, Benishangul-Gumuz.

Respondents' Practice of Blood Donation and Related Factors

Among all participants, 5.6% had been transfused with blood and 23% had family history of blood transfusion. Of all respondents, 40.5% and 67.2% had knowledge and favorable attitude about blood donation, respectively. Overall, 12.4% (95% CI: 9.5–15.5) of participants had donated blood at least once in their lifetime, of which, 62.7% were donated voluntarily. Fear of health

Table 2 Respondents' Practice of Blood Donation and Related Factors Among Health Science College Students in Dessie Town, 2019 (n = 412)

Variables	Frequency	Percent (%)
History of being transfused with blood		
Yes	23	5.6
No	389	94.4
History of blood transfusion in the family		
Yes	95	23.1
No	317	76.9
Attitude towards blood donation		
Favorable	277	67.2
Unfavorable	135	32.8
Knowledge about blood donation		
Yes	167	40.5
No	245	59.5
Had donated blood previously		
Yes	51	12.4
No	361	87.6
Type of blood donation(n=51)		
Voluntary	32	62.7
Replacement	19	37.3
Reasons for not donated blood (n=361)?		
Fear of health problem after screening	142	39.3
Lack of information	114	31.5
Fear of pain	56	15.5
Fear of weight loss	33	9.1
Others*	16	4.6
Willingness to donate blood voluntarily in the future		
Yes	244	59.2
No	168	40.8

Note: *Lack of time, non-remuneration, I do not think I am fit to donate.

problems after screening (39.3%) and lack of information (31.5%) were the main reasons identified by non-blood donors. In this study, 59.2% of students are willingto donate blood voluntarily in the future (Table 2).

Factors Associated with Practice of Blood Donation

A total of eleven independent variables were included in the bivariable logistic regression analysis. Those variables associated with the dependent variable at p-value of less than 0.2 were subjected into multivariable logistic analysis. Thus, after adjusted for confounding variables in multivariable analysis; older age (≥25 year) (AOR=2.30, 95% CI: 1.18–4.46), those who had a family history of blood transfusion (AOR=3.55, 95% CI: 1.71–7.36), knowledge (AOR=2.09, 95% CI: 1.04–4.17), and a favorable attitude (AOR=2.41, 95% CI: 1.01–5.75) about blood donation were significantly associated with the practice of donating blood (Table 3).

Discussion

Blood transfusion is a basic and vital tool in emergency lifesaving interventions. Globally, the need for blood and blood products has been increasing and evidence showed that the habit of donating blood in the population was not satisfactory. In our study, 12.4% of students had been donated blood at least once in their lifetime. This finding was comparable with a previously reported study done in Nigeria (15%).[25] However, it was higher compared to a study done in India (1.4%)[26] and it was lower compared to studies conducted in three settings of Ethiopia Ambo (23.6%),[27] Arsi (27.2%),[28] and Addis Ababa university students (24.3%).[29]

It was also lower compared to studies done in Africa; Tanzania (30%),[30] Sudan (27%),[31] Malaysia (29.7%),[32] Nigeria (59.5%),[33] Nepal (28.5%)[34] as well as in three setting of India (38.4%),[35] (17.3%),[36] (23%),[37] and Saudi Arabia (19%).[38] This discrepancy could be due to difference in socio demography and economical characteristics of study participants and the study period.

WHO is recommending all countries should obtain their blood from voluntary blood donors.[11] In this study, of the total respondents who had been donated blood, about 62.7% were donated voluntarily. In Ethiopia, voluntary blood donation showed a significant increase in the past decade.[10] Although the proportion of voluntary blood donors were moderate in the country, it is still not enough to meet the need required amount of blood so as to prevent the morbidity

Table 3 Bivariable and Multivariable Analysis for the Practice of Blood Donation Among Health Science College Students in Dessie Town, 2019 (n = 412)

Variables	Practice of Blood Donation		COR (95% CI)	AOR (95% CI)
	No (%)	Yes (%)		
Age (year)				
18–24	234(90.7)	24(9.3)	1	1
≥25	127(82.5)	27(17.5)	2.07(1.15–3.74)*	2.30(1.18–4.46)*
Sex				
Male	192(83.8)	37(16.2)	2.32(1.21–4.45)*	1.36(0.67–2.79)
Female	169(92.3)	14(7.7)	1	1
Residence				
Rural	136(93.8)	9(6.2)	1.000	1
Urban	225(84.3)	42(15.7)	2.82(1.33–5.97)**	1.34(0.55–3.26)
Ethnicity				
Amhara	313(87.7)	44(12.3)	0.98(0.12–8.19)	
Oromo	26(92.9)	2(7.1)	1.86(0.17–19.9)	
Tigray	15(78.9)	4(21.1)	0.53(0.04–6.83)	
Others	7(87.5)	1(12.5)	1.000	–
Religion				
Orthodox	160(86.0)	26(14.0)	2.13(0.89–5.11)	
Muslim	109(85.8)	18(14.2)	2.17(0.86–5.42)	
Protestant	92(92.9)	7(7.1)	1.000	1
Type of department				
Nursing	70(87.5)	10(12.5)	1.000	1
Health Officer	126(82.9)	26(17.1)	1.44(0.65–3.16)	1.05(0.55–3.79)
Medical laboratory	92(91.1)	9(8.9)	0.68(0.26–1.77)	0.47(0.25–2.83)
Midwifery	73(92.4)	6(7.6)	0.57(0.19–1.66)	0.16(0.45–5.94)
Year of study				
First	97(90.7)	10(9.3)	1.000	1
Second	99(83.2)	20(16.8)	1.96(0.87–4.40)	
Third	84(87.5)	12(12.5)	1.38(0.57–3.37)	
Forth	81(90.0)	9(10.0)	1.07(0.41–2.78)	
History of being transfused with blood				
Yes	16(69.6)	7(30.4)	3.43(1.33–8.79)*	2.51(0.81–7.79)
No	345(88.7)	44(11.3)	1	1
Family history of blood transfusion in the family				
Yes	68(71.6)	27(28.4)	4.87(2.63–8.92)***	3.55(1.71–7.36)**
No	293(92.4)	24(7.6)	1	1
Attitude towards VBD				
Favorable	233(84.1)	44(15.9)	3.43(1.51–7.88)**	2.41(1.01–5.75)*
Unfavorable	128(94.8)	7(5.2)	1	1
Knowledge about blood donation				
Yes	133(79.6)	34(20.4)	3.42(1.84–6.37)***	2.09(1.04–4.17)*
No	228(93.1)	17(6.9)	1	1

Notes: Asterisk shows significant associations at different P-value: 0.05–0.01*, 0.01–0.001**and < 0.001***.

and mortality of children and women, in particular. The most significant increases in the percentage of voluntary blood donation were reported in India, Bulgaria, Afghanistan, Belarus, Algeria, and Costa Rica.[3]

In our study, participants aged ≥ 25 years were 2.3 times more likely donated blood as compared to those who were found between 18 and 24 years. It was in line with the study done in Addis Ababa, Ethiopia.[29] The odds of blood donation practice was higher in those students who had family history of blood transfusion compared to those who had not family history. This was in line with the study done in Ambo, Ethiopia.[27] This could be explained by people voluntarily donating blood after experiencing a family member's health problems which required a blood transfusion. Furthermore, students who had knowledge about blood donation were twice as likely to donate blood compared to those who had no knowledge, which was similar to the studies done in Ambo, Ethiopia[27] and Ghana.[20] Students who had favorable attitudes towards blood donation were 2.4 times more likely to donate blood compared to their counterparts. In this study, 87.6% of participants had never donated blood and fear of health problems after screening and lack of information were the main reasons identified for not donating. However, a significant proportion of students are willingto donate blood in the future, which is an opportunity to increase the number of blood donors in the country.

A strength of this study is that we have assessed blood donation practices of health science college students who would be involved in direct patient care. The possible limitations of this study could be related to the nature of the cross-sectional study design used and there could be social desirability and recall bias. Additionally, since this study was carried out on selected health science college students of Dessie town, our findings may not be generalized to the overall college students of the town.

Conclusion

In this study, blood donation practices of health science college students was found to be low. Age, family history of blood transfusion, knowledge, and attitude towards blood donation were independent predictors of blood donation practice. Therefore, Red Cross societies, Dessie town health office, health science colleges and other stakeholders should enhance the awareness of college students regarding the importance of donating blood. Furthermore, frequent promotional campaigns should be conducted by media and health care providers.

Data Sharing Statement

The used data set and analyzed during this study are available from the corresponding author on reasonable request.

Ethical Approval and Consent

Ethical approval was obtained from ethical review committee of Wollo University, school of nursing and midwifery, department of nursing. Study participants were informed that participation was on a voluntary basis and they can leave the study at any time if they are not comfortable about the questionnaire. Verbal informed consent taken from study participants was approved by the Ethical Review Committee of Wollo University and that this study was conducted in accordance with the Declaration of Helsinki. Confidentiality was preserved for all data collected.

Acknowledgment

We would like to thank coordinators' of health science colleges in Dessie town for their cooperation and all study participants for this study.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors report no conflicts of interest for this work.

References

1. World Health Organization. *World Blood Donor Day. 2016*.
2. Blessing T, Oyeniyi E, Fox E, et al. Trends in 1029 trauma deaths at a level 1 trauma center: impact of a bleeding control bundle of care. *Int J Care Injury*. 2017;5(21).
3. World Health Organization. *Global Database on Blood Safety. 2011*.
4. WHO. *Safe Blood and Blood Products. 2009*.
5. Mohammed Y, Bekele A. Seroprevalence of transfusion transmitted infection among blood donors at Jijiga blood bank, Eastern Ethiopia: retrospective 4 years study. *BMC Res Notes*. 2016;9(129).
6. Cross, Red, and Red Crescent. *Key Data from 189 National Red Cross and Red Crescent Societies – A Baseline. 2014*.
7. WHO. *Blood Donor Selection: Guidelines on Assessing Donor Suitability for Blood Donation*. Luxembourg; 2012.
8. Africa Health. *Drop by Drop: Improving African Blood Services*. Africa Health; 2016.

9. Chevalier MS, Kuehnert M, Basavaraju SV, Bjork A, Pitman JP. Progress toward strengthening national blood transfusion services 14 countries, 2011–2014. *MMWR Morb Mortal Wkly Rep*. 2016;65 (5):115–119. doi:10.15585/mmwr.mm6505a4

10. Federal democratic republic of Ethiopia ministry of health. *Special Bulletin 16th Annual Review Meeting*. 2014.

11. Asif N, Hassan K. Voluntary blood donation. *J Islamabad Med Dent College*. 2015;5(1).

12. Adeloye D, Thompson JY, Akanbi MA, et al. The burden of road traffic crashes, injuries and deaths in Africa: a systematic review and meta-analysis. *Bull World Health Organ*. 2016;94(7):510–521A. doi:10.2471/BLT.15.163121

13. Mokdad AH, Cuchilla J, Zúñiga-Brenes P. Maternal mortality and morbidity burden in the Eastern Mediterranean region: findings from the global burden of disease 2015 study. *Int J Public Health*. 2017;62 (2):271–282. doi:10.1007/s00038-016-0859-z

14. GBD. 2015 Maternal mortality collaborators. Global, regional, and national levels of maternal mortality, 1990–2015: a systematic analysis for the global burden of disease study. *Lancet*. 2016;388.

15. Neal S, Mahendra S, Bose K, et al. The causes of maternal mortality in adolescents in low and middle income countries: a systematic review of the literature. *BMC Pregnancy Childbirth*. 2016;16(352). doi:10.1186/s12884-016-1120-8.

16. Maswime S, Buchmann E. A systematic review of maternal near miss and mortality due to post partum hemorrhage. *Int J Gynaecol Obstet*. 2017;137.

17. Legesse T, Abdulahi M, Dirar A. Trends and causes of maternal mortality in Jimma University specialized Hospital, southwest ethiopia: a matched case–control study. *Int J Womens Health*. 2017;9.

18. Mapunda OE, Msuya SE, Kapologwe NA, John B, Damian DJ, Mahande MJ. Assessment of maternal mortality and its associated causes at shinyanga regional hospital in Tanzania. *Womens Health Bull*. 2017;4(2).

19. Ala F, Allain J-P, Bates I, et al. External financial aid to blood transfusion services in sub-saharan africa: a need for reflection. *PLOS Med*. 2012;9(9).

20. Mohammed S, Essel HB. Motivational factors for blood donation, potential barriers, and knowledge about blood donation in first-time and repeat blood donors. *BMC Hematol*. 2018;18(36). doi:10.1186/s12878-018-0130-3

21. Jemberu YA, Esmael A, Ahmed KY. Knowledge, attitude and practice towards blood donation and associated factors among adults in Debre Markos town, Northwest Ethiopia. *BMC Hematol*. 2016;16 (23). doi:10.1186/s12878-016-0062-8

22. Melku M, Terefe B, Asrie F, et al. Knowledge, attitude, and practice of adult population towards blood donation in Gondar town, Northwest Ethiopia: a community based cross-sectional study. *J Blood Transfus*. 2016;2016:1–10. doi:10.1155/2016/7949862

23. Urgesa K, Hassen N, Seyoum A. Knowledge, attitude, and practice regarding voluntary blood donation among adult residents of Harar town, Eastern Ethiopia: a community-based study. *J Blood Med*. 2017;8:8. doi:10.2147/JBM.S121460

24. Addisu AG, Sultan H, Deginet T, Addishiwot Z, Samuel T. Assessment of knowledge, attitude and practice of voluntary blood donation and associated factors among residents of Birbir Town. *J Community Med Health Educ*. 2017;7(1).

25. Salaudeen AG, Odeh E. Knowledge and behavior towards voluntary blood donation among students of a tertiary institution in Nigeria. *Niger J Clin Pract*. 2011;14(3):303. doi:10.4103/1119-3077.86773

26. Gaonkar SR. Assessment of knowledge. *Int J Physiol*. 2015;3(2).

27. Nigatu A, Demissie DB. Knowledge, attitude and practice on voluntary blood donation and associated factors among ambo university regular students, Ambo Town, Ethiopia. *Community Med Public Health*. 2014;4(5).

28. Gebresilase HW, Fite RO, Abeya SG. Knowledge, attitude and practice of students towards blood donation in Arsi university and Adama science and technology university: a comparative cross sectional study. *BMC Hematol*. 2017;17(20). doi:10.1186/s12878-017-0092-x

29. Misganaw C, Tenkir M, Deresea A, Tesfaye M, Tessema TT, Taye H. The level and associated factors of knowledge, attitude and practice of blood donation among health science students of Addis Ababa University. *Int J Med Sci Public Health Res*. 2014;1(10).

30. Elias E, Mauka W, Philemon RN, Damian DJ, Mahande MJ, Msuya SE. Knowledge, attitudes, practices, and factors associated with voluntary blood donation among university students in Kilimanjaro, Tanzania. *Hindawi Publ Corp J Blood Transfus*. 2016;2016:1–8. doi:10.1155/2016/8546803

31. Mohammed H, Osman T. Voluntary blood donation among medical students in a resource-limited country. *J Public Health Res Dev Ctries*. 2016;2(3).

32. Elnajeh M, Ghazi HF, Abdalqader MA, Baobaid MF. Knowledge, attitude and practice towards blood donation and its associated factors among university students in Shah Alam, Malaysia. *Int J Community Med Public Health*. 2017;4(7):2230. doi:10.18203/2394-6040.ijcmph20172811

33. Nwabueze SA, Nnebue CC, Azuike EC, et al. Perception of blood donation among medical and pharmaceutical science students of Nnamdi Azikiwe University, Awka. *Open J Prev Med*. 2014;4.

34. Amatya M, Prajapati R, Yadav S. Knowledge and practice of blood donation: a comparison between medical and non-medical Nepalese students. *Nepal Med Coll J*. 2012;14(4).

35. Meinia SK, Kumar Y, Meinia A, Singh G, Dutt N. A study to assess the knowledge, attitude, and practices about voluntary blood donation amongst the undergraduate medical students in Solan, North India. *Int J Med Sci Public Health*. 2016;5(12):2550. doi:10.5455/ijmsph.2016.24052016523

36. Thakur A, Chauhan HS, Acharya B. Knowledge and practices of blood donation among the undergraduate students of district Una, Himachal Pradesh, India. *Glob J Med Public Health*. 2015;4(6).

37. Chopra D, Jauhari N, Bhattacharya S. Knowledge attitude & practices towards voluntary blood donation among medical students in Barabanki. *Indian J Community Health*. 2015;27(3):60–65.

38. Baig M, Habib H, Haji AH, Alsharief FT, Noor AM, Makki RG. Knowledge, misconceptions and motivations towardsblood donation among university students in Saudi Arabia. *Pak J Med Sci*. 2013;29 (6). doi:10.12669/pjms.296.4137

Value of the Direct Antiglobulin Test in Predicting the Need for Phototherapy in Newborns

Suzan A AlKhater, Rana A Albalwi, Sara A Alomar, Anfal A Alsultan, Halah R Almuhaidib, Rahaf A Almousa, Sarah M Alanezi, Raghad K Alghamdi & Hwazen A Shash

ORIGINAL RESEARCH

Value of the Direct Antiglobulin Test in Predicting the Need for Phototherapy in Newborns

Suzan A AlKhater [1,2]
Rana A Albalwi [2]
Sara A Alomar [1]
Anfal A Alsultan [1]
Halah R Almuhaidib [1]
Rahaf A Almousa [1]
Sarah M Alanezi [1]
Raghad K Alghamdi [1]
Hwazen A Shash [1,2]

[1]College of Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [2]Department of Pediatrics, King Fahad Hospital of the University, Al-Khobar, Saudi Arabia

Purpose: Guidelines for managing neonatal hemolytic disease of the newborn (HDN) recommend a selective approach in the use of direct antiglobulin test (DAT). In Saudi Arabia, many hospitals still perform routine DAT for all newborns. This study assessed the need for phototherapy in relation to DAT results in full-term healthy newborns.

Patients and Methods: A retrospective analysis of all healthy newborns admitted during 2018 was performed. The primary outcome was the association of positive DAT results with phototherapy.

Results: There were 1463 newborns born during the study period. The DAT was positive at 4.4%. The 24-hour bilirubin levels were higher in DAT-positive cases (P=0.06); however, peak bilirubin levels were not correlated with the DAT results (P=0.717). Thirty-six neonates (2.46%) required phototherapy, and the need was similar among DAT-positive and DAT-negative cases (P=0.271). The most common indication for phototherapy was clinical jaundice in 22 neonates (61.1%), followed by DAT positivity in 12 (33.3%) and hospital protocol in 2 patients (5.6%) (P <0.01 by chi-square overall comparison).

Conclusion: Our results indicate that factors other than DAT positivity are important in assessing the need for phototherapy in newborns. Clinical signs of jaundice were indicators of high serum bilirubin levels and subsequent phototherapy, further indicating that the DAT test was overused in predicting the need for phototherapy.

Keywords: hemolytic disease of the newborn, neonatal jaundice, neonatology, screening

Plain Language Summary

The direct antiglobulin test (DAT) is still used in many centers as a screening test to assess for the risk of hemolytic disease of the newborn. In this retrospective study of all healthy neonates admitted over a one-year period in a university teaching hospital, we correlated, in maternal-neonate pairs, the positive DAT results with the need for phototherapy in the neonates. The results of the study showed that the clinical evaluation of jaundice is more indicative of a need for phototherapy than the DAT result. In a multivariate analysis, only peak bilirubin and 24-hour bilirubin values were statistically significant for phototherapy. We suggest using American and European guidelines, which call for selective use of the DAT for the management of neonatal jaundice. Eliminating overuse will also result in considerable cost savings.

Introduction

The direct antiglobulin test (DAT), also known as the direct Coombs test, is a screening test used primarily to assess whether antibodies are attached to the patient's red blood cells (RBCs), which can lead to destruction of the cell and subsequent immune hemolytic anemia.[1] The predominant causes of positive DAT

Correspondence: Hwazen A Shash
Department of Pediatrics, King Fahd Hospital of the University, Al Khobar 31952, Saudi Arabia
Tel +966540353333
Email hashash@iau.edu.sa

results in neonates are fetal-maternal ABO incompatibility, maternal alloimmunization and, less frequently, maternal autoimmune hemolytic anemia.[2] Hemolytic disease of the newborn (HDN) arises when the mother is exposed to foreign antigens present on fetal RBCs. The mother's immune system recognizes them as foreign antigens and starts producing immunoglobulin G (IgG) antibodies that are able to cross the placenta. Consequently, these antibodies can destroy and shorten the lifespan of fetal RBCs, resulting in clinical consequences.[3] The clinical manifestations of hemolysis in newborns can range from mild anemia and hyperbilirubinemia to severe hemolysis and severe hyperbilirubinemia.[4] Although neonatal jaundice is common and usually harmless, high levels of unconjugated bilirubin may place the infant at risk of developing a form of neurotoxicity known as kernicterus.[4]

For many years, blood typing and DAT testing of cord blood samples were considered standard procedures and performed in many hospitals for all infants. A positive DAT result has been considered a component of the diagnosis of HDN for many years.[5] However, Levine and Meyer[6] found that DAT was not diagnostic and did not predict the severity of hemolytic disease and was therefore unwarranted for routine screening. Madlon-Kay[7] concluded that a selective approach limited to significantly jaundiced infants might be preferable in infants not discharged early. Dinesh[8] reported that clinical jaundice, rather than a positive DAT result, alerted the physician to the possible need for phototherapy. Early onset and the rapid progression of bilirubinemia are the clearest indications of potential HDN and the need for phototherapy.[5]

Given the evidence in favor of a more selective approach to screening, our objective was to determine the need for DAT screening in full-term healthy newborns by correlating clinical signs and DAT results with the need for phototherapy. We also intended to describe the current practices at our institution with the goal of assessing whether changes should be instituted to reduce the costs of newborn screening. We determined the one-year incidence of DAT positivity and studied the relationships between risk factors and the need for phototherapy. We also evaluated the indications for starting phototherapy within the first 48 hours of life and prior to discharge.

Patients and Methods

This study was a retrospective cohort chart review of all newborns admitted to the nursery between January 1 and December 31, 2018, at King Fahad Hospital of the University, Al-Khobar, Saudi Arabia. The study was approved by the University Institutional Review Board (lRB) Committee (lRB UGS-2019-01-349). Data were obtained for both the mothers and newborns from electronic medical records as well as blood bank records. The ABO/Rh status evaluation, DAT results and strength, and antibody identification were performed by the DiaMed gel testing method (Bio-Rad Laboratories, Cressier, Switzerland).

The data extracted for the mothers were age, parity, ABO group, and Rh status. The data collected for the newborns included gender, gestational age in weeks, birthweight in kilograms, ABO group, Rh status, and DAT results. The ABO/Rh type and DAT were determined from the cord blood for all babies and when a sample was clotted from the serum. Bilirubin levels were determined from the serum for all DAT-positive infants, or as per hospital protocol, which requires serum bilirubin to be assessed in infants of diabetic mothers and in infants small for gestational age, regardless of their clinical condition. In addition, the serum bilirubin level may have been assessed for clinical jaundice.

We compared the blood types of the mother with that of the neonate to determine ABO and RhD compatibility. In the newborns with a positive DAT result, we further determined the strength of DAT positivity. The strength of DAT positivity is graded on a scale from 0 to 4+ depending on the degree of agglutination, where 0 indicates no agglutination and 4+ indicates solid agglutination.

The bilirubin levels were determined from the serum in neonates at 3 hours of age if they were DAT positive, at 24 hours of age as per the hospital protocol for infants small for gestational age and infants of diabetic mothers, and at various ages as part of the evaluation for clinical jaundice. The neonates with clinical jaundice were included in analysis if the first bilirubin level was done at an age less than 48 hours and prior to discharge from the nursery. Additional bilirubin level assessments were performed as needed in follow-ups. We plotted bilirubin levels with neonatal age in hours. The need for phototherapy and the selection of the type (single phototherapy, double phototherapy, exchange transfusion) were decided after plotting the bilirubin levels according to an hour-specific bilirubin nomogram based on the National Institute for Health and Clinical Excellence (NICE) guidelines.[9] The assessment of the need for phototherapy was limited to that subset of patients.

Statistical Analysis

Categorical variables are expressed as frequencies and percentages. Continuous variables are summarized as the mean (standard deviation) or median (interquartile range), as appropriate, depending on the normality of the data distribution. Cross tabulation and statistical significance of variables were assessed by independent t-test or chi-square test. A separate multivariate logistic regression with possible phototherapy requirement as the dependent variable was performed to explore which variables were associated with the need for phototherapy. A p value of <0.05 was considered to indicate statistical significance. The software package used in the multivariate analysis was jamovi, version 1.1.9.0 (retrieved from https://www.jamovi.org). The data were plotted with the R package ggplot2 3.2.1.

Results

Sixty-five neonates (65/1463, 4.4%) were DAT positive and 59 of those (90.8%) were born to mothers with blood group O. ABO incompatibility was more frequent in cases with a positive DAT result (P <0.001), but there was no difference in relation to Rh incompatibility (P=0.239). The serum bilirubin level was assessed in 251 neonates for the following reasons: 65 (25.9%) due to DAT positivity, 106 (42.2%) due to hospital protocol, and 80 (31.9%) due to clinical jaundice. The median (25th percentile, 75th percentile, range) maternal age of the 251 mothers was 32.0 (28.0, 36.0, 19–49) years. The mean parity was 3.0.

The bilirubin levels in neonates with clinical jaundice were more commonly obtained after 24 hours of age, which was likely related to the timing of discharge examination. The 24-hour bilirubin levels were higher in DAT-positive cases (P=0.06); however, peak bilirubin levels were not correlated with the DAT results (P=0.717). The median age for the first bilirubin level for neonates with DAT positive results was 3.5 hours, while neonates assessed due to hospital protocol and neonates with clinical jaundice were tested at a median of 24 and 36 hours, respectively. The 24-hour bilirubin level was measured in 170 patients for the following reasons: 61 (35.9%) neonates were DAT positive, 10 (5.9%) neonates had clinical jaundice, and 99 (58.2%) neonates were assessed per hospital protocol. There were 4 patients who were DAT positive without 24-hour serum bilirubin data, as the treating physician deemed it not necessary to obtain these data

because the 3-hour serum bilirubin level was <59.85 µmol/L, and there were 9 patients in the hospital protocol group for whom testing was performed at variable times by request of the treating physician. The bilirubin levels in neonates with clinical jaundice were more commonly obtained after 24 hours of age, which was likely related to the timing of discharge examination.

Phototherapy was initiated in 36 (14.3%) of the 251 neonates in whom the need was assessed. The assessment was based on serum bilirubin levels and the hospital nomogram (based on the NICE guidelines) (Table 1). The most common indication for phototherapy was clinical jaundice in 22 neonates (61.1%), followed by DAT positivity in 12 (33.3%) and hospital protocol in 2 patients (5.6%) (P <0.01 by chi-square overall comparison). There was no difference in gender, gestational age, or birthweight between the groups (Table 1). There was also no difference in ABO/Rh incompatibility between the groups (Table 2). The need for phototherapy was similar in the DAT-positive cases and DAT-negative cases (P=0.271), with a sensitivity of 33.3% (95% CI 19.1–51.0) and positive predictive value of 18.5% (95% CI 10.3–30.4) for predicting phototherapy need. Phototherapy requirement did not differ in relation to the strength of DAT positivity (P=0.333) (Table 3). The lack of statistical significance emphasizes that DAT should not guide clinicians in their evaluation for the need of obtaining bilirubin levels to evaluate the need of phototherapy.

The 24-hour and first serum bilirubin levels were significantly higher in infants who were deemed to require phototherapy (Table 3) (P <0.001 for both comparisons). There were only two babies in which serum bilirubin was assessed for hospital protocol reasons and required phototherapy, indicating the possibility that the bilirubin level is assessed excessively per our hospital protocols.

The type of phototherapy was single in most cases (91.7%, n=33), and none required exchange transfusion. In the comparisons of the peak bilirubin level by age at sampling, the serum bilirubin values for neonates who underwent phototherapy clearly clustered among the neonates with higher peak bilirubin levels, regardless of the DAT results (Figure 1). Figure 1 reflects the difference in bilirubin levels by phototherapy requirement in Table 3. When neonates with a gestational age less than 38 weeks were compared with the others, the peak bilirubin levels for the neonates who underwent phototherapy were generally higher than in those who did not undergo phototherapy (Figure 2) (P=0.947).

Table 1 Demographics of the Patients Assessed for the Need for Phototherapy (n=251)

	No Phototherapy (n=215)	Required Phototherapy (n=36)	P value	Overall (n=251)
Gender			0.625	
Male	111 (51.6%)	17 (47.2%)		128 (51.0%)
Female	104 (48.4%)	19 (52.8%)		123 (49.0%)
Birthweight (kg)				
Median [25th, 75th percentile]	3.10 [2.80, 3.40]	2.90 [2.60, 3.30]	0.196	3.02 [1.40, 4.70]
Missing	27 (12.6%)	3 (8.3%)		30 (12.0%)
Gestational age (categorical)			0.782	
≤38+6	103 (51.0%)	19 (55.9%)		122 (51.7%)
39 to 40+6	84 (41.6%)	12 (35.3%)		96 (40.7%)
>41	15 (7.4%)	3 (8.8%)		18 (7.6%)
Gestational age (days)				
Mean (SD)	271 (10.3)	269 (11.6)	0.216	271 (10.5)
Missing	24 (11.2%)	2 (5.6%)		26 (10.4%)

Note: Comparisons of the mean (independent *t*-test) for the normally distributed data and the median (chi-square test) for the nonnormally distributed data.

Table 2 Maternal and Neonatal ABO Blood Groups of the Patients Assessed for the Need for Phototherapy (n=251)

	No Phototherapy (n=215)	Required Phototherapy (n=36)	P value	Overall (n=251)
Maternal blood group			0.441	
Blood group O	121 (56.3%)	22 (61.1%)		143 (57.0%)
Blood group A	46 (21.4%)	5 (13.9%)		51 (20.3%)
Blood group B	41 (19.1%)	9 (25.0%)		50 (19.9%)
Blood group AB	7 (3.3%)	0 (0%)		7 (2.8%)
Neonate blood group			0.082	
Blood group O	86 (40.0%)	9 (25.0%)		95 (37.8%)
Blood group A	52 (24.2%)	8 (22.2%)		60 (23.9%)
Blood group B	63 (29.3%)	18 (50.0%)		81 (32.2%)
Blood group AB	14 (6.5%)	1 (2.8%)		15 (6.0%)
Maternal/neonatal ABO blood compatibility			0.292	
Compatible	110 (51.2%)	15 (41.7%)		125 (49.8%)
Incompatible	105 (48.8%)	21 (58.3%)		126 (50.2%)
Maternal/neonatal Rh blood compatibility			0.5	
Compatible	193 (89.8%)	33 (91.7%)		226 (90%)
Incompatible	22 (10.2%)	3 (8.3%)		25 (10%)

Note: Comparisons of the mean (independent *t*-test) for the normally distributed data and the median (chi-square test) for the nonnormally distributed data.

Table 3 The DAT Results and Bilirubin Levels in Patients Assessed for Phototherapy (n=251)

	No Phototherapy (n=215)	Required Phototherapy (n=36)	P value	Overall (n=251)
DAT results			0.271	
Negative	162 (75.3%)	24 (66.7%)		186 (74.1%)
Positive	53 (24.7%)	12 (33.3%)		65 (25.9%)
DAT strength (In DAT-positive patients, n=65)			0.333	
1+	25 (47.2%)	3 (25%)		28 (43.1%)
2+	25 (47.2%)	8 (66.67%)		33 (50.8%)
3+	3 (5.6%)	0 (0%)		3 (4.6%)
4+	0	0		0
Missing	0	1 (8.3%)		1 (1.5%)
Total	53 (81.5%)	12 (18.46%)		65
First serum bilirubin level (μmol/L)				
Median [25th, 75th percentile]	88.9 [65.0, 118.0]	130 [94.05, 191.52]	<0.001	
Age at first serum bilirubin level (hours)				
Median [25th, 75th percentile]	24.0 [13.0, 25.0]	23.0 [4.75, 33.8]	0.629	
24-hour serum bilirubin level (μmol/L)				
Median [25th, 75th percentile]	88.9 [75.8, 110.6]	149.6 [115.9, 186.4]	<0.001	
Peak bilirubin level				
Median [25th, 75th percentile]	104.31 [85.5, 141.9]	210.3 [181.3, 239.4]	<0.001	
Age at peak serum bilirubin level (hours)				
Median [25th, 75th percentile]	25.0 [24.0, 48.0]	58.5 [48.0, 72.0]	<0.001	31.0 [24.0, 52.0]

Note: Comparisons of the mean (independent *t*-test) for the normally distributed data and the median (chi-square test) for the nonnormally distributed data.

Several factors were assessed to explore relationships with the need for phototherapy. In the univariate analyses, the serum bilirubin values were the only statistically significant variables. Other variables, such as ABO compatibility, gestational age, birthweight, and DAT results, were included in the multivariate analysis for clinical reasons or because of known or suspected associations with the need for phototherapy. However, only the peak bilirubin and 24-hour bilirubin values were statistically significant.

A total of 64 eluates were detected in the DAT-positive neonates. The most commonly detected antibodies were anti-B (45.3%) followed by anti-A (32.8%). There were 13 DAT-positive neonates with a positive maternal antibody screen. The most common maternal antibody detected was anti-D in 5 mothers (38.5%). The remaining antibodies detected included anti-E (n=3), anti-M (n=1), and anti-c (n=1) antibodies. There

was no antibody titer reported for the mothers or newborns. Eleven (84.6%) of the neonatal eluates had antibodies that were the same as in the mothers. The two newborns that had different eluates than the mothers were a nonspecific antibody in a neonate born to a mother with anti-D antibody, and anti-B antibody detected in a mother with anti-M.

Discussion

Our results indicate that factors other than a positive DAT are important in assessing the need for phototherapy in Saudi newborns potentially at risk for HDN. As expected, clinical signs of jaundice were found to be an indicator of high serum bilirubin levels. The neonates with positive DAT results did not have higher peak bilirubin levels or any other indication for phototherapy requirement compared to those with a negative DAT.
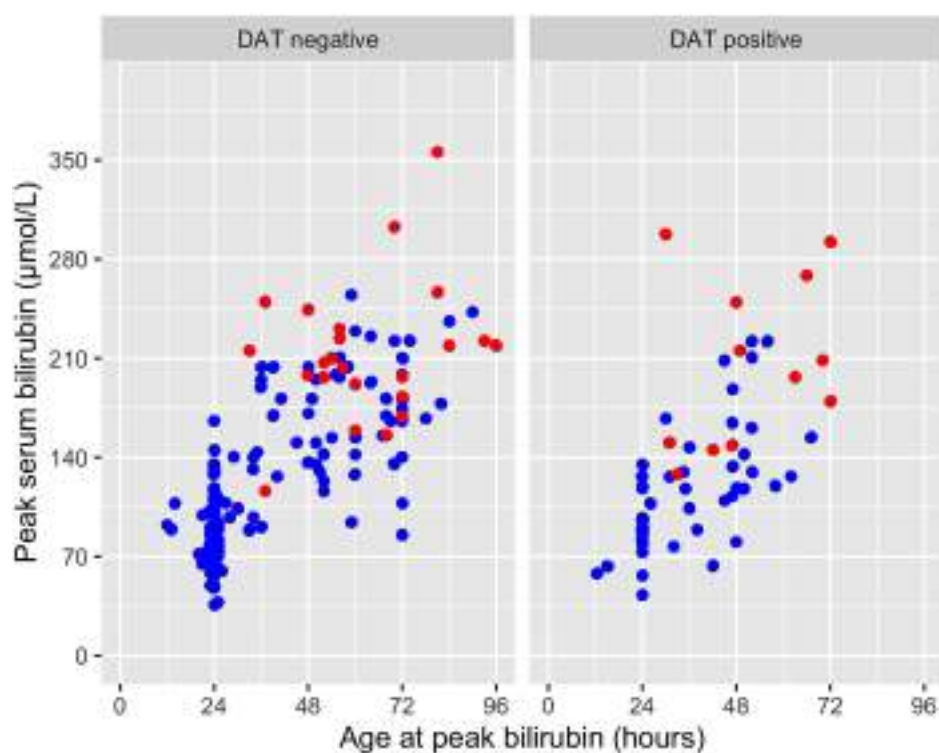
Figure 1 Peak serum bilirubin by age at sampling in neonates by DAT results. Neonates that underwent phototherapy are red points and neonates that did not undergo phototherapy are blue points (n=251) by DAT results.
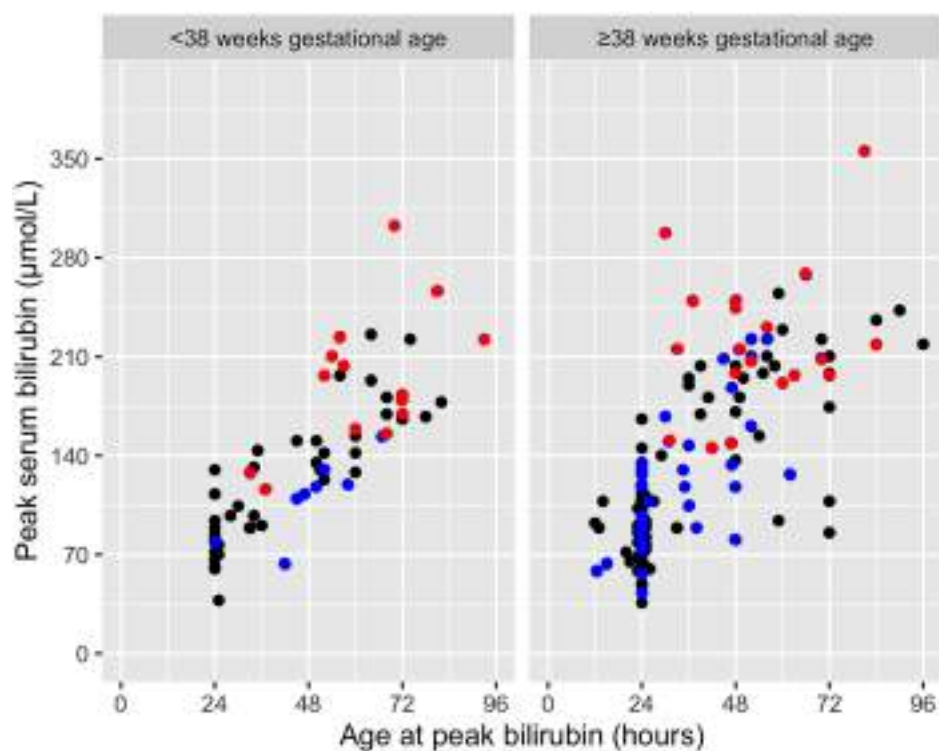
Figure 2 Peak serum bilirubin by age at sampling and gestational age in weeks < 38 weeks and > 38 weeks in neonates who underwent phototherapy and were DAT positive (red) or DAT negative (blue) (n=225).

Our results are consistent with other reports in the literature. As noted by Keir et al[1], hyperbilirubinemia alone does not indicate a need for the DAT in the absence of anemia, reticulocytosis, or other signs of hemolysis. The positive predictive value of the test for significant hyperbilirubinemia was poor, ranging from 12 to 53%, as shown in our results.[8,10,11] A third of neonates of ABO incompatible mothers may have a positive DAT result, but few develop HDN.[12]

Studies have shown that unselective DAT testing for all infants is not cost-effective.[12,13] The authors of these studies concluded that selective newborn cord testing of neonates born to mothers with blood group O is feasible and can decrease hospital costs without increasing the risk of HDN. Resources and costs, as a result, would be saved without increasing the risk of clinically significant hyperbilirubinemia.[12] Such selective testing in fact has been implemented in many institutions, and many recommendations advocate for testing according to the clinical evaluations of newborns.[14,15] In Saudi Arabia, there is no unified approach in screening for HDN. While some centers are performing routine DAT testing for all neonates, others perform only selective testing for blood group O or testing of symptomatic neonates only.

In our study, we examined the issue in terms of the need for phototherapy and found that the decision to use phototherapy in neonates did not differ between DAT-positive and DAT-negative infants. As expected, serum bilirubin levels, regardless of the age of testing, were significantly higher in neonates who required phototherapy and unrelated to DAT results. In the plot of the peak serum bilirubin levels as a function of the DAT results, the bilirubin levels are similar in the infants who did and did not undergo phototherapy. In the DAT-negative neonates, clinical signs of jaundice were indicators of high serum bilirubin levels, further indicating that the DAT test is not of value in predicting which infants might be in need of phototherapy. The neonates requiring phototherapy were more commonly identified by clinical evaluations, emphasizing the importance of clinical assessments and the likelihood that serum bilirubin assessments and the DAT are being overused in our institution.

Studies that evaluated the effectiveness of the DAT in predicting hyperbilirubinemia demonstrated sensitivity values ranging from 15% to 64%, which is consistent with our results.[8,10,11] Maisels and Watchko[16] suggested in 2013 that routine testing for blood type and the DAT in infants of blood group O mothers "can safely be abandoned". The recommendations of the American Academy of Pediatrics (AAP) published in 2004 considered the tests optionally provided optimal follow-up.[14] In multivariable analyses,[17,18] bilirubin levels were the one factor most predictive of the need for phototherapy and future development of hyperbilirubinemia. Our multiple logistic regression results were consistent with these findings.

There are some limitations in our studies that warrant future follow-up investigations. Although we included all maternal-neonate pairs over one year, the size of the sample was relatively small, being from one institution. However, we had sufficient data to evaluate the important variables in univariate analyses, namely, the serum bilirubin levels in the early ages of life, and associate those data with the need for phototherapy to prevent potential severe hyperbilirubinemia. We were not able to evaluate other factors that may cause an increased risk of HDN, such as hemoglobin H disease or glucose-6-dehydrogenase deficiency, as they are not assessed as part of neonatal screening and not routinely evaluated in neonates started on phototherapy. In addition, one aspect we were unable to evaluate was the strength of DAT positivity in relation to the need for phototherapy. Strength was measured in only 58 patients (89.2% of DAT-positive neonates), and of those patients, high values for strength were observed in only two patients who had DAT strength values of 3+, and neither required phototherapy. We did not observe any patients with DAT values of 4+, which has been reported to be strongly associated with the need for a prolonged duration of phototherapy.[8,19] The NICE guidelines recommend that when the DAT is performed in infants who undergo a formal assessment for significant hyperbilirubinemia, the strength of the reaction is considered.[9] The results should be interpreted with consideration of whether the mother has received anti-D prophylaxis during pregnancy, as maternal anti-D is associated with positive DAT in neonates.[20] Furthermore, there may have been selection bias regarding the infants from whom a serum bilirubin sample was taken after clinical evaluation, since the practice was to assess the bilirubin level in all DAT-positive infants. As the results show, clinical jaundice rather than DAT positivity was indicative of the need for phototherapy. We also do not have the complete records of the post discharge follow-ups of babies who may have later required phototherapy since some neonates may have gone to other hospitals. Of the 27 babies that presented to our emergency department for the assessment of clinical jaundice, all of them were DAT negative, and five (18.5%) required phototherapy.

Despite these shortcomings, our study supports the notion that unselective DAT testing in newborns should not be the standard care for HDN prevention and management. In this regard, the NICE guidelines also recommend against the use of DAT and provide a set of nomograms and guidance for initiating phototherapy based solely on the serum bilirubin level.[9] For neonates of gestational age 38 weeks or more, they recommend that phototherapy be considered when bilirubin levels are higher than 250 μmol/L at 24 hours of age. In many cases, this recommendation is followed but is not universally applied in our institution. All newborns in our study underwent direct antiglobulin testing. We recommend a change from this unselective use of the DAT to a policy that follows the American and European guidelines for managing neonatal jaundice to avoid the costs of routine DAT testing for all full-term healthy newborns. A prospective study evaluating selective DAT and bilirubin testing in cases of clinical jaundice in our center is warranted, and follow-ups post discharge are necessary to study the risk factors that may indicate an increased risk of readmission for neonatal jaundice.

Conclusions

Our paper confirmed that the DAT is not useful alone for screening for HDN, and there are other factors important in assessing the need for phototherapy. The test is overused in our center due to unselective testing. The most useful indicator for the need for phototherapy is clinical jaundice. A clinical evaluation of neonates is essential and will likely provide the first indication for the need for phototherapy.

Abbreviations

DAT, direct antiglobulin test; NICE, National Institute for Health and Clinical Excellence; HDN, hemolytic disease of the newborn; RBC, red blood cells; NICU, neonatal intensive care.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

The study was approved by the University Institutional Review Board (lRB) and Ethical Committee (lRB UGS-2019-01-349). This is a retrospective study conducted on electronic records of patients with no patient identity being disclosed. Therefore, informed consent was not applicable. This study was conducted in accordance with the Declaration of Helsinki.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Keir A, Agpalo M, Lieberman L, Callum J. How to use: the direct antiglobulin test in newborns. *Arch Dis Child Educ Pract Ed.* 2015;100(4):198–203. doi:10.1136/archdischild-2013-305553
2. Valsami S, Politou M, Boutsikou T, Briana D, Papatesta M, Malamitsi-Puchner A. Importance of Direct Antiglobulin Test (DAT) in cord blood: causes of DAT (+) in a cohort study. *Pediatr Neonatol.* 2015;56(4):256–260. doi:10.1016/j.pedneo.2014.11.005
3. Klein HG, Anstee DJ. *Mollison's Blood Transfusion in Clinical Medicine.* Oxford: Wiley-Blackwell; 2014.
4. Hendrickson JE, Delaney M. Hemolytic disease of the fetus and newborn: modern practice and future investigations. *Transfus Med Rev.* 2016;30(4):159–164. doi:10.1016/j.tmrv.2016.05.008
5. Murray NA, Roberts IA. Haemolytic disease of the newborn. *Arch Dis Child Fetal Neonatal Ed.* 2007;92(2):F83–F88. doi:10.1136/adc.2005.076794
6. Levine DH, Meyer HB. Newborn screening for ABO hemolytic disease. *Clin Pediatr.* 1985;24(7):391–394. doi:10.1177/000992288502400707
7. Madlon-Kay DJ. Identifying ABO incompatibility in newborns: selective vs automatic testing. *J Fam Pract.* 1992;35(3):278–280.
8. Dinesh D. Review of positive direct antiglobulin tests found on cord blood sampling. *J Paediatr Child Health.* 2005;41(9–10):504–507. doi:10.1111/j.1440-1754.2005.00692.x
9. National Institute for Health and Care Excellence (NICE). Jaundice in newborn babies under 28 days; 2020. Available from: https://www.nice.org.uk/guidance/cg98/evidence. Accessed August 30, 2020.
10. Herschel M, Karrison T, Wen M, Caldarelli L, Baron B. Evaluation of the direct antiglobulin (Coombs') test for identifying newborns at risk for hemolysis as determined by end-tidal carbon monoxide concentration (ETCOc); and comparison of the Coombs' test with ETCOc for detecting significant jaundice. *J Perinatol.* 2002;22 (5):341–347. doi:10.1038/sj.jp.7210702
11. Meberg A, Johansen KB. Screening for neonatal hyperbilirubinaemia and ABO alloimmunization at the time of testing for phenylketonuria and congenital hypothyreosis. *Acta Paediatr.* 1998;87(12):1269–1274. doi:10.1111/j.1651-2227.1998.tb00950.x
12. Shahid R, Graba S. Outcome and cost analysis of implementing selective Coombs testing in the newborn nursery. *J Perinatol.* 2012;32(12):966–969. doi:10.1038/jp.2012.26
13. Leistikow EA, Collin MF, Savastano GD, de Sierra TM, Leistikow BN. Wasted health care dollars. Routine cord blood type and Coombs' testing. *Arch Pediatr Adolesc Med.* 1995;149 (10):1147–1151. doi:10.1001/archpedi.1995.02170230101015
14. American Academy of Pediatrics Subcommittee on Hyperbilirubinemia. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics.* 2004;114(1):297–316. doi:10.1542/peds.114.1.297
15. Newman J. Guidelines for detection, management and prevention of hyperbilirubinemia in term and late preterm newborn infants (35 or more weeks' gestation) - Summary. *Paediatr Child Health.* 2007;12 (5):401–418. doi:10.1093/pch/12.5.401

16. Maisels MJ, Watchko JF. Routine blood typing and DAT in infants of group O mothers. *J Perinatol*. 2013;33(7):579. doi:10.1038/jp.2013.6

17. Bhutani VK, Stark AR, Lazzeroni LC, et al. Predischarge screening for severe neonatal hyperbilirubinemia identifies infants who need phototherapy. *J Pediatr*. 2013;162(3):477–482.e1. doi:10.1016/j.jpeds.2012.08.022

18. Maisels MJ, Deridder JM, Kring EA, Balasubramaniam M. Routine transcutaneous bilirubin measurements combined with clinical risk factors improve the prediction of subsequent hyperbilirubinemia. *J Perinatol*. 2009;29(9):612–617. doi:10.1038/jp.2009.43

19. Oztekin O, Kalay S, Tezel G, et al. Is the strength of direct antiglobulin test important for the duration of phototherapy? *J Matern Fetal Neonatal Med*. 2014;27(5):534–536. doi:10.3109/14767058.2013.819335

20. Dillon A, Chaudhari T, Crispin P, Shadbolt B, Kent A. Has anti-D prophylaxis increased the rate of positive direct antiglobulin test results and can the direct antiglobulin test predict need for phototherapy in Rh/ABO incompatibility? *J Paediatr Child Health*. 2011;47(1–2):40–43. doi:10.1111/j.1440-1754.2010.01888.x

Autoimmune Acquired Factor XIII Deficiency: A Case Report

Ana Marco & Pascual Marco

CASE REPORT

Autoimmune Acquired Factor XIII Deficiency: A Case Report

Ana Marco [iD][1,2]
Pascual Marco [iD][1–3]

[1]Thrombosis and Hemostasis Department, Hematology Service, University General Hospital in Alicante, Alicante, Spain; [2]Biomedical Health Research Institute, University General Hospital in Alicante, Alicante, Spain; [3]Department of Clinical Medicine, Miguel Hernández University, Alicante, Spain

Abstract: Autoimmune acquired factor XIII (FXIII) deficiency is a rare disorder characterized by severe spontaneous hematomas and autoantibodies against FXIII. High mortality rates have been reported (18% within a year of diagnosis). We present a 70-year-old patient with recurrent muscular hematomas. The basic hemostasis study and the coagulation factors were within normal ranges. The aggregation platelet study was also normal and von Willebrand disease was excluded. Bearing in mind the recurrent bleeding history and the described laboratory results, we considered a FXIII deficiency, that was confirmed (FXIII<10%). In addition, we suspected an acquired FXIII deficiency since the patient did not report a personal or family history of bleeding and FXIII gene sequencing study was normal. Non-immune causes were ruled out, and plasma autoantibodies against FXIII were detected. Immunosuppression was rapidly initiated to eradicate inhibitor as was hemostatic treatment to obtain bleeding control. Currently, the patient is asymptomatic, but a low level of FXIII inhibitor remains.

Keywords: acquired disease, autoantibodies, factor XIII deficiency, hematoma, spontaneous

Introduction

Coagulation factor XIII (FXIII) is a hetero-tetrameric zymogen that plays a key role in clot stabilization. FXIII is comprised of two A and two B subunits and is activated by thrombin and calcium.[1] However, other additional functions have been described. Although FXIII-B is generated in the liver, FXIII-A is produced by hematopoietic cells. Therefore, FXIII contributes to pregnancy maintenance, bone and cartilage growth and wound healing.[1]

FXIII deficiency is characterized by variable bleeding episodes depending on FXIII levels. Congenital FXIII deficiency is an autosomal recessive rare disease with an incidence of 1.5 cases per 2 million people. It is characterized by severe and spontaneous bleeding, including umbilical and intracranial hemorrhage at birth, usually in case of severe deficiency (FXIII<1%). Other symptoms include soft tissue bleeding, recurrent miscarriages, bruising and hemarthroses.[2] Inhibitor development against FXIII in inherited FXIII deficiency in patients receiving FXIII replacement therapy is also infrequent but may induce severe bleeding.[3] However, acquired FXIII deficiency is more common and can be classified as immune and non-immune disease. Acquired immune-mediated FXIII deficiency implies the presence of an autoantibody targeting FXIII epitopes which may lead to severe bleeding. The clinical presentation is variable from mucocutaneous and intramuscular bleedings to life-threatening hemorrhages such as intracranial, intra-thoracic or intra-peritoneal.[4] In addition, clinical bleeding in any

Correspondence: Ana Marco
University General Hospital in Alicante, Pintor Baeza 12, Alicante, 03010, Spain
Tel +34 965913863
Fax +34 965913869
Email marco_anaric@gva.es

acquired immune factor deficiency does not correlate with factor level or inhibitor titer.[5] Ichinose et al described the largest series in the literature, including 93 patients with a large proportion of patients from Japan (59).[6] Around 20% died within the first year of diagnosis. Non-immune causes are more common but rarely cause bleeding and are related to excessive consumption or decreased production of FXIII.[7]

An early diagnosis is essential, as acquired immune FXIII deficiency can be fatal if proper treatment is not rapidly provided.

Case Report

A 70-year-old male was admitted to our clinic for assessment of a spontaneous hematoma in the lower right extremity. Antiplatelet and anticoagulant treatment was not reported. The ultrasound revealed a 14 cm quadriceps hematoma. The patient also referred the presence of provoked hematomas in upper and lower extremities in the last 6 months, which evolved adequately with tranexamic acid. The initial blood test including a basic hemostasis study and a complete blood count was within normal ranges. The platelet function study and the von Willebrand profile were also normal. All coagulation factors were normal, except for the FXIII antigen of 9% confirmed in 2 different samples. Fibrinogen and alfa2-antiplasmin were in the range of normality. Factor activity assays (extrinsic and intrinsic pathways) were performed with a one-stage coagulant assay using factor-deficient plasma in automated coagulometer (ACL-Top, IL, Bedford, USA). Von Willebrand factor antigen and ristocetin cofactor activity were performed by chemiluminescence (HemosIL AcuStar, IL, Bedford, USA) in automated coagulometer (ACL-AcuStar, IL, Bedford, USA) in poor platelet plasma. Fibrinogen was measured using the coagulative Clauss method (STA-Liquid-Fib, Diagnostica Stago, Paris, France) in poor platelet plasma (STA-Rac, Diagnostica Stago, Paris, France) in automated coagulometer and alfa2-antiplasmin was determined by chromogenic assay (ACL-Top, IL, Bedford, USA) in poor platelet plasma, following the manufacturer´s recommendations. FXIII antigen was determined using the automated latex immunoassay (FXIII antigen latex reagent, IL, Bedford, USA) in automated coagulometer (ACL-Top, IL, Bedford, USA), following the manufacturer´s instructions.

We considered an acquired immune FXIII deficiency, confirmed by Bethesda assay (inhibitor titer of 12 BU) and absence of mutation in FXIII gene sequencing study. The Bethesda assay quantifies the amount of inhibitor that neutralizes 50% residual factor in an equal mixture of normal plasma and patient plasma in 2 hours at 37º.[8]

We also excluded any underlying disease and immune dysregulation. Initial weight-adjusted treatment (weight of 70 Kg) with prednisone 1 mg/kg/day (70 mg daily) for 15 days and immunoglobulins 1g/kg/day (70 g daily) for 2 days was prescribed. The patient presented an appropriate clinical and analytical improvement, with a remarkable decrease in the autoantibody titer. We monitored FXIII levels and performed the thrombin generation test before starting steroids, 1 week and 2 weeks after. The patient had an increase in FXIII levels throughout the scheduled visits associated with an enlargement in the endogenous thrombin potential and a higher thrombin peak (Figure 1). This improvement was confirmed by the thromboelastometry, which showed an increase in clot firmness and a shortened clot formation time (Figure 2). Thrombin generation was determined by the fluorometric method described by Hemker,[9] modified and automated (STA Genesia, Diagnostica Stago, Paris, France) in poor platelet plasma. We used the STG-Bleedscreen reagent (Diagnostica Stago, Paris, France) that contains a mixture of phospholipids and a low recombinant tissue factor concentration. Thrombin generation assay provides information on the lag time of thrombin generation, the endogenous thrombin potential, the peak of thrombin concentration, the time to reach the maximum peak of thrombin and the time to thrombin neutralization. Rotational thromboelastometry (ROTEM®, IL, Bedford, USA) provides global information about the dynamics of clot development, stabilization and dissolution using citrated whole blood. The following ROTEM® parameters were measured in EXTEM (provides similar information to prothrombin time) and FIBTEM (analyzes the functional fibrinogen component): clotting time, clot formation time and maximum clot firmness. EXTEM and FIBTEM are fibrinogen and FXIII dependent.

However, the patient had poor tolerance to corticosteroids with hypertension and anxiety, so we decided their discontinuation. No new bleeding episodes were reported. Due to persistence of the inhibitor, rituximab was then proposed, but since the patient refused, immunosuppressive therapy was changed to azathioprine 50 mg daily. One week later the patient referred vomiting and diarrhea, so azathioprine was discontinued. The patient remained clinically stable. One month after discontinuation of immunosuppression, the patient was admitted to the emergency department with thoracic oppressive pain and minimal
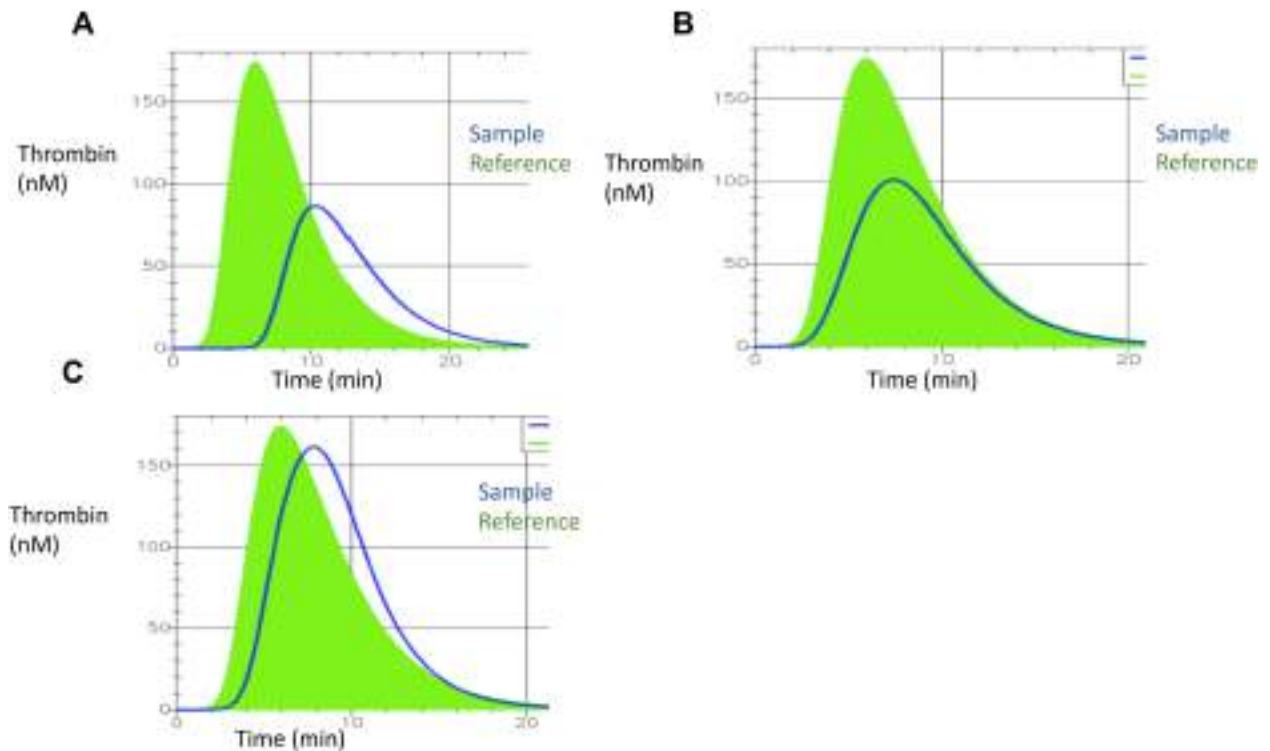
Figure 1 Changes in thrombin generation depending on FXIII antigen. (**A**) Before starting steroids. Baseline FXIII of 5%. (**B**) The patient received steroids for 1 week. Baseline FXIII of 34%. (**C**) The patient received steroids for 2 weeks. Baseline FXIII of 45%. Reference within normal parameters is shown in green and patient´s sample in blue.
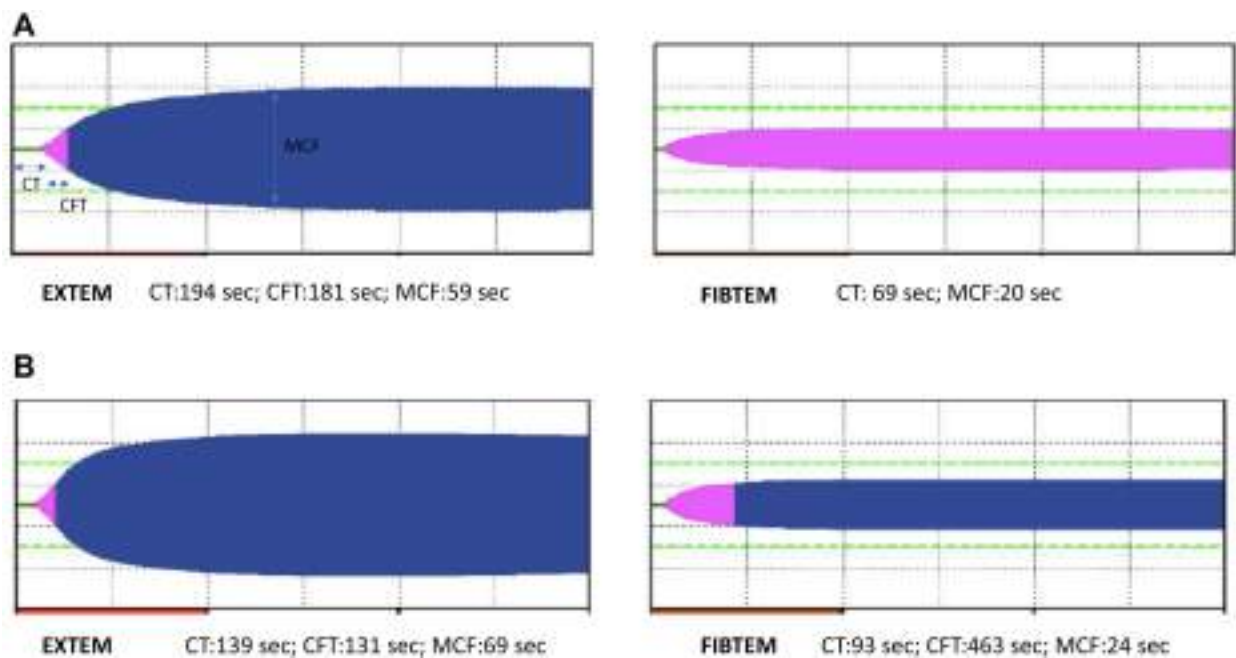
Figure 2 Changes in thromboelastography. (**A**) The patient received corticosteroids for 1 week. (**B**) The patient received steroids for 2 weeks.
Abbreviations: CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness.

Table 1 Changes in Hemostatic Parameters Throughout Time

	FXIII Antigen (%)	VWF Antigen (%)	VWF Activity (%)	Fibrinogen (mg/dl)	Bethesda Units (Inhibitor Titer, BU)	APTT Ratio	INR
At diagnosis	9	116	127	240	12	1.25	1.2
Corticosteroids discontinuation	35	145	130	290	1.4	1.15	1.3
Admission for mediastinal hematoma	20	180	160	270	3	1.28	1.2
Last blood sample	55	190	200	310	1	1.12	1.1

Notes: References values: FXIII>60%, VWF antigen:45–150%, VWF activity:45–150%, Fibrinogen:200–450 mg/dl, Bethesda Units<0.6 BU, APTT R: 0.8–1.25, INR: 0.8–1.30.
Abbreviations: VWF, Von Willebrand factor; APTT, activated partial thromboplastin time; INR, international normalized ratio.

effort dyspnea for 24 hours. The blood count revealed mild leukocytosis with neutrophilia (12.27 x10^3/μL leukocytes and 10.53 x10^3/μL neutrophils), hemoglobin of 14 gr/dl (previous was 16 gr/dl) and platelets of 248 x10^3/μL. The basic hemostatic study was normal, the baseline FXIII level was 20% and the inhibitor titer was 3 BU. The chest x-ray and CT scan objectified the presence of a mediastinal hematoma. Hemostatic treatment with fresh frozen plasma (15 mL/kg) and tranexamic acid (500 mg/8 hours) as well as immunosuppressive therapy with ritux-imab (375 mg/m^2 weekly for 4 weeks, 678 mg weekly) were administered (body surface of 1.81 m^2). Factor XIII concentrate was not necessary, due to clinical stability. No further bleedings were reported. The patient is currently receiving corticosteroids at low doses (20 mg daily), with an excellent clinical evolution. The patient is asympto-matic with no relevant side effects. The last blood sample (5 months after initial diagnosis) was taken 1 month after corticosteroids 20 mg daily were initiated and revealed a FXIII of 55% and an antibody titer of 1 BU. Changes in hemostatic parameters are shown in Table 1.

Discussion

Acquired immune FXIII deficiency is a rare disease caused by the presence of an autoantibody which targets FXIII epitopes and may cause severe and life-threatening bleed-ing. About 50% of the cases are idiopathic. Autoimmune diseases and malignancies are the most prevalent under-lying conditions. Intramuscular and subcutaneous bleeding events are the most frequently reported.[4]

Evaluating differential diagnosis, congenital FXIII defi-ciency was ruled out, as the patient referred a negative personal and familiar bleeding tendency and no mutation in FXIII gene sequencing study was detected. Non-immune-mediated causes of acquired FXIII deficiency including increased consumption or decreased production like recent surgery, liver disease, cancer, sepsis or interfering medication were also discarded. In these cases, life-threatening bleeding events rarely appear, FXIII levels normally range between 20% and 70% and anti-bodies against FXIII are not detected. Here we report a major bleeding with FXIII level below 10% and presence of anti-bodies against FXIII. Finally, other autoimmune coagulation factor deficiencies such as acquired hemophilia or acquired Von Willebrand disease were not considered because all coa-gulation factors and von Willebrand factor (antigen and activ-ity) were within normal range except for FXIII.

Our patient met the diagnostic criteria for immune-mediated acquired FXIII deficiency proposed by the ISTH.[4] He referred no previous personal or family bleeding history and was not receiving anticoagulant or antiplatelet treatment. In our laboratory, the identification of neutraliz-ing autoantibodies was confirmed using a 1:1 mixing test of patient plasma and normal control plasma, with quantifica-tion of the inhibitor by the Bethesda assay. Most inhibitors against FXIII are directed against A subunit.[4,10] In our patient, it was not possible to identify the inhibitor specifi-city although the reagent to determine FXIII antigen is based on an antibody highly specific for A subunit.

The sequencing study of FXIII gene detected no muta-tion, supporting the final diagnosis of acquired immune FXIII deficiency.

Thrombin generation and thromboelastometry could be useful global hemostasis tests for monitoring clinical and biological evolution. To our knowledge, these global tests in acquired immune FXIII deficiency have not been pre-viously reported. Our patient presented a significant decrease in thrombin generation compared with normal reference plasma at diagnosis that improved as FXIII increased and inhibitor titer decreased (Figure 1). In agreement with Gosh et al, we described a reduction in the peak height of thrombin

and endogenous thrombin potential at diagnosis although they only determine basal Factor XIII levels in patients with inherited FXIII deficiencies.[11] We suggest that as FXIII levels increase, more thrombin is generated that binds to FXIII, promoting a positive feedback and activation of the coagulation cascade.[12] These results were endorsed by the thromboelastography that showed an increase in maximum clot firmness and a shortened clot formation time (Figure 2A and B). Theusinger et al obtained similar results in critical patients in the postoperative period of a major surgery (acquired non-immune FXIII deficiencies).[13]

Regarding the management of these patients, hemostatic and antibody eradication therapy should be rapidly initiated. However, given the rarity of this disease, evidence-based recommendations are lacking.[14] High-dose FXIII concentrates (50–100 IU/kg) or FXIII-containing blood products like fresh frozen plasma or cryoprecipitate could be an optimal approach to achieve hemostatic control. However, they could show no effectiveness in the presence of high titer inhibitor.[14] Other hemostatic treatments including antifibrinolytics like tranexamic acid together with FXIII administration have been associated with favorable effects.[14] Recombinant factor VII has been rarely reported probably due to effectiveness of FXIII concentrates or fresh frozen plasma. However, it should be considered in severe bleedings with no response to FXIII-containing products.[15] Immunosuppressive treatments include corticosteroids alone or in combination with cyclophosphamide. Rituximab, ciclosporin or immunoglobulins have also presented successful results.[15,16] Our patient received first-line immunosuppressive treatment with corticosteroids and immunoglobulins and hemostatic treatment with tranexamic acid. Although immunosuppression was initially effective, steroids were discontinued due to side effects. Although no new bleeding events appeared, a second-line immunosuppression treatment with azathioprine was initiated, that was also discontinued due to side effects. Four weeks later, the patient developed a mediastinal hematoma that evolved favorably with rituximab and hemostatic treatment with fresh frozen plasma and tranexamic acid.

A fatal intracranial hemorrhage in an adult patient with comorbidities diagnosed of an acquired FXIII deficiency has been recently published. This patient received rituximab as immunosuppression and hemostatic treatment with fresh frozen plasma and rFVIIa.[17]

Our patient developed a major bleeding episode. Neither a triggering disease nor an underlying drug was identified. However, the patient evolved favorably with an appropriate response to immunosuppression and hemostatic treatment. Generally speaking, even with this atypical favorable presentation, we suggest, in agreement with the literature, an early diagnosis and management to prevent further morbidity and mortality.

Long-term outcomes are variable. Around 50–68% achieved antibody eradication without bleeding recurrence.[14,18] If no bleeding episodes are reported, there is no evidence to support the administration of FXIII prophylaxis in patients with recurrent or persistent antibodies. A long-term follow-up is necessary, as this is a chronic disease and relapse may occur.[7,14] Our patient currently receives corticosteroids 20 mg daily and remains asymptomatic with a low inhibitor titer.

Ethics and Consent

The study participant has given written informed consent to participate and publish the data. Institutional approval was not required for publication.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Komaromi I, Bagoly Z, Muszbeck L. Factor XIII: novel structural and functional aspects. *J Thromb Haemost*. 2011;9:9–20. doi:10.1111/j.1538-7836.2010.04070.x
2. Levy JH, Greenberg C. Biology of factor XIII and clinical manifestations of factor XIII deficiencies. *Transfusion*. 2013;53:1120–1131. doi:10.1111/j.1537-2995.2012.03865.x
3. Muszbek L, Penzes K, Katona E. Auto and alloantibodies against factor XIII: laboratory diagnosis and clinical consequences. *J Thromb Haemost*. 2018;16:822–832. doi:10.1111/jth.13982
4. Ichinose A, Kohler HP, Philippou H; on behalf of the Factor XIII and Fibrinogen SSC Subcommittee of the ISTH. Recommendations for ISTH/SSC criterion 2015 for autoimmune acquired factor XIII/13 deficiency. *Thromb Haemost*. 2016;116:772–774. doi:10.1160/TH-16-05-0362
5. Baudo F, Colllins P, Huth-kuhne A, et al. Management of bleeding in acquired hemophilia A: results from the European Acquired Haemophilia (EACH2) registry. *Blood*. 2012;120:39–46. doi:10.1182/blood-2012-02-408930
6. Ichinose A; on behalf of the Japanese Collaborative Research Group on AH13. Autoimmune acquired factor XIII deficiency due to anti-factor XIII/13 antibodies: a summary of 93 patients. *Blood Rev*. 2017;31:37–45. doi:10.1016/j.blre.2016.08.002
7. Muszbek L, Katona E. Diagnosis and management of congenital and acquired FXIII deficiencies. *Semin Thromb Hemost*. 2016;42:429–439. doi:10.1055/s-0036-1572326
8. Duncan E, Collecutt M, Street A. Nijmegen-bethesda assay to measure factor VIII inhibitors. *Methods Mol Biol*. 2013;992:321–333.
9. Hemker H, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb*. 2003;33:4–15. doi:10.1159/000071636
10. Kohler HP, Ichinose A, Seitz R, Ariens AR, Muszbek L. on behalf of the factor XIII and fibrinogen SSC subcommittee of the ISTH. *J Thromb Haemost*. 2011;9:1404–1406. doi:10.1111/j.1538-7836.2011.04315.x

11. Ghosh K, Mota L, Shetty S, Kulkarni B. Spectrum of changes in endogenous thrombin potential due to heritable disorders of coagulation. *Blood Coagul Fibrinolysis*. 2008;19:577–580. doi:10.1097/MBC.0b013e328307089d

12. Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice.*Lippincott Williams & Wilkins.*2006*. Vol. 319. 1078–1079.

13. Theusinger OM, Baulig W, Asmis LM, Seifert B, Spahn DR. In vitro factor XIII supplementation increases clot firmness in Rotation Thromboelastometry (ROTEM). *Thromb Haemost*. 2010;104:385–391. doi:10.1160/TH09-12-0858

14. Sheng Yan MT, Rydz N, Goodyear D, Sholzberg M. Acquired factor XIII deficiency: a review. *Transfus Apher Sci*. 2018;57:724–730. doi:10.1016/j.transci.2018.10.013

15. Boehlen F, Casini A, Chizzolini C, et al. Acquired factor XIII deficiency: a therapeutic challenge. *Thromb Haemost*. 2013;109:479–487. doi:10.1160/TH12-08-0604

16. Franchini M, Frattini F, Crestani S, Bonfanti C. Acquired FXIII inhibitors: a systematic review. *J Thromb Thrombolysis*. 2013;36:109–114. doi:10.1007/s11239-012-0818-3

17. Di Micco P, Gussoni G, Pieralli F, Campanini M, Dentali F, Fontanella A. Acquired factor XIII deficiency inducing recurrent and fatal bleeding, description of a case. *J Blood Med*. 2020;11:43–45. doi:10.2147/JBM.S232115

18. Kessel R, Hu C, Shore-Lesserson L, Rand J, Manwani D. A child with acquired factor XIII deficiency: case report and literature review. *Haemophilia*. 2013;19:814–826. doi:10.1111/hae.12145

Antithrombotic and Anti-Inflammatory Effects of Fondaparinux and Enoxaparin in Hospitalized COVID-19 Patients: The FONDENOXAVID Study

Giuseppe Cardillo, Giuseppe Vito Viggiano, Vincenzo Russo, Sara Mangiacapra, Antonella Cavalli, Giampiero Castaldo, Federica Agrusta, Annamaria Bellizzi Snr, Maria Amitrano Snr, Mariateresa Iannuzzo, Clara Sacco, Corrado Lodigiani, Andrea Fontanella, Pierpaolo Di Micco & For The FondenoxavidStudy Group

ORIGINAL RESEARCH

Antithrombotic and Anti-Inflammatory Effects of Fondaparinux and Enoxaparin in Hospitalized COVID-19 Patients: The FONDENOXAVID Study

Giuseppe Cardillo[1]
Giuseppe Vito Viggiano[2]
Vincenzo Russo[3]
Sara Mangiacapra[4]
Antonella Cavalli[5]
Giampiero Castaldo[2]
Federica Agrusta[2]
Annamaria Bellizzi Snr[5]
Maria Amitrano Snr[4]
Mariateresa Iannuzzo[6]
Clara Sacco[7]
Corrado Lodigiani [7]
Andrea Fontanella [6]
Pierpaolo Di Micco[6]

For The FondenoxavidStudy Group

[1]Medylab, Biochimica Avanzata Laboratory, Naples, Italy; [2]UO Pronto Soccorso e Medicina D'urgenza, Semintensiva Covid, Ospedale Ramazzini Di Carpi — AUSL Modena, Modena, Italy; [3]Department of Translational Medical Sciences, University of Campania Luigi Vanvitelli Monaldi Hospital, Piazzale Ettore Ruggeri, Naples 80131, Italy; [4]UOC Medicina, AO Moscati, Avellino, Italy; [5]UOC Medicina-Covid, Presidio Opsedaliero Frangipane Di ArianoIrpino (AV), Naples, Italy; [6]UOC Medicina, Ospedale Fatebenefratelli di Napoli, Naples, Italy; [7]Humanitas Clinical and Research Center IRCCS, Rozzano, Italy

Correspondence: Pierpaolo Di Micco
Email pdimicco@libero.it

Background: Since the outbreak of novel coronavirus SARS-CoV2 around the world, great attention has been paid to the effects of such antithrombotic drugs as heparinoids, because they have antiviral action in vitro and antithrombotic actions in vivo. We conducted a retrospective analysis in inpatients with confirmed COVID-19 on the anti-inflammatory and antithrombotic effects of enoxaparin and fondaparinux at prophylactic doses.

Methods: This retrospective cohort study used patients with confirmed COVID-19 during the first months of the Italian outbreak from February 18 to April 30, 2020. Our aim was to compare clinical characteristics, prophylactic treatment, markers of inflammation, and thrombotic outcomes in inpatients positive for SARS-CoV2 during hospitalization associated with thromboprophylaxis with enoxaparin (40 mg or 60 mg once daily) or fondaparinux (2.5 mg once daily). Statistical analysis was conducted with using MatLab R2016B and ad hoc functions.

Results: There were no significatant differences in clinical characteristics between patients that used enoxaparin or fondaparinux as thromboprophylaxis for SARS-CoV2. No differences were found in D-dimer and fibrinogen levels either, which were used as markers of inflammation during the infection at testing on admission and after 3 weeks.Significant differences in CRP, IL6, and LDH were found in patients after 21 days' treatment.

Discussion: Increased levels of fibrinogen and D-dimer in patients with confirmed COVID-19 have been reported in several studies. Our results showed that anti-inflammatory effects of fondaparinux and enoxaparin after 3 weeks of prophylactic treatment were similar when levels of fibrinogen and D-dimer were considered. Furthermore, levels of CRP showed a decrease in patients treated with enoxaparin and fondaparinux, although the decrease in the fondaparinux group seems to be more relevant.

Keywords: venous thromboembolism, fibrinogen, D-dimer, COVID-19, SARS-CoV2, fondaparinux, enoxaparin

Introduction

Following the COVID-19 outbreak,[1] which has spread rapidly from China to other countries in the world with alarming morbidity and mortality,[2] several therapeutic strategies have been reported, including oxygen support[3] and experimental antiviral therapies.[4] Based on the coexistence of inflammation and hypercoagulable state[5] leading to increased risk of venous thromboembolism events (VTEs),[6–8] the use of low–molecular weight heparins (LMWHs) is recommended as part of standard

therapy in hospitalized COVID-19 patients.[9,10] LMWHs, such as enoxaparin, seem to act with a specific antithrombotic action and also with additional anti-inflammatory and antiviral activities in vitro against SARS-CoV2.[11,12]

Despite the antithrombotic role of enoxaparin and also fondaparinux in preventing VTEs in COVID-19 patients,[13–15] few data are available on their antithrombotic and anti-inflammatory effects in vivo. The aim of our study was to evaluate the antithrombotic and anti-inflammatory effects of fondaparinux and enoxaparin among COVID-19 patients according to selected laboratory markers found in the FONDENOXAVID study.

Methods
FONDENOXAVID Study
Inclusion Criteria
Clinical data of selected patients with symptomatic, confirmed COVID-19 were analyzed by a retrospective analysis in order to understand incidence and mortality for VTEs and also to understand trends of inflammatory and clotting markers.

Selected clinical records of these patients were chosen according to their thromboprophylaxis with fondaparinux or enoxaparin at doses suggested by international guidelines for VTE prevention.

Oral or written informed consent were arranged in agreement with the Ethical Committee of the University of Campania and in accordance with the Declaration of Helsinki and given to each patient for participation in this retrospective analysis — FONDENOXAVID. The ethical committee approved the registry on anticoagulant treatments (751/2019).

Physicians participating in the FONDENOXAVID retrospective analysis made all efforts to select consecutive patients.

Data were recorded onto a computer-based report at each participating hospital and sent to a centralized coordinating center through a secure system.

Several Italian Hospitals agreed to perform this analysis: Ospedale Ramazzini di Carpi, Italy; Moscati Hospital, Avellino, Italy, Ospedale Fatebenefratelli di Napoli, Italy, Thrombosis Center, IRCCS Humanitas Clinical and Research Center, Rozzano, Italy, and Presidio Ospedaliero Frangipane di Ariano Irpino, Italy.

All hospital provided clinical data derived from records from specialistic nonintensive and subintensive COVID wards.

FONDENOXAVID also used electronic data monitoring to detect inconsistencies or errors, and attempted to resolve discrepancies by contacting the local coordinators.

Exclusion Criteria
Patients aged <18 years were excluded from the retrospective analysis, as were those anticoagulants at therapeutic doses before SARS-CoV2 diagnosis for any medical reason patients positive for SARS-CoV2, but with recent bleeding (ie, within 30 days of hospital admission).

Patients were also excluded if they were currently participating in any clinical trials regarding COVID-19 or VTEs.

Design
This retrospective cohort study used patients with SARS-CoV2 during the Italian outbreak from February 18 to April 30, 2020.

Our aim was to compare clinical characteristics, laboratory markers, prophylactic treatment, and rate of VTEs in inpatients positive for SARS-CoV2 on primary thromboprophylaxis with enoxaparin (40 mg or 60 mg once daily) or fondaparinux (2.5 mg once daily).

Inflammatory markers to detect progression of prothrombotic state were recorded: levels of fibrinogen, D-dimer, LDH, IL6, and CRP were sequentially tested. In particular, D-dimer testing was performed at admission, day 21 after admission, or in cases of worsening of lung performance. In order to monitor lung performance in COVID-19, SIRS criteria were adopted.[16]

Main outcomes were rate of VTEs (ie, deep-vein thrombosis DVT, superficial VT, pulmonary embolism), mortality for VTEs, and levels of fibrinogen, IL6, CRP, LDH, and D-dimer as markers of inflammatory and prothrombotic state during the disease.

Study Population
In total, 100 consecutive symptomatic patients with laboratory-proven COVID-19 admitted to internal medicine units of five Italian hospitals from February 18 to April 30, 2020 were selected for this retrospective analysis. All selected COVID-19 patients were receiving VTE prophylaxis according to the current international guidelines.[9]

The institutional ethical committee (FBGD-90320) approved the study, and patients gave standard written consent to the use of their data.

Table 1 Demographic, clinical, and laboratory characteristics of the study population

	Enoxaparin, n=62	Fondaparinux, n=38	*p*-value
Male, n (%)	40 (65%)	23 (61%)	0.76
Age <40 years, n (%)	3 (5%)	6 (16%)	0.09
Age 40–60 years, n (%)	34 (34%)	17 (45%)	
Age ≥60 years, n (%)	38 (61%)	15 (39%)	
VT	5 (8%)	2 (5%)	0.76
PE	4 (6%)	0	0.12
Deaths	6 (9%)	4 (10%)	0.82

Note: All variables were analyzed with Barnard's test, except age (Fisher's test).
Abbreviations: VT, vein thrombosis; PE, pulmonary embolism.

Serum levels of fibrinogen, D-dimer, LDH, IL6, and CRP were evaluated at admission and after 3 weeks to evaluate changes in inflammatory and prothrombotic clotting markers during treatment.

All VTEs of the lower limbs with or without associated pulmonary embolism diagnosed with objective methods were recorded.

Statistical Analysis

The Anderson–Darling test was used to analyze data normality. Continuous variables are reported using median and IQRs. Categorical variables are expressed as frequency counts and percentages. Differences between unpaired data were evaluated using two-tailed Fligner–Policello and Fligner–Killeen tests and paired data using the one-tailed Wilcoxon test and Hodges–Lehmann estimator of mean difference. Differences among three groups were evaluated using the two-tailed Dunn multiple-comparison test with Bonferroni correction. Categorical data

Table 2 Classes of drugs used in the described cohort of patients treated for SARS-CoV2

Patients (n=100)	n
Antibiotics	64
Biologics (tocilizumab or others)	12
Antivirals	21
Steroids	55
Immunomodulants (hydroxychloroquine or others)	65
Heparinoids (enoxaparin or fondaparinux)	100
NSAIDs, daily use	20
Acetaminophen	68

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs.

were evaluated using Barnard's or Fisher's test. Statistical comparisons were performed using MatLab R2018a.

Results

Baseline clinical and laboratory characteristics of the study population are shown in Table 1. No significant differences between enoxaparin and fondaparinux group has been shown.

Table 2 reports all categories of drug used to treat SARS-CoV2 in our cohort, in which heparinoids (ie, enoxaparin and fondaparinux) were the only drugs to reach 100% of patients.

In Table 3, all VTEs that we detected with objective methods during the analysis are reported. VT of the lower limbs was detected in five patients in the enoxaparin group vs two patients in the fondaparinux group: three DVT, one isolated distal VT, and one superficial VT for the enoxaparin group and two DVTs for the fondaparinux group. Pulmonary embolism was detected in 4 patients in the enoxaparin group while no pulmonary embolism was found in the fondaparinux group. Three events of proximal symptomatic DVT were found in the group on enoxaparin, and two events of symptomatic proximal DVT were found in the groupon fondaparinux. Furthermore two asymptomatic VTs were found in the patients on enoxaparin: one superficial VT of the lower limbs and one isolated distal DVT. In the enoxaparin group, four thrombotic events were associated with pulmonary embolisms. pPulmonary embolisms were detected with lung CT scans. Data on distribution of thrombotic eventsaere summarized in Tables 1 and 3. No statistical differences were found between groups.

In Table 4, differences in of D-dimer, fibrinogen, CRP, and IL6 levels at hospital admission and after 3 weeks of treatment are reported.

Table 3 Different doses of enoxaparin and fondaparinux in patients with COVID-19 at baseline with occurrence of vein-thrombosis events (VTEs)

	n	Type of VTE	Clinical signs of suspected VTE
Enoxaparin twice daily after VTE diagnosis	5	Three proximal DVT, one IDVT, one SVT, four f them with PE	Twosymptomatic DVT, two asymptomatic IDVT and SVT
Fondaparinux therapeutic dosage after VTE diagnosis	2	2 proximal DVT	Two symptomatic DVT

Abbreviations: DVT, deep-vein thrombosis; IDVT, isolated DVT; SVT, superficial VT; PE, pulmonary embolism.

Table 4 Distributions of inflammatory markers between enoxaparin and fondaparinux groups at baseline and after 3 weeks

D-dimer, µg/dL	Enoxaparin, n=62	Fondaparinux, n=38	FP test *p*-value	FK test *p*-value
Admission	710.5 (520–1,208)	643.5 (502–919)	0.0972	0.1362
3 weeks later	602 (428–1,230)	606 (450–810)	0.3783	0.2059
HL mean difference	−151 (−292.5 to 16.5)	−42.75 (−210.75 to 133)		
Wilcoxon test *p*-value	0.015217	0.21036		
Fibrinogen, mg/dL				
Admission	600 (478–734.5)	569.5 (503–632)	0.1501	0.0758
3 weeks later	631 (497–722.5)	535 (450–630)	0.0114	0.0695
HL mean difference	14.8 (−37.0 to 59.5)	−16.5 (−67 to 38)		
Wilcoxon test *p*-value	0.28607	0.28219		
CRP, mg/dL				
Admission	11.5 (3.6–22)	44 (15–52)	0.000004	0.0011
3 weeks later	13 (5–40.5)	15 (9–21)	0.3051	0.1652
HL mean difference	6 (0.5–13.8)	−22.5 (−34.5 to 11)		
Wilcoxon test *p*-value	0.0063151	0.00050565		
LDH, U/L				
Admission	252.5 (209–343)	301 (232–349)	0.2085	0.7407
3 weeks later	212.5 (202–255)	239 (203–264)	0.0889	0.2446
HL mean difference	−62 (−88.5 to 38)	−55 (−140 to 4)		
Wilcoxon test *p*-value	0.0000002	0.0128		
IL6, pg/mL				
Admission	16 (13.2–20)	15 (13–20)	0.4891	0.9692
3 weeks later	6 (4–9.8)	6 (4–9.8)	0.4946	0.9692
HL mean difference	−9 (−11.0 to 6.5)	−9 (−11.0 to 6.5)		
Wilcoxon test *p*-value	0.0000002	0.00007		

Abbreviations: FP, Fligner–Policello; FK, Fligner–Killeen; HL, Hodges–Lehmann.

No differences in medians or IQRs for unmatched D-dimer were found. Hodges–Lehmann mean-difference estimators were not significant (95% CIs including 0) for fondaparinux or enoxaparin, and the one-tailed Wilcoxon test showed the same results. We can conclude that fondaparinux is not effective in reducing D-dimer, but enoxaparin is effective.

No differences in medians or IQRs on unmatched fibrinogen distributions were found. Hodges–Lehmann mean-difference estimators were not significant (95% CIs including 0), and the one-tailed Wilcoxon test did not reveal differences between groups. As such, we can conclude that neither drug had a significant effect on fibrinogen.

Data on CRP distributions were found to be different: the fondaparinux group showed a higher median and IQR (maybe more severe cases were treated with fondaparinux) than the enoxaparin group. However, 3 weeks later, both groups had reached the same median and IQR. In the enoxaparin group, the Hodges–Lehmann estimator was >0 while in the fondaparinux group it was <0. We can conclude (from the IQR too) that enoxaparin is not effective in reducing CRP, while fondaparinux is effective.

No differences in medians or IQRs on unmatched LDH distributions were found Hodges–Lehmann mean-difference estimators and one-tailed Wilcoxon test results were found to be significant in both groups. As such, we can conclude that both drugs were effective in reducing LDH.

Finally, no differences in medians or IQRs for unmatched IL6 were found. Hodges–Lehmann mean-difference estimators and one-tailed Wilcoxon test results were found to be significant in both groups. Therefore, we can conclude that both drugs were effective in reducing IL6.

Discussion

Previous reports on the COVID-19 outbreak have focused on the prognostic roles of laboratory markers regarding their involvement in the pathophysiology of infectious or inflammatory disease. The prognostic roles of D-dimer, fibrinogen, CRP, LDH, IL6, and other tests during COVID-19 outbreak have been underlined.[2,5]

D-dimer testing is one of the laboratory procedures used to investigate or exclude venous thromboembolism,[17] and its levels may also have prognostic value after confirmation of a VTE.[18] Usually, D-dimer ha negative predictive value, because an increase may also be present in other diseases, such as inflammations and/or infections.[19,20] COVID-19 is in fact a specific infection that is associated with increased D-dimer levels per se.[4,5]

In the same way, increased fibrinogen levels have been associated with increased rates of VTEs and severe infections due to SARS-CoV2.[4,5]

On the other hand, CRP is one of the most used inflammatory markers to screen acute and chronic infections and inflammation, and LDH is one of the most common markers of chronic disease associated with cytolysis. Retrospective analysis of COVID-19 cohort has confirmed these roles.[21]

Also, IL6 levels have been associated with differing evolution of lung inflammation[21,22] in patients with confirmed COVID-19.

The main objective and findings of our study were the reduction of inflammatory markers after thromboprophylaxis with enoxaparin or fondaparinux. The findings point to the ancillary properties of these drugs in vivo.

Anti-inflammatory effects of heparins have been reported in vitro and in vivo, and are based on their role as a coadjuvant of serpins.[23] Reductions in inflammatory markers during administration of enoxaparin has also been reported in animal models.[24] These mechanisms also have a role in reduction of IL6 levels during inflammation induced by SARS-CoV2.[10,25–27]

Standard treatment for COVID-19 in fact is based on the administration of several kind of drugs with multiple actions, in order to reduce inflammatory damage (ie, antivirals, immunomodulants, antibiotics, steroids, antithrombotics). Due to their pharmacological properties, heparinoids can fight SARS-CoV2 in multiple ways: a specific antiviral action of heparin toward SARS-CoV2 has been found in vitro and in vivo,[10,23–27] as well a specific anti-inflammatory action of heparinoids and fondaparinux.[28,29] Of course, these additional properties should be added to the well-known antithrombotic actions of both drugs. As the specific action of heparin is due to the pentasaccharide sequence present in heparins and fondaparinux,[30] we postulated that all therapeutic actions exerted by enoxaparin could also be achieved by fondaparinux.

We found anti-inflammatory effects for enoxaparin and fondaparinux in levels of D-dimer, fibrinogen, CRP, LDH, and IL6 at admission and after 3weeks of treatment.

A progressive reduction in all values was recorded in both groups for D-dimer, fibrinogen, CRP, LDH, and IL6 levels.

Reduction in D-dimer levels after 3 weeks of prophylaxis with heparinoids was similar for patients treated with enoxaparin or fondaparinux, although enoxaparin seems to be more effective in reducing D-dimer levels.

Both drugs were also effective in reducing median LDH and IL6 levels.

On the contrary, neither drug seemed to be effective for fibrinogen reduction, though the fondaparinux group showed a lower median.

As such, we can assume that the use of such heparinoids as enoxaparin or fondaparinux has multiple clinical advantages for patients with confirmed COVID-19, based on the reductions in inflammatory markers and VTE rate found.

Interestingly, as shown in Table 2, heparinoids were the only drugs used in all patients, so we can postulate that results from the clinical and laboratory points of view are also associated with their use. In particular, their anti-inflammatory effects contributed to improvements in levels of inflammatory markers. Based on these data, we can speculate on specific actions of enoxaparin or fondaparinux on specific inflammatory markers, but this topic would be better investigated on a large population and include a composite clinical evaluation (eg, clinical performance, laboratory evaluation, and specific outcomes). We also emphasize that prolonged administration of enoxaparin or fondaparinux in inpatients with COVID-19 may be associated with ancillary anti-inflammatory responses to the known antithrombotic action of these drugs.

We found that the incidence of VTEs in our cohort was lower than other studies (nearly 10%), although abnormal and increased values of D-dimer were present in >75% of patients. This could be related to the difficulty in investigating DVT in patients with COVID-19.[31] In this clinical setting, the incidence of thrombotic events was similar in both groups, confirming that both pharmacological approaches were valid in terms of support for these patients.

Therefore, in this specific field we have added to and enlarged the knowledge base concerning the use of enoxaparin and fondaparinux in this clinical setting. Studies have provided more data on the use of enoxaparin or other heparins than fondaparinux in VTE prevention of COVID-19 patients and reduction of anti-inflammatory markers.

Study Limitations

Our study has several limitations. First of all, of patient selection was retrospective. The number of patients that received thromboprophylaxis with fondaparinux 2.5 mg daily in the hospitals involved was very low. Furthermore, the fondaparinux has a unique dosage for thromboprophylaxis, while different dosages are available for enoxaparin. We selected only patients that received the standard dosage of 4,000 U daily.

The small number of thrombotic events that we noted in the retrospective analysis could be related to the fact that ultrasound scans for VT were performed between days 5 and 8 of hospitalization. The best timing for ultrasound scans for VT in these patients is still a matter of discussion in daily clinical management.

Another limitation could be related to the minority of patients that developed VTEs, because in these patients heparinoid dose was changed. However, the number of patients with VTEs was very small, and statistical analysis did not reflect this limitation.

Last but not least, outcomes of events were limited to 4 weeks. More detailed and prolonged studies should improve on this first study in thise field.

Conclusion

This retrospective analysis on inpatients with confirmed COVID-19 was based on the ancillary properties of the heparinoids enoxaparin and fondaparinux, which have anti-inflammatory effects in vitro and in vivo and antithrombotic effects.

We performed a combined analysis on the incidence of VTEs confirmed by objective methods and anti-inflammatory actions in patients treated with prophylactic doses of enoxaparin or fondaparinux.

Both drugs were able to exert anti-inflammatory effects, as shown by reduction in all markers. Although fondaparinux showed little advantage in reducing CRP levels, enoxaparin did with regard to D-dimer levels. These additional aspects that we found in our analysis should always be joined to clinical aspects.

Funding

Disclosure

The authors certify that they have no conflicts of interest to declare for this report.

References

1. Sohrabi C, Alsafi Z, O'Neill N, et al. World Health Organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). *Int J Surg*. 2020;76:71–76. doi:10.1016/j.ijsu.2020.02.034
2. Juul S, Nielsen N, Bentzer P, et al. Interventions for treatment of COVID-19: a protocol for a living systematic review with network meta-analysis including individual patient data (The LIVING Project). *Syst Rev*. 2020;9(1):108. doi:10.1186/s13643-020-01371-0
3. Cao B, Wang Y, Wen D, et al. A trial of Lopinavir-Ritonavir in adults hospitalized with severe Covid-19. *N Engl J Med*. 2020;382(19):1787–1799. doi:10.1056/NEJMoa2001282

4. Di Micco P, Russo V, Carannante N, et al. Clotting factors in COVID-19: epidemiological association and prognostic values in different clinical presentations in an Italian cohort. *J Clin Med*. 2020;9(5):1371. doi:10.3390/jcm9051371

5. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost*. 2020;18(4):844–847. doi:10.1111/jth.14768

6. Scudiero F, Silverio A, Di Maio M, et al. Cov-IT network. Pulmonary embolism in COVID-19 patients: prevalence, predictors and clinical outcome. *Thromb Res*. 2021;198:34–39. doi:10.1016/j.thromres.2020.11.017

7. Klok FA, Kruip MJHA, van der Meer NJM, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res*. 2020; 191:145–7.

8. Cattaneo M, Bertinato EM, Birocchi S, et al. Pulmonary embolism or pulmonary thrombosis in COVID-19? Is the recommendation to use high-dose heparin for thromboprophylaxis justified? *Thromb Haemost*. 2020.

9. Bikdeli B, Madhavan MV, Jimenez D, et al. COVID-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up. *J Am Coll Cardiol*. 2020;75 (23):2950–73.

10. Shastri MD, Stewart N, Horne J, et al. In-vitro suppression of IL-6 and IL-8 release from human pulmonary epithelial cells by non-anticoagulant fraction of Enoxaparin. *PLoS One*. 2015;10(5): e0126763. doi:10.1371/journal.pone.0126763

11. Di Micco P, Di Micco G, Russo V, et al. Blood targets of adjuvant drugs against COVID19. *J Blood Med*. 2020;11:237–241. doi:10.2147/JBM.S256121

12. Tiwari V, Beer JC, Sankaranarayanan NV, Swanson-Mungerson M, Desai UR. Discovering small-molecule therapeutics against SARS-CoV-2. *Drug Discov Today*. 2020;25(8):1535–1544. doi:10.1016/j.drudis.2020.06.017

13. Russo V, Cardillo G, Viggiano GV, et al. Fondaparinux use in patients with COVID-19: a preliminary multicenter real-world experience. *J Cardiovasc Pharmacol*. 2020;76(4):369–371. doi:10.1097/FJC.0000000000000893

14. Russo V, Cardillo G, Viggiano GV, et al. Thromboprophylaxis with fondaparinux versus enoxaparin in hospitalized covid-19 patients: a multicenter Italian observational study. *Front Med (Lausanne)*. 2020;7. doi:10.3389/fmed.2020.569567

15. Pesavento R, Ceccato D, Pasquetto G, et al. The hazard of (sub) therapeutic doses of anticoagulants in non-critically ill patients with Covid-19: the Padua province experience. *J Thromb Haemost*. 2020;18(10):2629–2635. doi:10.1111/jth.15022

16. Raith EP, Udy AA, Bailey M, et al. Prognostic accuracy of the SOFA score, SIRS criteria, and qSOFA score for in-hospital mortality among adults with suspected infection admitted to the intensive care unit.. *JAMA*. 2017;317(3):290–300. doi:10.1001/jama.2016.20328

17. Streiff MB, Agnelli G, Connors JM, et al. Guidance for the treatment of deep vein thrombosis and pulmonary embolism. *J Thromb Thrombolysis*. 2016;41(1):32–67. doi:10.1007/s11239-015-1317-0

18. Lobo JL, Zorrilla V, Aizpuru F, et al. RIETE investigators. D-dimer levels and 15-day outcome in acute pulmonary embolism. Findings from the RIETE registry. *J Thromb Haemost*. 2009;7(11):1795–1801. doi:10.1111/j.1538-7836.2009.03576.x

19. Schutte T, Thijs A, Smulders YM. Never ignore extremely elevated D-dimer levels: they are specific for serious illness. *Neth J Med*. 2016;74(10):443–448.

20. Di Micco P, D'Uva M, Strina I, et al. The role of D-dimer as first marker of thrombophilia in women affected by sterility: implications in pathophysiology and diagnosis of thrombophilia induced sterility. *J Transl Med*. 2004;2(1):38. doi:10.1186/1479-5876-2-38

21. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet*. 2020;395(10233):1340. doi:10.1016/S0140-6736(20)30869-2

22. Herold T, Jurinovic V, Arnreich C, et al. Elevated levels of IL-6 and CRP predict the need for mechanical ventilation in COVID-19. *J Allergy Clin Immunol*. 2020;146(1):128–136.e4. doi:10.1016/j.jaci.2020.05.008

23. Beinrohr L, Murray-Rust TA, Dyksterhuis L, et al. Serpins and the complement system. *Methods Enzymol*. 2011;499:55–75.

24. Vicci H, Eblen-Zajjur A, López M, Crespo G, Navarro M. Enoxaparin pretreatment effect on local and systemic inflammation biomarkers in the animal burn model. *Inflammopharmacology*. 2019;27(3):521–529. doi:10.1007/s10787-018-0444-6

25. Shi C, Wang C, Wang H, et al. The potential of low molecular weight heparin to mitigate cytokine storm in severe COVID-19 patients: a retrospective cohort study [published online ahead of print, 2020 Sep 3]. *Clin Transl Sci*. 2020;13(6):1087–1095. doi:10.1111/cts.12880

26. Marietta M, Vandelli P, Mighali P, et al. Randomised controlled trial comparing efficacy and safety of high versus low low-molecular weight heparin dosages in hospitalized patients with severe COVID-19 pneumonia and coagulopathy not requiring invasive mechanical ventilation (COVID-19 HD): a structured summary of a study protocol. *Trials*. 2020;21(1):574.

27. Paschoa AF. Heparin: 100 years of pleiotropic effects. *J Thromb Thrombolysis*. 2016;41(4):636–643. doi:10.1007/s11239-015-1261-z

28. Keshari RS, Silasi R, Popescu NI, et al. Fondaparinux pentasaccharide reduces sepsis coagulopathy and promotes survival in the baboon model of Escherichia coli sepsis. *J Thromb Haemost*. 2020;18 (1):180–190. doi:10.1111/jth.14642

29. Iba T, Okamoto K, Ohike T, et al. Enoxaparin and fondaparinux attenuates endothelial damage in endotoxemic rats. *J Trauma Acute Care Surg*. 2012;72(1):177–182. doi:10.1097/TA.0b013e31821a83f0

30. Tan KT, Lip GY. Fondaparinux. *Curr Pharm Des*. 2005;11(4):415–419. doi:10.2174/1381612053382089

31. Di Micco P, Russo V, Lodigiani C. Venous thromboembolism and its association with COVID-19: still an open debate. *Medicina (Kaunas)*. 2020;56(10):506. doi:10.3390/medicina56100506

Evaluation of Hematological Parameters of *Helicobacter pylori*-Infected Adult Patients at Southern Ethiopia: A Comparative Cross-Sectional Study

Kassahun Haile & Abebe Timerga

ORIGINAL RESEARCH

Evaluation of Hematological Parameters of *Helicobacter pylori*-Infected Adult Patients at Southern Ethiopia: A Comparative Cross-Sectional Study

Kassahun Haile [ID][1]
Abebe Timerga[2]

[1]Department of Medical Laboratory Science, College of Medicine and Health Sciences, Wolkite University, Wolkite, Ethiopia; [2]Department of Biomedical Science, Wolkite University, Wolkite, Ethiopia

Background: *Helicobacter pylori* (*H. pylori*) infection is a global public health problem, a higher burden of the infection was reported in developing countries including Ethiopia. It has been associated with several gastrointestinal diseases, and recently implicated in some hematological abnormalities. Despite the high prevalence of *H. pylori* infection in Ethiopia, there was limited data regarding the relationship between hematological parameters with *H. pylori* infection. Therefore, this study aimed to evaluate selected hematological parameters of *H. pylori*-infected patients attending Wachemo University Nigist Eleni Mohammed Memorial Referral Hospital (WUNEMMRH), Hosanna, Southern, Ethiopia.

Methods and Materials: A comparative cross-sectional study was conducted from January to May 2019 among 374 (187 *H. pylori*-infected patients and 187controls) study participants. Data on socio-demographic characteristics were collected using a structured questionnaire. A five-milliliter venous blood sample was collected for hematological parameter analysis. Approximately two gram of stool specimen was collected to assess the presence of *H. pylori* antigen. Data were entered and analyzed by using SPSS version 21. Pearson correlation analysis and independent sample T-test was performed, and P-value < 0.05 was considered statistically significant.

Results: Mean value of Hgb (p<0.001), RBC count (p<0.001), HCT (p<0.001), MCV (p=0.003), MCH (p=0.008), and MCHC (p=0.006) of *H. pylori*-infected patients were significantly lower than control group. However, the mean value of RDW (p=0.003) in *H. pylori*-infected patients was significantly higher than in the control group. About 13.3%, 7%, 6.4%, and 18.2% of *H. pylori*-infected patients showed reduced Hgb concentration, RBC count, HCT, and MCV values, respectively.

Conclusion: The study showed a statistically significant difference in the mean value of Hgb, RBC count, HCT, MCV, MCH, MCHC, and RDW of *H. pylori*-infected patients and controls. Thus, hematological parameters should be considered for proper diagnosis and management of *H. pylori*-infected patients and eradication of this microorganism from infected patients, determination of hematological parameters for *H. pylori*-infected patients were recommended.

Keywords: hematological parameters, *H. pylori* infection, southern, Ethiopia

Correspondence: Kassahun Haile
Department of Medical Laboratory Science, College of Medicine and Health Sciences, Wolkite University, P.O. Box: 07, Wolkite, Ethiopia
Tel +25-926-07-43-74
Fax +251 11 322 0041
Email kassahaile07@gmail.com

Introduction

Helicobacter pylori (*H. pylori*) infection is a global public health problem affecting both developed and developing countries,[1,2] with a higher burden reported from developing countries; a study showed 50.8% in developing countries as compared to 34.7% in developed countries.[3] In Ethiopia,52.2% of the population were suffering

from this infection.[4] Besides, different studies reported 52.4%, 36.8% 60.5%, and 83.3% burden of *H. pylori* infection in Butajira,[5] Addis Abebe,[6] Dessie,[7] and Hawwassa,[8] respectively.

H. pylori infection is one of the most common bacterial infections in humans and possesses several mechanisms to colonize the host; flagella-mediated motility, surviving in an acidic stomach environment, attaching to host receptors by adhesions, and causing tissue damage by releasing toxin.[9,10] It causes gastritis, peptic ulcer disease, gastroduodenal ulcer, atrophic gastritis, gastric cancers, and dyspeptic symptom.[11,12] A study conducted in Addis Ababa, Ethiopia, reported a significant number of gastric cancer cases in Ethiopia.[13] Furthermore, *H. pylori* infection has been implicated in some hematological manifestations such as anemia and micronutrient deficiency (iron, and vitamin B12).[14,15]

Red blood cell synthesis is regulated by many factors, like erythropoietin, iron, vitamin B12, folic acid, and vitamin C. Iron and vitamin B12 are especially important for hemoglobin synthesis and maturation of red blood cells, lack of any of the nutrients resulted in alteration of RBC parameter.[16–18] However, different studies indicated *H. pylori* infection was independently associated with vitamin B12 deficiency,[17,19] iron deficiency,[20,21] and iron-deficiency anemia,[22] and the presence of *H. pylori* infection is associated with a poorer response to oral iron therapy.[23,24] It has been suggested that *H. pylori* eradication therapy in addition to iron therapy, might show improvements in ferritin and hemoglobin levels in infected patients.[23,25]

H. pylori infection is related to iron deficiency and iron-deficiency anemia by impairing iron absorption as a result of chronic gastritis which causes gastric hypochlorhydria, leading to impair reduction of the dietary iron from the ferric to ferrous form.[26] Because most dietary iron is in the ferric form, needs acidic intra-gastric pH and ascorbic acid to reduce into ferrous form for absorption.[27] Hence, *H. pylori* is a major cause of chronic superficial gastritis leading to atrophy of gastric glands, resulting in reducing gastric acid secretion.[28,29]

H. pylori impair iron uptake which can compete with the host,[30] and increased hepcidin production secondary to *H. pylori* infection decreases the release of iron from macrophages of the reticuloendothelial system and entrecote, which impairs hemoglobin synthesis.[26] Because hepcidin acts as an acute phase reactant in response to the inflammation produced in the gastric mucosa.[14]

Hemorrhagic gastritis and active bleeding peptic ulcers were other possible ways for iron loss.[31]

Also, *H. pylori* infection causes the deficiency of vitamin B12 by producing chronic gastritis and atrophic gastritis;[32] *H. pylori* infection causes hypochlorhydria, which leads to increased bacterial colonization. Bacteria compete with the host for vitamin B12. Gastric acid in the stomach and pepsin was required to release protein bounded vitamin B12 from food; thereby vitamin B12 cannot bind to R-protein and then with intrinsic factor, lead to malabsorption of vitamin B12. Also, decreased production of an intrinsic factor due to atrophic gastritis may result in vitamin B12 deficiency and anemia.[31–33]

H. pylori infection diagnosis was performed by invasive (rapid urease test, culture, endoscopy, and endoscopic biopsy for histopathology) and non-invasive (urea breath tests, stool antigen test, and serological tests) methods.[34] However, the choice of diagnostic methods general depends on population prevalence of infection, differences in test performance, availability, affordability, and clinical situation.[35]

Several epidemiological studies reported a higher burden of H.pylori infection in Africa including Ethiopia.[1,4] Despite the high prevalence of H.pylori infection in Ethiopia, data regarding the association of hematological parameters with H.pylori infection is scarce, evaluating the relationship between hematological parameters with H. pylori infection was important to aid effective intervention measures to reduce its public health burden and related complication in adult patients. Also, it aids in the proper diagnosis and management of *H. pylori*-infected patients. Therefore, this study aimed to determine the effect of *H. pylori* infection on selected hematological parameters in Southern Ethiopia.

Methods and Materials
Study Design, Period, and Area
A comparative cross-sectional study was conducted among adults patient from January 1, to May 30, 2019, at Wachemo University Nigist Eleni Mohammed Memorial Referral Hospital (WUNEMMRH), Hosanna town, Hadiya Zone, Southern Ethiopia. The town is located about 232 km far from the capital city of Ethiopia, Addis Ababa. WUNEMMRH is the largest public referral hospital in the Hadiya Zone, as a teaching hospital, it plays an important role in providing teaching, research, and community service and provides services for more than

3.2 million inhabitants. All adult patients (≥18years) who visited the hospital with signs and symptoms suggestive of H.pylori infections were considered as a source population for the *H. pylori*-infected group.

Study Participants

A total of 374 study participants; 187 *H. pylori*-infected patients (94 females and 93 males) and 187 control groups (94 females and 93 males) were included in the study. Control groups were age and sex-matched healthy individuals who had no previous history of chronic diseases that affect hematological parameters. Also, the control groups were negative for H. pylori infection. Control groups were WUNEMMRH staff, patient's relatives or guardians, and Wachemo University students.

From those patients with presumptive signs and symptoms suggestive of H.pylori infections, stool antigen confirmed consecutive H.pylori infected patients were taken as *H. pylori*-infected groups. *H. pylori*-infected patients who took treatment within the last three months, who had previous stomach or small bowel surgery, donate blood within the last three months and on treatment for anemia before data collection, bleeding manifestations, pregnant women, severely ill and who had other chronic diseases were excluded from the study.

Sample Size Determination and Sampling Technique

Two population mean formulae were used to calculate the sample size using G-power, version 3.1, by considering the following assumptions: 95% confidence interval (two-sided), 80% power and the ratio of cases to control group was 1:1. Taking the mean and standard deviation (SD) of RBC for H.pylori infected patients and control group from the previous study,[5] 4.63 and 0.59 for H.pylori infected patients and 4.83 and 0.72 for the control group. We got a total sample size of 374 with 187 for each group. All consecutively identified controls and *H. pylori*-infected cases were included in the study.

Data Collection and Laboratory Method

Data on socio-demographic characteristics were collected using a structured questionnaire by trained nurses.

Blood Sample Collection and Analysis

A five-milliliter venous blood sample was collected from each study participant by laboratory technologists for hematological parameter analysis. Hematological parameters (RBC, Hgb, HCT, MCV, MCH, MCHC, and RDW) were determined using Mindray BC-3000 plus (Shenzhen Mindray Bio-Medical Electronics, China) automated blood analyzer. Hematological parameters (RBC, Hgb, HCT, MCV, MCH, MCHC, and RDW) were categorized into low, normal, and high based on the hematological reference range conducted in adults in Ethiopia.[36]

Stool Specimen Collection and Analysis

After explaining how to collect representative stool specimens clean cupped plastic container was given to the participants. Approximately two gram of stool specimen was collected from each study participant and checked for the presence of *H. pylori* antigen by wondfo one step *H. pylori* feces test (Guangzhou Wondfo Biotech, China).

From all study participants; (height, weight, and blood pressure) were measured and body mass index (BMI) was calculated as weight in kilogram divided by the square of height in meter and categorized into four groups; BMI<18.5 kg/m^2 as underweight, BMI = 18.5–24.9 kg/m^2 as normal weight, BMI = 25–29.9 kg/m^2 as overweight, and BMI ≥ 30 kg/m^2 as obese.

Data Management and Quality Assurance

To ensure the quality of data, all laboratory tests were done by following the standard operating procedures and manufacturer instructions, reagents and test kits were checked for their expiry date, half-day training was given to data collectors and completeness of each questionnaire was checked regularly. The performance of the hematology analyzer was checked before running the patients' samples by performing normal, low, and high blood controls.

Data Analysis and Interpretation

Data were entered and analyzed by using SPSS version 21 (SPSS, Chicago, IL, USA). Frequency tables and descriptive summaries like mean and standard deviation (SD) were used to describe the study variables. Data were tested for the normality of its distribution by Kolmogorov–Smirnov test. The difference in the mean (SD) values of RBC parameters between *H. pylori*-infected and control groups were explored using an independent sample T-test. Pearson correlation analysis was performed. A P-value, < 0.05 was considered statistically significant.

Results

Socio-Demographic Characteristics of Study Participants

A total of 374 study participants; 187 *H. pylori*-infected patients and 187 control groups were included in this study. The mean age of *H. pylori*-infected patients was 31.89 with a standard deviation of ±7.8 years. There was no statistically significant difference between the mean age (p=0.29) of the *H. pylori*-infected patient and the control group. Among the study participants, 93 (49.7%) and 94 (50.3%) were males and females in both groups respectively. More than half of the study participants; 109 (58.3%) in the *H. pylori*-infected group and 107 (57.2%) in the control group were rural dwellers (Table 1). Among *H. pylori*-infected study participants 7(3.5%),151 (80.7%), and 29 (15.5%) had underweight, normal weight and overweight, respectively.

Comparisons of RBC Parameters in Study Participants

The mean (SD) of the parameters related to red blood cells were also compared between H.pylori infected and the control group. Accordingly statistically significant lower mean value of Hgb (p<0.001), RBC (p<0.001), HCT (p<0.001), MCV (p=0.003), MCH (p=0.008), and MCHC (p=0.006) were observed in *H. pylori*-infected patients compared to control group. However, the mean value of RDW in *H. pylori*-infected patients was higher than the

Table 1 Socio-Demographic Variables Distribution of *H. pylori*-Infected Patients and Controls at Wachemo University Nigist Eleni Mohammed Memorial Referral Hospital, 2019

Variables	Categories	*H. pylori*-Infected Group	Controls Group
Gender	Female Male	94(50.3%) 93(49.7%)	94(50.3%) 93(49.7%)
Age in years	18–27 28–37 38–47 ≥48	62(33.2%) 72(38.5%) 49(26.2%) 4(2.1%)	76(40.6%) 66(35.3%) 42(22.5%) 3(1.6%)
Residence	Urban Rural	78(41.7%) 109(58.3%)	80(42.8%) 107(57.2%)
Educational status	Illiterate Primary Secondary Higher level	56(29.9%) 31(16.6%) 54(28.9%) 46(24.6%)	44(23.5%) 24(12.8%) 65(34.8%) 54(28.9%)

Abbreviation: *H. pylori, Helicobacter pylori.*

Table 2 Comparisons of Mean Values of RBC Parameters of *H. pylori*-Infected Patients and Controls at Wachemo University Nigist Eleni Mohammed Memorial Referral Hospital, 2019

RBC Parameters	*H. pylori*-Infected Group Mean ±SD	Control Group Mean ±SD	p-value
Hgb g/dl	13.32 ±1.56	14.25 ±1.89	<0.001
RBCx10⁶/μL	4.39 ±0.46	4.79 ±0.61	<0.001
HCT (%)	40.38 ±4.40	45.71 ±5.31	<0.001
MCV(fl)	88.03 ±5.44	89.64±4.84	0.003
MCH(pg)	28.33 ±2.18	28.94 ±2.19	0.008
MCHC g/dl	31.99 ±1.30	32.38 ±1.45	0.006
RDW (%)	14.31±1.45	13.92±1.12	0.003

Note: P-value <0.05 is considered statistically significant.
Abbreviations: fl, femtoliters; g/dl, gram per deciliter; Hgb, hemoglobin; *H. pylori, Helicobacter pylori*, RBC, red blood cell; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; μL, microliters; %, percentage; pg, pictogram; RDW, red blood cell distribution width; SD, standard deviation.

control group, which was also statistically significant (p=0.003) (Table 2).

Hematologic Abnormalities in *H. pylori*-Infected Patients

Among 187 H.pylori infected patients; about 13.3%, 7%, 6.4% and 18.2% of patients showed reduced Hgb concentration, RBC count, HCT, and MCV values, respectively (Table 3).

Correlation Analysis of RBC Parameters with Predictors Among *H. pylori*-Infected Patients

Haemoglobin concentration showed statistically positive correlation with systolic blood pressure (r: 0.14, p: 0.006), and negative correlation with residence (r: −0.12, p=0.01). In addition HCT shows negative correlation with residence (r: −0.11, p: 0.002) and positive correlation with gender (r: 0.15, p: 0.003) (Table 4).

Discussion

H. pylori infection is a major cause of morbidity and mortality worldwide[2]. More than 50% of the global population is estimated to be infected.[1,37] It causes chronic gastritis, peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma.[12]

Table 3 The Proportion of *H. pylori*-Infected Patients (n=187) with Low, Normal, and High Values of RBC Parameters at Wachemo University Nigist Eleni Mohammed Memorial Referral Hospital, 2019

RBC Parameters	Categories	Frequency (%)	Reference Range
Hgb (g/dl)	Low Normal	26(13.9%) 161(86.1%)	Male 11.5–18 Female 11–16.7
RBCx10^6/μL	Low Normal	13(7%) 174(93%)	Male 3.53–6.93 Female 3.45–6.25
HCT (%)	Low Normal	12(6.4%) 175(93.6%)	Male 36.2–58.6 Female 32.1–56.6
MCV(fl)	Low Normal High	34(18.2%) 148(79.1%) 5(2.7%)	Male 85–100 Female 85–100
MCH(pg)	Low Normal High	27(14.4%) 159(85%) 1(0.5)	Male 26.6–33.3 Female 25.8–32.8
MCHC g/dl	Low Normal High	4(2.1%) 179(95.7%) 4(2.1%)	Male 29.5–34.4 Female 28.5–34.4
RDW (%)	Low Normal High	1(0.5%) 180(96.3%) 6(3.2%)	Male 12–17 Female 12–17

Abbreviations: fl, femtoliters; g/dl, gram per deciliter; Hgb, hemoglobin; RBC, red blood cell; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; μL, microliters; %, percentage; pg, pictogram; RDW, red blood cell distribution width.

Different studies suggested that *H. pylori* infection was associated with some hematological abnormalities.[17] Therefore, the current study aimed to assess the relation between RBC parameters and *H. pylori* infection in adult patients at WUNEMMRH.

Alteration of RBC parameters in *H. pylori*-infected adult patients associated with different mechanisms; consumption of iron by the *H. pylori*[30] decreased iron absorption secondary to chronic gastritis,[20] iron loss via hemorrhagic gastritis, and active bleeding peptic ulcers, deficiency of iron and vitamin B12 secondary to chronic and atrophic gastritis,[27] which might contribute to the alteration of RBC parameters in *H. pylori*-infected patients as compared to control.

Besides, *H. pylori* infection is associated with deficiency of iron and vitaminB12,[20,21,30] which was

Table 4 Correlation Analysis of RBC Parameters with Predictors Among *H. pylori*-Infected Patients at Wachemo University Nigist Eleni Mohammed Memorial Referral Hospital, 2019

Predictors	RBC		Hgb		HCT		MCV		MCH		MCHC		RDW	
	r	p	R	p	R	p	r	p	r	p	r	p	r	p
Age	0.01	0.8	0.05	0.26	0.02	0.6	0.02	0.6	0.02	0.5	0.2	0.6	0.05	0.3
Gender	0.01	0.9	0.06	0.18	0.15	0.003	−0.1	0.01	0.07	0.1	0.5	0.2	0.1	0.04
Residence	0.08	0.1	−0.12	0.01	−0.11	0.02	0.05	0.2	0.08	0.1	0.1	0.5	0.1	0.04
BMI	0.03	0.4	0.08	0.1	0.03	0.5	0.04	0.4	0.11	0.02	0.1	0.03	0.02	0.5
SBP	0.13	0.01	0.14	0.006	0.07	0.14	0.02	0.6	−0.1	0.02	0.7	0.1	0.03	0.4
DBP	0.001	0.9	0.02	0.6	0.02	0.6	0.01	0.7	0.01	0.8	0.7	0.1	−0.1	0.04

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; Hgb, hemoglobin; RBC, red blood cell; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; p, the p-value for correlation; RDW, red blood cell distribution width; r, Pearson correlation coefficient.

important micronutrients for hemoglobin synthesis and maturation of red blood cells; their deficiency may result in variation in size and shapes of RBC, and defects in hemoglobin maturation. Consequently may contribute to the alteration of RBC parameters.

This study revealed a significant decrement of mean hemoglobin concentration (p<0.001) of *H. pylori*-infected patients as compared to the control. This finding was in line with other similar studies conducted in Palestine in adult *H. pylori*-infected patients,[17] in Egypt[22] and Karachi.[38] Consumption of iron by the *H. pylori*,[30] decreased iron absorption secondary to chronic gastritis,[20] iron loss via hemorrhagic gastritis, and active bleeding peptic ulcers,[27] which might contribute to decrement of blood hemoglobin concentration in *H. pylori*-infected patients. Several studies reported that depletion of iron in *H. pylori*-infected patients,[30] and eradication of *H. pylori* infection shows improvements in blood hemoglobin concentration.[22,23] However, our finding contradicts the study conducted in Dhaka, Bangladesh,[39] which reported there was no significant difference in hemoglobin concentration between the two groups. This might be due to a difference in sample size and methodology.

The mean values of RBC count were lower in *H. pylori*-infected patients compared to the control group in our study, which was statistically significant. This finding is supported by previous studies conducted in Turkey,[40] Palestine[17] and Sudan.[41] The decrement of RBC count among *H. pylori*-infected patients might be because of blood loss due to chronic erosive gastritis and active bleeding peptic ulcers.

In the current study, the mean level of HCT was showed a statistically significant difference between *H. pylori*-infected patients and control groups. A similar observation was reported from Butajira, Ethiopia,[5] Sudan,[41] and Turkey.[40] Change in hematological parameters might be due to *H. pylori* infection. Different studies also reported that eradication of *H. pylori* infection shows improvements in hematological parameters.[22,25]

The mean cell volume value was significantly lower in *H. pylori*-infected patients as compared to the control group in this study. This finding is supported by a study conducted in Sudan,[16] Turkey,[40] Dhaka, Bangladesh,[39] Butajira, Ethiopia.[5] Whereas, another similar study done in Palestine[17] and Sudan[41] was reported no significant difference in MCV value between *H. pylori*-infected patients and the control group. The observed difference

may be due to the variation in the sample size between studies.

Statistically, a significant mean difference in MCH value was observed between *H. pylori*-infected patients and the control group in our study. This finding is in agreement with a study conducted in Butajira, Ethiopia,[5] Kosti Teaching Hospital, Sudan,[16] and Bangladesh.[39] However, a study conducted in Palestine[17] and Sudan[41] reported no significant differences were found in the MCH value, which was contradicting our finding. Several studies indicated that *H. pylori* infection was associated with impairments of micronutrients essential for hemoglobin synthesis,[17,22,30] which may contribute to the observed difference.

In the current study, the mean level of MCHC was showed a statistically significant difference between *H. pylori*-infected patients and control groups. Contrary results were reported from Sudan,[16,41] but the supporting observation was reported from Ethiopia.[5]

H. pylori infection is associated with deficiency of iron and vitaminB12,[20,21,30] which was important micronutrients for hemoglobin synthesis and maturation of red blood cells; their deficiency may result in variation in size and shapes of RBC. A significantly higher mean value of RDW was found in *H. pylori*-infected patients as compared to the control group in this study. This finding is supported by a study conducted in Palestine.[17] This is might be due to fact that high RDW indicates impairment of erythropoiesis, reflecting chronic inflammation and deficiency of micronutrients, both of which are significant signs of *H. pylori* infection that result in the RBC size variation.

In the current study about 13.3%, 7%, 6.4%, and 18.2% of *H. pylori*-infected patients showed reduced blood Hgb concentration, RBC count, HCT, and MCV values respectively. Reduction in hematological indices might be due to blood loss via hemorrhagic gastritis and active bleeding peptic ulcers, impairing iron absorption as a result of chronic gastritis, deficiency of iron and vitamin B12 secondary to chronic and atrophic gastritis.

Concerning the correlation of hematological indices, RBC count and hemoglobin level showed a statistically positive correlation with systolic blood pressure. A similar observation was reported from Ethiopia,[42] China,[43] and Netherland.[44]

Conclusion

In this study, the mean value of Hgb, RBC count, HCT, MCV, MCH, and MCHC were showed significant decrements in *H. pylori*-infected patients compared to the

control group. However, the mean value of RDW in *H. pylori*-infected patients was significantly higher than in the control group. Therefore, hematological parameters showed a significant difference should be considered for proper diagnosis and management of *H. pylori*-infected patients, and eradication of this microorganism from infected patients, determination of hematological parameters for the patients who had *H. pylori* infection, prevention and control of this infection, and performing large longitudinal community-based studies were recommended.

Limitation of the Study

The limitation of this study is it does not show a cause-effect relationship between variables and *H. pylori* infection because of the cross-sectional nature of the study design. We had not assessed micronutrient level and neutrophil-lymphocyte ratio and platelet lymphocyte ratio due to logistic constraints.

Data Sharing Statements

The original data for this study is available from the corresponding author on a reasonable request.

Ethical Considerations

Ethical clearance was obtained from Wolkite University Ethical Review Board. A letter of cooperation was written to WUNEMMRH and permission was obtained from the hospital administration. Written informed consent was obtained from each study participant after explaining the purpose and procedures of the study. The data were kept confidential by using codes rather than any personal identifier, and the results were communicated to the physicians for proper management. The study was carried out following the Declaration of Helsinki.

Acknowledgments

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors declared that they have no competing interests.

References

1. Hooi JKY, Lai WY, Ng WK, et al. Global Prevalence of Helicobacter pylori Infection: systematic Review and Meta-Analysis. World J of Gastroenterology. 2017;153(2):420–429. doi:10.1053/j.gastro.2017.04.022
2. Sjomina O, Pavlova J, Niv Y, Leja M. Epidemiology of Helicobacter pylori infection. John Wiley. 2018;13:6–11.
3. Zamani M, Alizadeh- WHMR. Systematic review with meta-analysis: the worldwide prevalence of Helicobacter pylori infection. Aliment Pharmacol Ther. 2018;868–876.
4. Melese A, Genet C, Zeleke B, Andualem T. Helicobacter pylori infections in Ethiopia; prevalence and associated factors: a systematic review and meta-analysis. BMC Gastroenterol. 2019;19(8):1–15.
5. Kibru D, Gelaw B, Alemu A, Addis Z. Helicobacter pylori infection and its association with anemia among adult dyspeptic patients attending Butajira Hospital, Ethiopia. BMC Infect Dis. 2014;14:1–7.
6. Shiferaw G, Abera D. Magnitude of Helicobacter pylori and associated risk factors among symptomatic patients attending at Jasmin internal medicine and pediatrics specialized private clinic in Addis Ababa city, Ethiopia. BMC Infect Dis. 2019;5:1–6.
7. Seid A, Demsiss W. Feco-prevalence and risk factors of Helicobacter pylori infection among symptomatic patients at Dessie Referral. BMC Infect Dis. 2018;18:1–9.
8. Tadesse E, Daka D, Yemane D, Shimelis T. Seroprevalence of Helicobacter pylori infection and its related risk factors in symptomatic patients in southern Ethiopia. BMC Res Notes. 2014;7(1):1–5.
9. Kao C, Sheu B, Wu J. Helicobacter pylori infection: an overview of bacterial virulence factors and pathogenesis. Biomed J. 2016;39(1):14–23.
10. Camilo V, Sugiyama T, Touati E. Pathogenesis of Helicobacter pylori infection. John Wiley. 2017;22:1–6.
11. Chmiela M, Kupcinskas J. Review: pathogenesis of Helicobacter pylori infection. John Wiley. 2019;24:1–5.
12. De BBB, Antônio F, Soares AS, et al. Pathogenesis and clinical management of Helicobacter pylori gastric infection. World J Gastroenterology. 2019;25(37):5578–5589.
13. Gebresillasse HW, Tamrat G, Abule T. Gastric cancer features and outcomes at a tertiary teaching hospital in Addis Ababa, Ethiopia: a 5-year retrospective study. East Cent Afr J Surg. 2019;24(2):105–109.
14. Augusto J, Díaz U, Regino WO, Zuleta MG. Helicobacter pylori and hematologic diseases. Rev Col Gastroenterol. 2013;28(4):323–331.
15. Campuzano-Maya G. Hematologic manifestations of Helicobacter pylori infection. World J Gastroenterology. 2014;20(36):12818–12838.
16. Ali E, Elamin I, Suliman MA, et al. Effect of helicobacter pylori infection on haematological parameters in kosti teaching hospital, sudan. Iran Red Crescent Med J. 2018;20(2):1–6.
17. Mwafy SN, Afana WM. Hematological parameters, serum iron, and vitamin B 12 levels in hospitalized Palestinian adult patients infected with Helicobacter pylori. Hematol Transfus Cell Ther. 2018;40(2):160–165.
18. Victor Hoffbrand A. Daniel Catovsky, Edward GD Tuddenham ARG. Postgraduate Haematology. sixth ed. Wiley-Blackwell; 2011.

19. Humeida AT, Abdalla MHA. Association of helicobacter Pylori Infection and Vitamin B 12 Level among Sudanese Patients. IOSR J Dent Med Sci. 2017;16(3):12–14.

20. Jasem MA, Al-ubaidi AA, Daood NM, Muhsin JA. Iron deficiency in Helicobacter pylori-infected patients in Baghdad. J Microbiol Infect Dis. 2011;1(3):114–117.

21. Fraser AG, Scragg R, Schaaf D, Metcalf P, Grant CC. Helicobacter pylori infection and iron deficiency in teenage females in New Zealand. J New Zeal Med Assoc. 2010;123(1313):38–45.

22. Abdel Y, Abdel L, Mohamed R, Mohamed R, Ahmed M. Helicobacter pylori and its hematological effect. Egypt J Intern Med. 2019;31:332–342.

23. Huang X, Qu X, Yan W, et al. Iron deficiency anemia can be improved after eradication of Helicobacter pylori. Postgrad Med J. 2010;86:272–278.

24. Demerdash DME, Ibrahim H, Hassan DM, Moustafa H, Tawfik NM. Helicobacter pylori-associated to unexplained or refractory iron deficiency anemia: an Egyptian single-center experience. Hematol Transfus Cell Ther. 2018;40(3):219–225.

25. Hudak L, Jaraisy A, Haj S, Muhsen K. An updated systematic review and meta-analysis on the association between Helicobacter pylori infection and iron deficiency anemia. Wiley. 2017;22:1–16.

26. Sato Y, Yoneyama O, Azumaya M, Takeuchi M, Sasaki S. The relationship between iron deficiency in patients with helicobacter pylori -infected nodular gastritis and the serum prohepcidin level. John Wiley. 2014;20:11–18.

27. Miguel A. Hematological Extradigestive Manifestations of Helicobacter pylori Infection in Childhood. InTech. 2016;76–110.

28. Ansari S, Yamaoka Y. Survival of Helicobacter pylori in the gastric acidic territory. HHS Public Access. 2018;22(4):1–25.

29. Mitchell H, Dipped H, Katelaris P, Frcp F. Epidemiology, clinical impacts and current clinical management of Helicobacter pylori infection. Med J Aust. 2016;204(10):376–386.

30. Ciacci C, Sabbatini F, Cavallaro R, et al. Helicobacter pylori impairs iron absorption in infected individuals. Dig Liver Dis. 2004;36:455–460.

31. Campuzano-Maya G. Helicobacter pylori and Hematologic Diseases. InTech. 2016;33–52.

32. Tsay F, Hsu P. H. pylori infection and extra-gastroduodenal diseases. J Biomed Sci. 2018;1–8.

33. Campuzano-Maya G. Helicobacter pylori and Hematologic Diseases. World J Gastroenterology. 2016;33–39.

34. Kalali B, Formichella L, Gerhard M. Diagnosis of Helicobacter pylori: changes towards the Future. Open-Access Dis. 2015;3:122–135.

35. Hunt RH, France FM. Helicobacter pylori in developing countries. world gastroenterology organisation global guideline. J Gastrointestin Liver Dis. 2011;20(3):299–304.

36. Yalew A, Terefe B, Alem M, Enawgaw B. Hematological reference intervals determination in adults at Gondar university hospital, Northwest Ethiopia. BMC Res Notes. 2016;9(483):1–9.

37. Eusebi LH, Zagari RM, Bazzoli F. Epidemiology of Helicobacter pylori Infection. John Wiley. 2014;19:1–5.

38. Zuberi BF, Afsar S, Qadeer R, Baloch I. Muhammad Saeed Quraishy AK, and NA. hemoglobin, ferritin, vitamin B12, and helicobacter pylori infection: a study in patients who underwent upper gi endoscopy at civil hospital Karachi. JCPSP. 2007;17(9):546–549.

39. Rahman A, Karim ME, Saeed A, Siddique AR, Sadat SMA. Association between helicobacter pylori infection and iron deficiency anemia: a cross-sectional study. J Bangladesh Coll Phys Surg. 2020;38(2):68–78.

40. Saler T, Özgür F, Ke G, Sibel KJ, Ahbab S. L GO. H. pylori may not be associated with iron deficiency anemia in patients with normal gastrointestinal tract endoscopy results. Hindawi Publ Corp Adv Hematol. 2015;1–5.

41. Abass A, Mohamed KO, Yosif AA, Fatima A, Elfadil M. Evaluation of serum vitamin B12 and ferritin levels in h. pylori- associated gastritis. IOSR J Pharm Biol Sci. 2016;11(1):1–5.

42. Enawgaw B, Adane N, Terefe B, Asrie F, Melku M. A comparative cross-sectional study of some hematological parameters of hypertensive and normotensive individuals at the University of Gondar hospital, Northwest Ethiopia. BMC Hematol. 2017;17:1–7.

43. Yan X, Junli Zuo SZ. Association of hemoglobin and blood pressure in a chinese community-dwelling population. Pulse. 2018;6:154–160.

44. Atsma F, Veldhuizen I, De KW, Van KM, Jong PP, Deinum J. Hemoglobin level is positively associated with blood pressure in a large cohort of healthy individuals. Hypertension. 2012;60:936–941.

Practice of Blood Donation and Associated Factors Among Adults of Gondar City, Northwest Ethiopia: Bayesian Analysis Approach [Retraction]

RETRACTION

Practice of Blood Donation and Associated Factors Among Adults of Gondar City, Northwest Ethiopia: Bayesian Analysis Approach [Retraction]

Kassie A, Birara S. *J Blood Med*. 2020;11:525–532.

The Editor-in-Chief and Publisher of the *Journal of Blood Medicine* wish to retract the published article.

It has come to our attention that a very similar version of this article was also published in *PLOS ONE*, Mar 2, 2020. https://doi.org/10.1371/journal.pone.0228929. The authors failed to properly attribute the source of their previous publication or disclose this to the editor. Thus, it has

been deemed to be a redundant publication and the editor has requested for the paper to be retracted.

Our decision-making was informed by our policy on publishing ethics and integrity and the COPE guidelines on retraction.

The retracted article will remain online to maintain the scholarly record, but it will be digitally watermarked on each page as "Retracted".

Duration of Storage Reduced Erythrocytes Profiles and *Plasmodium* Viability in Donor Blood

Nelly Al Audhah, Eko Suhartono, Teguh Wahju Sardjono & Loeki Enggar Fitri

Published online: 22 Feb 2021.

Submit your article to this journal ☑

Article views: 183

View related articles ☑

View Crossmark data ☑

ORIGINAL RESEARCH

Duration of Storage Reduced Erythrocytes Profiles and *Plasmodium* Viability in Donor Blood

Nelly Al Audhah[1,2]
Eko Suhartono[3]
Teguh Wahju Sardjono[4]
Loeki Enggar Fitri [4]

[1]Doctoral Program of Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia; [2]Department of Parasitology, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia; [3]Department of Chemistry/Biochemistry, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia; [4]Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia

Video Abstract

Correspondence: Loeki Enggar Fitri
Department of Parasitology, Faculty of Medicine Universitas Brawijaya, Jl. Veteran, Malang, East Java, Indonesia
Email lukief@ub.ac.id

Background: Malaria screening for blood derived from any donors prior to transfusions is a standard procedure that should be performed; but, in fact, it is not routinely conducted. In case of the blood is infected with *Plasmodium* spp., the survival of parasites may be depending on, or even influencing, the profile of red blood cells (RBCs).

Methods: This observational longitudinal study was conducted upon 55 bags of donor blood that randomly selected. Malaria infections were detected using Rapid Diagnostic Test/RDT with thin and thick blood smear confirmation. The changes of *Plasmodium* spp. viability and RBCs profiles, as well as other hematological parameters, were observed from the results of routine hematological examinations which were performed on days 1,7,14 and 21 of storage.

Results: Among 55 blood samples, there were 17 and 38 bags, respectively, positive and negative for malaria, then used for analysis as the case and control groups. There were significant decreasing values (p<0.05) of all routine blood examination parameters of donor blood, started from days 1, 7, 14, 21, and 28. There were no differences in decreasing profiles between those infected and non-infected donor blood (p>0.05). On days 21 and 28 none of the positive samples still contained parasites.

Conclusion: Erythrocytes profiles of donor blood significantly decreased with the duration of storage, but were not influenced by the presence of *Plasmodium* spp.

Keywords: donor blood, *Plasmodium* viability, erythrocyte profile, storage

Introduction

Malaria is one of the leading life-threatening infectious diseases, especially in endemic regions, such as Sub Sahara, South and Central America, and some regions in Asia. Besides the natural cases, transfusion-transmitted malaria (TTM) may commonly occur in an endemic area, through the transfusion of blood from asymptomatic donors. WHO has already recommended following the procedure of blood screening for malaria as well as for HIV, hepatitis, and VDRL, but in fact, those were not routinely conducted,[1,2] even in Indonesia as well.[3]

The bloods derived from the donors are collected in the bags containing anticoagulant, and if not immediately given to the recipients, they will be stored in certain conditions, such as a temperature of −4°C.[4] In those specific conditions, the anticoagulant is needed to maintain the viability of blood, together with its containing components, before the transfusion.[5]

In normal condition, the viability of blood especially red blood cells, as long as they are still in the circulation, is around 120 days, before they then are metabolized by the hematopoietic system. But if the RBCs are infected by *Plasmodium* spp., their viability will be shorter, as they will be damaged by the parasite inside the

RBCs. The rate of RBC damage will be relevant to the species of *Plasmodium*, and therefore will influence the RBC profiles also.[6]

There are several hematological parameters routinely used to express the quality of blood components, especially the RBCs profiles; ie, the level of hemoglobin (Hb), hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC).[7] The quality of the RBCs will also affect the growth of the parasites and indirectly the rate of transfusion-transmitted malaria. For that reason, this study was conducted in order to observe the influence of duration of blood storage prior to transfusion on RBCs profiles, in the blood infected and not infected by *Plasmodium* spp., as well as the viability of parasites in the infected blood.

Research Design

This study is an observational longitudinal study using blood from the donation as samples. The bloods were examined for the presence or absence of *Plasmodium* spp., and divided into two groups *Plasmodium* spp. (+) and *Plasmodium* spp. (-). Donor blood had been analyzed for erythrocyte profiles using a hematology analyzer for six parameters. This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Health Research Faculty of Medicine, Lambung Mangkurat University, through the issuance of an ethical clearance no. 521/KPK-FK UNLAM/EC/X/2017. The donors were informed about the possibility that their blood would not be given to recipients, instead to be used as a research sample, and they understood about it. If they agreed, they must sign an informed consent.

Materials and Methods

After receiving ethical approval from the Ethics Committee, blood samples were collected at the same time as blood donor activities on Blood Bank, Banjar District and Blood Bank, Tanah Laut District and Blood Bank, from October 2017 to February 2018. We collected 1281 bags of donor blood. Results of malaria screening using RDT and blood smear examinations showed that 17 were positive. As sample size calculation needed 55 samples, we randomly determined 38 malaria negative samples as control with the consideration of matching of sex and age. Prior to the blood donation activity, hemoglobin level, blood pressure examination and a brief interview

about donor history were conducted to select donors based on exclusion criteria, such as the age over 60 years, suffering from Diabetes Mellitus, hypertension, asthma, heart disease and congenital blood disorders. The bloods in the bags were taken from donors who were positive and negative for malaria, sent in a cooler box, and then stored at −4°C until they were used for further examinations and analysis, as study and control groups, respectively.

When examining hemoglobin, the residual blood at the fingertips was immediately screened for malaria using blood smear (thin and thick blood smear preparations) and using Rapid Diagnostic Test (Pakar Biomedika Indonesia, catalog number: AUC-01D07) to identify the presence of *Plasmodium falciparum* histidine-rich protein-2 (*Pf*HRP-2) and all species of pan lactate dehydrogenase. The donor blood bags that were used to collect samples contained CPDA-1 anticoagulant (JMS Single Blood Bag 350 mL Catalog Number 901396401). During the experimental study, the blood was stored at −4°C in the faculty refrigerator and checked daily to ensure the consistency of the temperature. Furthermore, screening for HIV/AIDS, syphilis, and hepatitis was carried out on blood transfusions as a routine screening in accordance with Government Regulation No.7 of 2011.[3] The changes of erythrocytes (RBCs) profile and other hematological parameters were observed from the results of routine hematological examinations using a hematological analyzer (Rayto Web lab, Type WP-330, catalog no. 42000000-AKS-000458665) which were performed on days 1,7,14, and 21 of storage. The viability of *Plasmodium* spp. was observed using standard Giemsa stain and was determined based on the morphology of the parasite that still contained nucleus and cytoplasm without any form of crisis form.

Erythrocyte profile examination results were tabulated and then calculated for the average and their distribution. Statistical analyses utilized to evaluate the erythrocyte profiles based on the day of observation were the Repeated ANOVA test and post-hoc Paired Wise Comparison test for RBC, HCT and MCH, and Friedman test and post-hoc Wilcoxon test for Hb, MCV and MCHC with a significance level of $p<0.05$, while erythrocyte profiles based on groups infected with *Plasmodium* spp. and not infected with *Plasmodium* spp. were analyzed using the Independent *T*-test for RBC, Hb, HCT, and MCH and Mann–Whitney test for MCV and MCHC with significance level $p<0.05$.

Results

The characteristics of the research subjects are based on age, gender, education, employment and the blood group classification as mention in Table 1.

The mean value of all parameters of erythrocyte blood profile in all donor blood tended to decrease through the days of observation (Table 2). The statistical results showed that there were significant differences in the profile of RBCs among five different days of observations (p=0.000, using Repeated ANOVA test and Friedman test).

Observations of the presence and the survival of the parasite in the donor blood based on the morphology of *Plasmodium* spp. were carried out using thin and thick blood smear preparations. Three species of *Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale* and mixed infections of *Plasmodium vivax, Plasmodium falciparum*, were identified as shown in Figures 1 and 2.

Until day 14, *Plasmodium falciparum, Plasmodium vivax* and mix infection still can be detected as immature and mature trophozoite stage with normal morphology containing a regular nucleus and clear cytoplasm without any form of crisis form. Unfortunately, the longer storage makes reduction in the presence of the parasites (Figure 3).

During storage, a decrease in the number of RBCs from donor blood containing *Plasmodium* spp. was compared to those in the RBCs from donor blood that did not contain *Plasmodium* spp. There was no significant difference in the results (Independent *T*-test showed the first day, p= 0.226; seventh day, p= 0.066; fourteenth day, p= 0.067; twenty-first day, p= 0.050; twenty-eighth day, p= 0.375). The mean of RBCs numbers decreased significantly with storage time based on day observation (7th day lower than those at 1st day, 14th day lower than those at 7th day, 21st day lower than those at 14th day, 28th day lower than those at 21st day, all p value = 0.000; Repeated ANOVA test and post-hoc Paired Wise Comparison test). The longer storage led to more erythrocyte lysis, causing more decrease in RBCs number in the two groups. Figure 4 shows there were more than 50% of RBCs lysis on the 21st day of observation in both donor blood groups.

The following graph shows a hemoglobin level in serial observations of both groups (Figure 5). There was no significant different about the results (the first day, p=

Table 1 Demographic Characteristics of Research Subjects

Characteristics	Category	*Plasmodium* spp. (+) (N = 17)		*Plasmodium* spp. (-) (N = 38)	
		Σ	Percentage	Σ	Percentage
Sex	Man	8	47,06%	17	44,74%
	Women	9	52,94%	21	55,26%
Age	< 30 years	3	17,65%	14	36,84%
	30–40 years	9	52,94%	14	36,84%
	41–50 years	4	23,53%	4	10,53%
	51–60 years	1	5,88%	6	15,79%
Employment	Traders/Seller	1	5,88%	3	7,89%
	The farmers	11	64,7%	7	18,42%
	The miners	3	17,65%	1	2,63%
	Company employees	-		19	50%
	Government employees	-		2	5,26%
	Not work or students	2	11,76%	6	15,79%
Education	No school	6	35,29%	2	5,26%
	Primary school	6	35,29%	5	13,16%
	junior high school	4	23,53%	13	34,21%
	Senior high school	-		16	42,11%
	University	1	5,88%	2	5,26%
Blood group	A	5	29,41%	13	34,21%
	B	4	23,53%	6	15,79%
	AB	-		3	7,89%
	O	8	47,06%	15	39,47%

Table 2 Erythrocyte Profiles (Mean, Median, Standard Deviation, Minimum–Maximum)

Parameters	Day of Observation	Plasmodium spp. (+) (N=17)		Plasmodium spp. (-) (N = 38)	
		Median (Minimum-Maximum)	Mean ± SD	Median (Minimum-Maximum)	Mean ± SD
RBC (× 10^6/ul)	1st day	5.31 (4.08–6.85)	5.35 ± 0.78[a]	4.90 (3.86–6.86)	5.08 ± 0.73[a]
	7th day	3.63 (2.90–5.14)	3.73 ± 0.59[b]	3.31 (2.62–4.66)	3.41 ± 0.51[b]
	14th day	2.27 (1.74–3.34)	2.34 ± 0.41[c]	2.09 (1.64–2.94)	2.12 ± 0.33[c]
	21st day	1.24 (0.00–2.00)	1.29 ± 0.42[d]	1.20 (0.00–1.71)	1.03 ± 0.52[d]
	28th day	0.00 (0.00–1.10)	0.13 ± 0.35[e]	0.00 (0.00–1.09)	0.42 ± 0.19[e]
Hb (g/dl)	1st day	14.20 (11.40–16.40)	14.20 ± 1.09[a]	14.30 (9.30–16.30)	13.80 ± 1.78[a]
	7th day	9.90 (8.60–12.30)	9.89 ± 0.95[b]	9.60 (6.00–11.10)	9.31 ± 1.24[b]
	14th day	5.90 (5.20–8.00)	6.20 ± 0.76[c]	5.90 (3.60–7.00)	5.79 ± 0.81[c]
	21st day	3.40 (2.80–4.80)	3.58 ± 0.55[d]	3.40 (2.00–4.10)	3.31 ± 0.50[d]
	28th day	1.80 (0.00–2.60)	1.81 ± 0.58[e]	1.80 (0.00–2.20)	1.56 ± 0.66[e]
HCT (%)	1st day	42.90 (36.50–49.60)	43.06 ± 3.35[a]	42.75 (29.20–51.80)	42.31 ± 5.25[a]
	7th day	29.70 (24.20–37.20)	30.04 ± 3.17[b]	29.05 (19.00–35.20)	28.41 ± 3.68[b]
	14th day	18.40 (14.50–24.20)	18.85 ± 2.56[c]	18.05 (11.40–22.20)	17.67 ± 2.45[c]
	21st day	10.70 (8.00–14.50)	10.92 ± 1.86[d]	10.40 (6.30–12.90)	10.11 ± 1.5 2[d]
	28th day	5.70 (4.00–8.00)	5.79 ± 1.21[e]	5.50 (3.10–6.80)	5.28 ± 0.88[e]
MCV (fL)	1st day	79.30 (56.90–89.10)	77.77 ± 9.00[a]	84.00 (55.80–89.40)	80.74 ± 9.05[a]
	7th day	54.30 (42.70–66.00)	54.19 ± 7.24[b]	56.4 (37.90–60.80)	54.18 ± 6.19[b]
	14th day	34.30 (25.00–42.90)	34.03 ± 5.35[c]	34.65 (23.00–38.30)	33.82 ± 4.09[c]
	21st day	18.90 (13.30–25.70)	19.71 ± 3.67[d]	19.95 (12.70–22.20)	19.35 ± 2.53[d]
	28th day	9.40 (6.40–14.20)	10.44 ± 2.32[e]	10.55 (6.30–11.80)	10.09 ± 1.48[e]
MCH (pg)	1st day	6.70 (5.20–8.60)	6.69 ± 1.00[a]	7.05 (4.50–10.80)	7.09 ± 1.24[a]
	7th day	4.70 (3.50–5.80)	4.66 ± 0.70[b]	4.60 (2.90–6.70)	4.68 ± 0.79[b]
	14th day	2.90 (2.10–3.80)	2.94 ± 0.49[c]	2.90 (1.70–4.20)	2.96 ± 0.55[c]
	21st day	1.70 (1.20–2.30)	1.71 ± 0.32[d]	1.70 (0.90–2.40)	1.69 ± 0.33[d]
	28th day	0.90 (0.60–1.30)	0.90 ± 0.21[e]	0.90 (0.50–1.30)	0.89 ± 0.19[e]
MCHC (g/dl)	1st day	32.40 (27.30–35.40)	32.49 ± 1.84[a]	32.85 (28.90–45.20)	33.51 ± 2.65[a]
	7th day	22.80 (17.70–26.60)	22.66 ± 2.11[b]	22.25 (19.70–30.70)	22.49 ± 1.87[b]
	14th day	13.80 (10.60–17.30)	14.24 ± 1.77[c]	13.95 (12.00–19.40)	13.99 ± 1.30[c]
	21st day	7.90 (5.80–10.40)	8.25 ± 1.32[d]	8.10 (6.60–11.30)	8.00 ± 0.87[d]
	28th day	4.20 (2.90–5.70)	4.36 ± 0.88[e]	4.30 (3.30–6.00)	4.18 ± 0.53[e]

Note: Different notations in superscript letters mean significant difference (p< 0.05).

0.402; seventh day, p= 0.061; fourteenth day, p= 0.081; twenty-first day, p= 0.098; twenty-eighth day, p= 0.178; Independent *T*-test). From the observation that was carried out from the first day to the twenty-eighth day of storage, the Hb levels were significantly different between day observations with all p value = 0.000 (Friedman test and Post-hoc Wilcoxon test) both in two groups.

The following graph shows a hematocrit level in serial observation of both groups (Figure 6). Both hematocrit levels from donor blood containing *Plasmodium* spp. and did not contain *Plasmodium* spp. decreased over a long period of storage. The trend was similar to that in Figure 4 which shows the decrease of erythrocyte

number. Statistical analysis showed there was no difference in the level of hematocrit between the two donor blood groups (p = > 0.05). Independent *T*- test showed the first day, p = 0.525; seventh day, p = 0.105; fourteenth day, p = 0.118; twenty-first day, p = 0.127; twenty-eighth day, p = 0.127. The longer storage caused more decrease in all of the hematocrit levels in the two groups, which showed there was a difference (all p value = 0.000, Repeated ANOVA test and post-hoc Paired Wise Comparison test).

All-day observations of MCV levels from donor blood containing *Plasmodium* spp. and that not containing *Plasmodium* spp. were similar and there was no difference
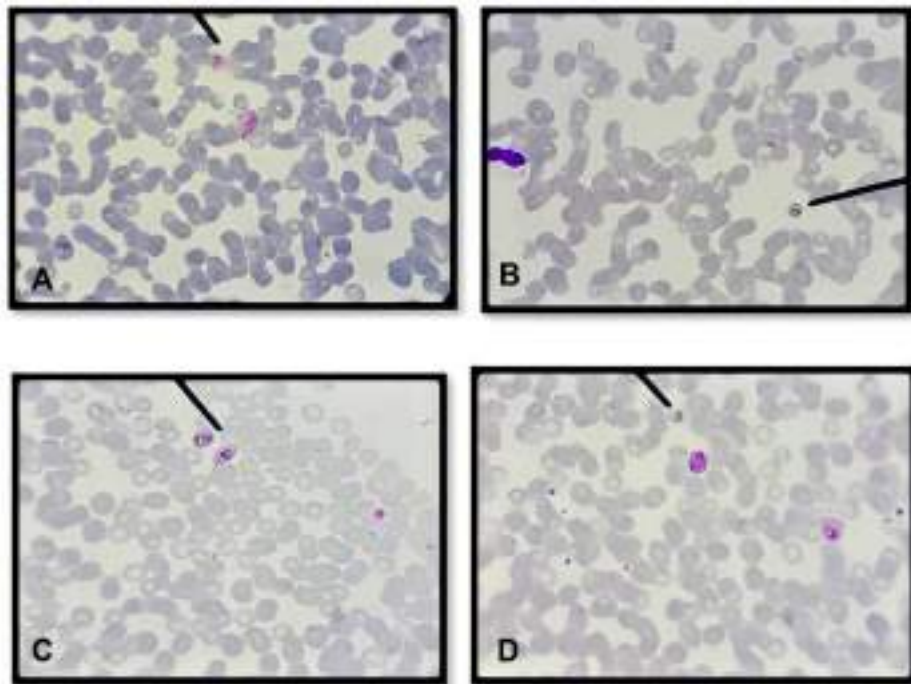
Figure 1 Species of *Plasmodium* identified using Giemsa stain on the day of blood collection. (**A**) *Plasmodium vivax*; (**B**) *Plasmodium falciparum*; (**C**) *Plasmodium ovale*; (**D**) mix infection (*Plasmodium falciparum* and *Plasmodium vivax*) were identified in donor blood samples. All the parasites show normal morphology characterized by regular ring form, normal volume of cytoplasm and no halo formation both in trophozoite and gametocyte stages.
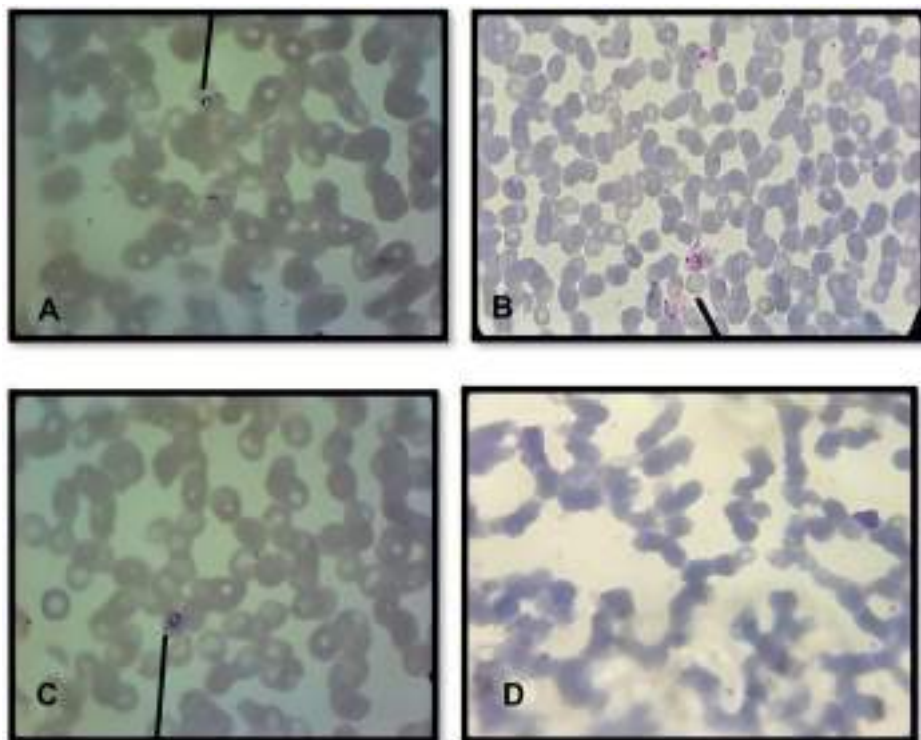
Figure 2 The presence of *Plasmodium* spp. identified using Giemsa stain on day 1, 7 and 14. (**A**) Several ring form of *Plasmodium falciparum* characteristic by acole form from day 1; (**B**) Amoeboid form of *Plasmodium vivax* from day 7; (**C**) Viable trophozoite of *Plasmodium falciparum* characteristic by ring with tick cytoplasm and Maurer's dot from day 14; (**D**) On day 21 no parasite was found. All the parasites show normal morphology characterized by regular nucleus, normal cytoplasmic volume and no halo formation representing live parasite.
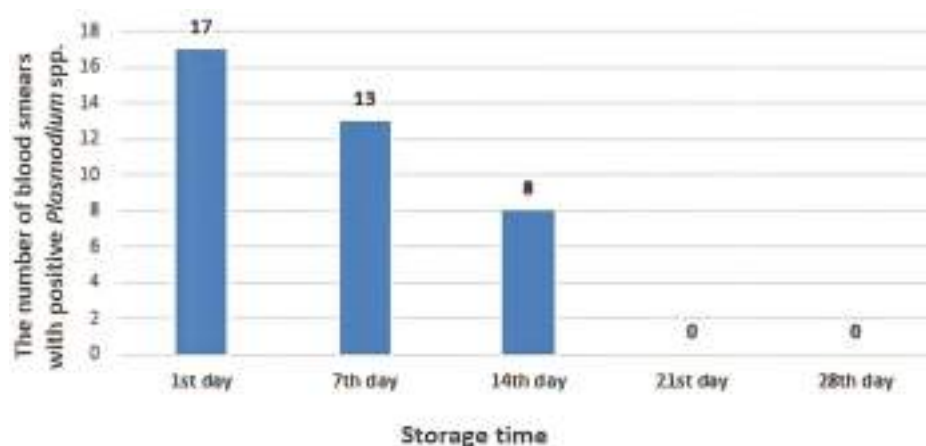
Figure 3 The number of positive samples in blood smears preparations from day 1 until day 28. The presence of parasites was reduced through the day observations.
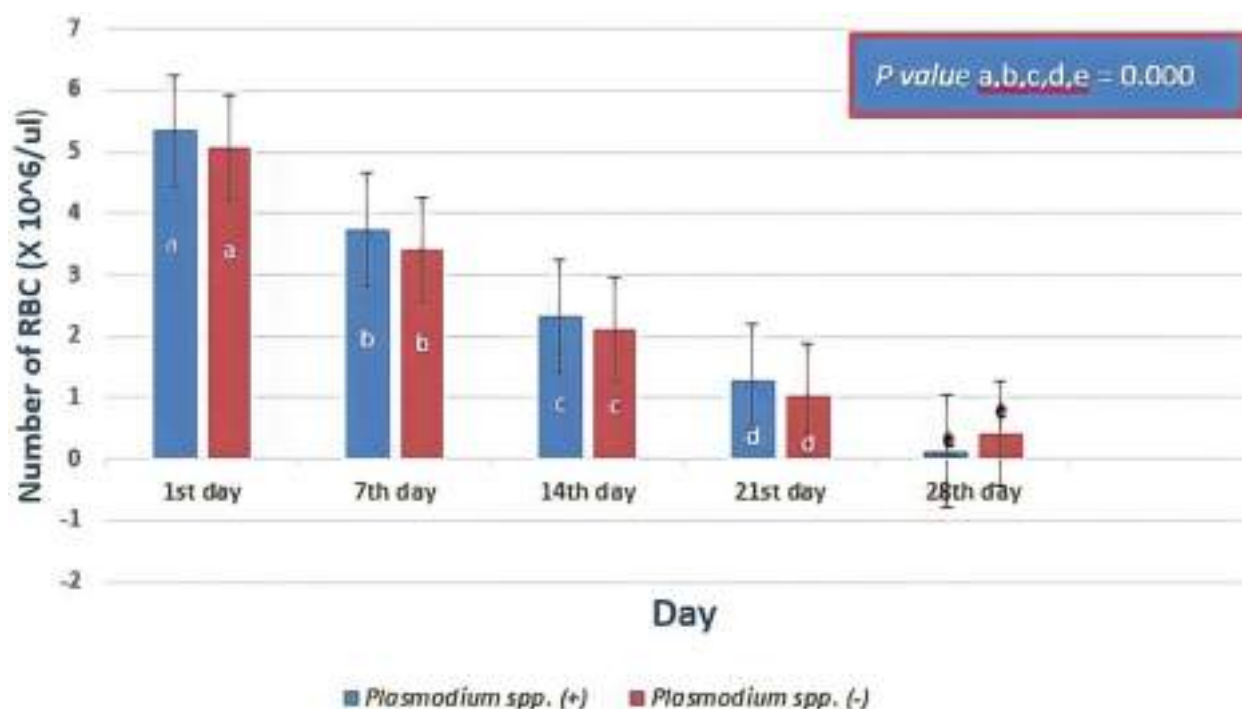
Figure 4 The number of red blood cells (RBCs) based on days of observation. In serial day observations, there was no significant difference in the decrease of number of RBCs between donor blood containing *Plasmodium* spp. and those of donor blood that did not contain *Plasmodium* spp. (p> 0.05; Independent *T*-test). However, the means of RBCs number through the day observations were reduced significantly with all p value = 0.000; Repeated ANOVA test and post-hoc Paired Wise Comparison test) in both groups. Different notations mean significant difference (p< 0.05).

between the two groups (Figure 7). Mann–Whitney test showed the p-value from the first day was 0.178, the seventh day was 0.942, the fourteenth day was 0.920, the twenty-first day was 0.792 and, the twenty-eighth day was 0.566, respectively. However, based on statistics, there was a difference in the average level of MCV in serial observations of both groups (all p value = 0.000, Friedman test and post-hoc Wilcoxon test).

Figure 8 shows, from all days of observation, the MCH of erythrocytes from donor blood containing *Plasmodium* spp., and those that did not contain *Plasmodium* spp. were not different. Statistical analysis using Independent *T*-test showed on the first day p= 0.211; on seventh day p= 0.935, on fourteenth day p= 0.881, on twenty-first day p= 0.814; and on twenty-eighth day p= 0.858. However, based on the Repeated ANOVA test and post-hoc Paired Wise
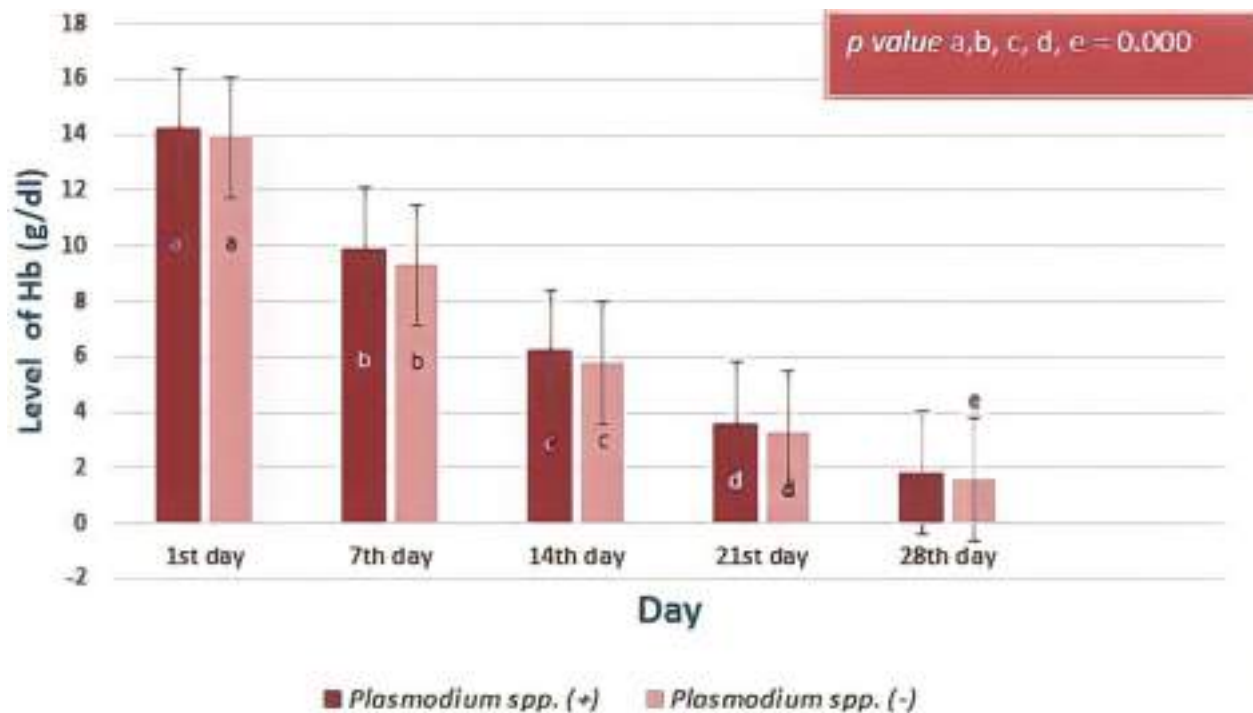
Figure 5 The level hemoglobin (Hb) levels based on day of observation. The decrease in the level of hemoglobin in donor blood containing *Plasmodium* spp. compared to that of donor blood did not contain *Plasmodium* spp. was not significantly different (p value > 0.05 Independent *T*-test). However, statistical results showed that there was a significant difference in the Hb profile at the five-day observation (p value <0.000, Friedman test and post-hoc Wilcoxon test) between the two groups. Different notations mean significant difference (p< 0.05).
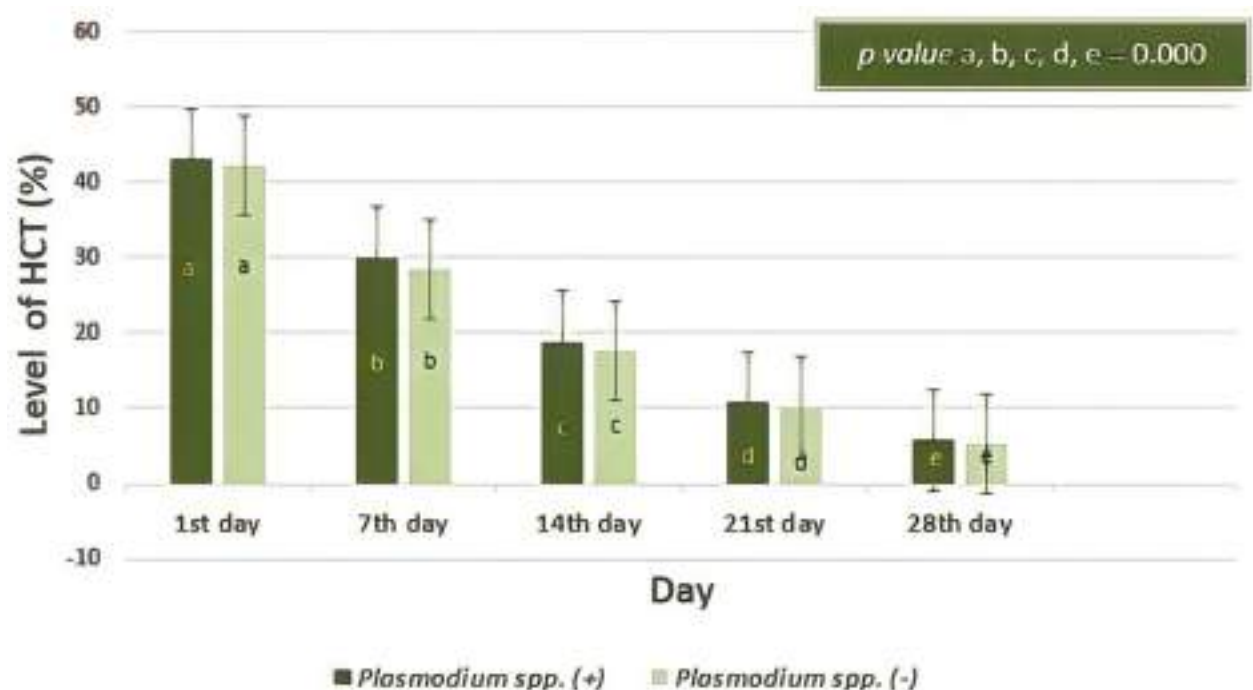
Figure 6 The percentage of Hematocrit (HCT) based on days of observation. Decreases in the percentage of hematocrit in donor blood containing *Plasmodium* spp. was not significantly different to those of donor blood that did not contain *Plasmodium* spp. (p> 0.05; Independent *T*-test). Among day observations, the level of hematocrit percentage were significantly different (all p value= 0.000, Repeated ANOVA test and post-hoc Paired Wise Comparison test) both in two groups. Different notations mean significant difference (p< 0.05).
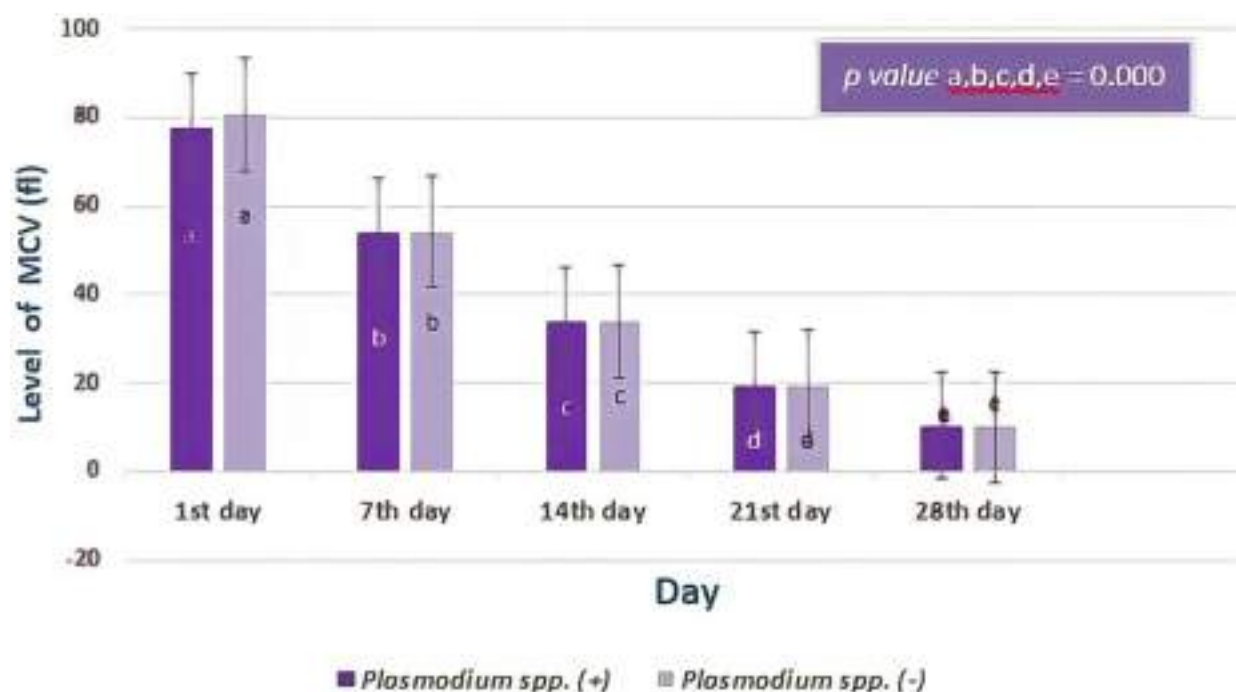
Figure 7 The level of Mean Corpuscular Volume (MCV) based on day of observation. The average level of MCV in donor blood containing *Plasmodium* spp. showed no significant difference compared to those in donor blood that did not contain *Plasmodium* spp. (p> 0.05; Mann–Whitney test). However, observation carried out from the first day to the twenty-eighth day of storage of level MCV showed a significant difference (all p value= 0.000, Friedman test and post-hoc Wilcoxon test) both in two groups. Different notations mean significant difference (p< 0.05).
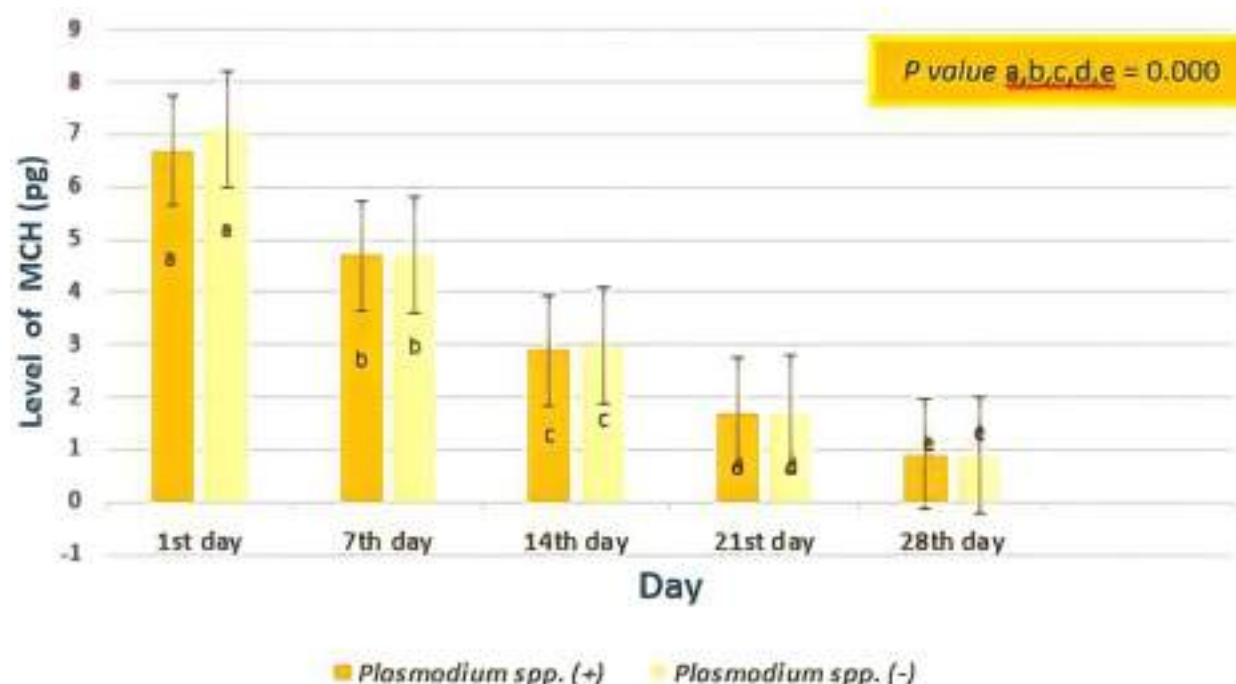
Figure 8 The level of Mean Corpuscular Hemoglobin (MCH) based on days of observation. The MCH of erythrocytes from donor blood containing *Plasmodium* spp. and those did not containing *Plasmodium* spp. was not different (all p value > 0.05; Independent *T*-test), but analysis on the level of MCH based on storage time showed a significant difference (all p value < 0.05; Repeated ANOVA test and post-hoc Paired Wise Comparison test) both in two groups. Different notations mean significant difference (p< 0.05).

Comparison test, there was a difference in the average level of MCH based on storage time (7th day lower than those at 1st day, 14th day lower than those at 7th day, 21st day lower than those at 14th day, 28th day lower than those at 21st day).

The decrease of the MCHC levels from donor blood that did not contain *Plasmodium* spp. was not different from those in donor blood that contained *Plasmodium* spp. (Figure 9). However, in the twenty-first day to twenty-eighth day examination, the decrease in the MCHC level of this group was very rapid, using Mann–Whitney test there were no significant differences in the results (the first day, p= 0.229; seventh day, p= 0.477; fourteenth day, p= 0.548; twenty-first day, p= 0.449; twenty-eighth day, p= 0.499). Statistical analysis showed MCHC levels were different based on longer storage, 7th day was lower than at 1st day, 14th day was lower than the 7th day, 21st day was lower than the 14th day, 28th day was lower than the 21st day (all p value = 0.000, Friedman test and post-hoc Wilcoxon test).

Discussion

Banjar Regency and Tanah Laut Regency are parts of malaria-endemic districts in South Kalimantan Province (Routine Data from the Malaria Sub Directorate, Ministry of Health, Republic of Indonesia, 2017).[8] However, malaria screening through microscopic examination and RDT has never been carried out during donor blood activities.

Donors who had a history of suffering from malaria but during donation activities showed no symptoms of malaria and met the criteria as a donor were allowed to donate blood. This might be the possible causes of malaria transmission through blood transfusions because although they felt healthy, their blood still had a risk to harbor *Plasmodium* parasites.

In this study, some of *Plasmodium* spp. was still found in RBCs until 14 days of storage. A Previous study revealed that cold storage of RBCs was able to support the growth of *P. falciparum*. The study also showed a decrease in the number of blood smear slides containing *Plasmodium* spp. during the sequential days of observation. It was assumed that the parasites have the capability to survive with minimal energy sources, such as in blood in the transfusion bags containing anticoagulant and stored at a certain temperature for many days. The limitation of cold storage environment might affect the parasite growth because some parasites are more likely to infect young RBCs.[9] In the early days, *Plasmodium* in the blood may be
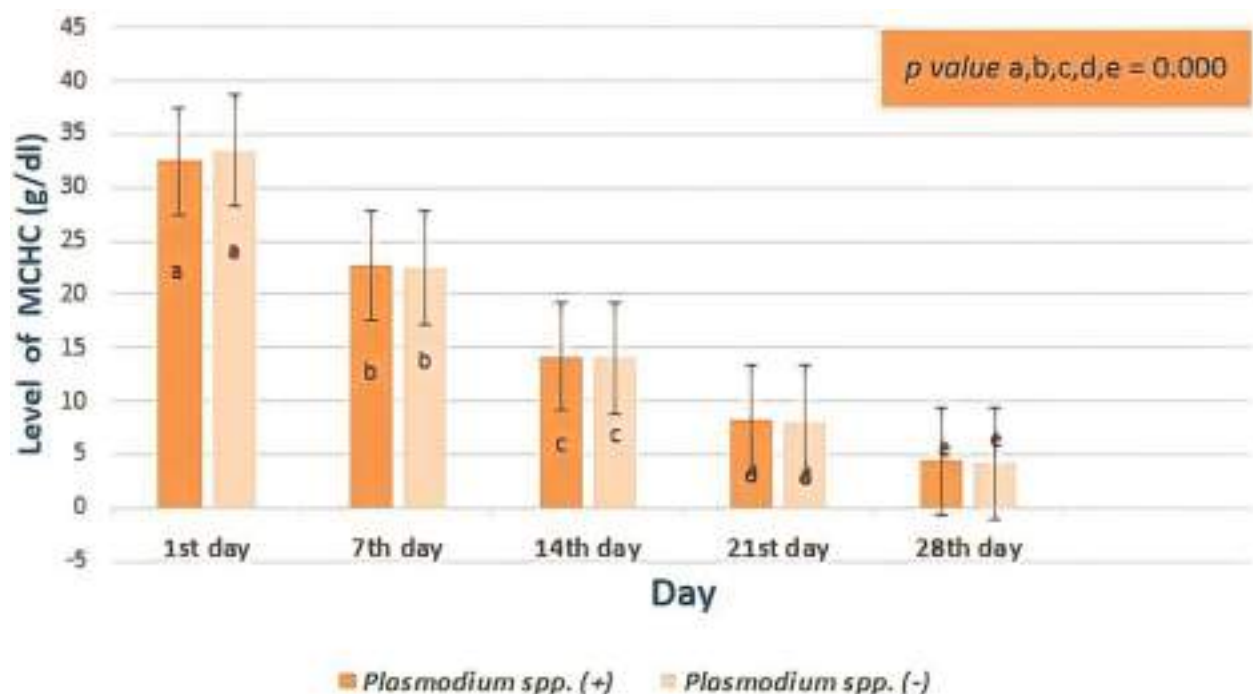
Figure 9 The levels of Mean Corpuscular Hemoglobin Concentration (MCHC) based on days of observation. There was no difference in the reduction of MCHC levels in donor blood containing *Plasmodium* spp. and those in donor blood that did not contain *Plasmodium*spp. However, levels of MCHC based on storage time showed significant difference (all p value < 0.05, Repeated ANOVA test and post-hoc Wilcoxon test). Different notations mean significant difference (p<0.05).

arrested for replication in terms of cold storage but after that, it was very likely it will die and disappear because of the microenvironment that did not support it. The survival mechanism of *Plasmodium* in infected donor blood during 14 days storage might be closely related to the regulation of the redox system by the *Plasmodium* through performing a new permeation pathway to obtain nutrient, antioxidant, and glucose from outside. However, it might be possible that *Plasmodium* is trying to maintain its viability in RBCs, a condition that later is suspected of potentially transmitting malaria.[10]

The profile of erythrocytes and the presence of parasites change starting at the first day of storage and decreased based on observation on the following day. Various factors cause a decrease in the level of erythrocyte profile parameters of the blood donor during storage times. According to Gregory et al, 2018, in addition to the decreased RBCs count, the leukocytes, the neutrophils, and lymphocytes count also decreased significantly (p<0.05). During storage, there will be biochemical changes, biomechanical damage and immunological reactions occur and cause hemolysis.[11,12]

Cooling stimulates the formation of sodium and the release of potassium so that there is a buildup of sodium and a decrease of potassium in RBCs. The mechanism of hemolysis begins with the metabolism of glucose into lactate with minimal energy sources, causing a decrease in the pH. The RBCs surface becomes fragile and partially destroyed (lysis). The concentration of hemoglobin in the plasma increases while the Adenosine Tri Phosphate (ATP) concentration and 2,3 Diphosphoglycerides (2,3 DPG) levels decrease progressively. Erythrocytes (RBCs) lose their ability to synthesize amino acids and red blood cell fatty acids,[13] hemoglobin (Hb) is released directly into the liquid, causing the loss of lipid-containing micro-vesicles into the plasma,[14] and making hemoglobin levels low.[15] In fact, hemoglobin serves as the main source of nutrients in the form of amino acids for *Plasmodium* spp. in maintaining its survival, especially in hypotonic solutions.[16]

Transfused blood storage is usually done for up to 35 days, depending on the anticoagulant used.[4] According to observation in this study, we recommend donor blood is not feasible to be given to the recipient after the 21st day of storage. However, a number of factors influence this event, including the storage temperature, the length of transportation (ie, when moving places from blood banks to laboratories), and the kind of anticoagulant.

During storage, ionized calcium levels increase causes blood to clot quickly. Anticoagulants in blood transfusions will slow the occurrence of storage lesions. The anticoagulant that is often used in transfusion is Citrate Phosphate Dextrose Adenine 1 (CPDA 1). The components in CPDA 1 (citrate, phosphate, dextrose, and adenine) are formulated to maintain the viability of blood cells and to minimize storage lesions that can happen because the mechanism of hemopoiesis does not occur outside the body.[17,18]

Citrate will work by inhibiting the activation of coagulation by binding to the calcium in the transfused blood, preventing cascade coagulation activation by inducing local hypocalcaemia.[19,20] The calcium content of the donor most probably gives a part of the explanation for this interesting observation. It is thought that citrate functioned as an antioxidant, but in research by Erman et.al, (2016), citrate does not have a direct effect on oxidative stress.[21] Citrate promotes and exacerbates the formation of ROS caused by the formation of hydrogen peroxide in cells.[22]

Similarly, organic phosphate works by maintaining levels of 2,3 DPG so that the production of ATP as an energy source continues and blood cell viability is maintained, resulting in blood cell damage go slow.[23] Dextrose is useful as an additional source of energy for blood cells during storage, which allows RBCs to be able to carry out glycolysis so that ATP is produced. Glycolysis can be slowed down by storing transfused blood at temperatures of 2–4°C, while adenine will save the use of other substrates needed during ATP synthesis.[19] The buffer function in anticoagulants can overcome the decrease in hydrogen levels due to temperatures that are too cold so that the pH is maintained.[24,25]

In blood donors, whole blood and plasma, when stored at −4°C, *Plasmodium* spp. was found to survive up to 18 days, and detectable parasites can present even up to 28 days when frozen.[16] This study has shown the RBCs count from donor blood both containing or did not contain *Plasmodium* spp. decreased on the first day until the 21st day of examination and was not a significant difference between them in all day observation. This shows that there is damage to RBCs that immediately occur when outside the blood vessels. These changes result in hemolysis resulting in nitric oxide [NO] reduced bioactivity due to scavenging, morphological changes, accumulation of lactic acid and potassium/calcium, a decrease in 2,3-DPG

and ATP, decrease in pH and glycolysis rate, and an accumulation of shed bioactive proteins, lipids, and RBC-derived micro-particles or micro-vesicles.[26] Anticoagulants and storing in hypothermic conditions are expected to slow down cell metabolism, so biochemical reactions and accumulation of the remaining metabolism do not cause blood cell death. It is suspected that *Plasmodium* spp. maintains its host cell (RBCs) so that it does not quickly damage (lysis)[27] possibly by regulating its redox system.

Unlike the case with RBCs that are not infected with *Plasmodium* spp. oxidative stress in infected RBCs increases when outside the lumen of the blood vessels resulting in their eventual damage by hemolysis.[28] However, the infected RBCs increase more in their permeability to obtain substantial low-molecular-weight solutes (glucose, anti-oxidant compared to the uninfected erythrocyte.[29,30] The forming of new permeation pathways (NPP), an increase in permeability can be attributed to a single type of permeation pathway with characteristics quite distinct from those of the host.[31,32] A channel on the parasitophorous vacuole membrane (PVM) has been described[33,34] and projections from the PVM, called the tubulo-vesicular membrane network (TVM), have been implicated in the acquisition of nutrients.[34,35] These mechanisms proposed a direct connection of the *Plasmodium* spp. to the plasma during prolonged storage.

Erythrocyte (RBCs) contains a conjugate protein derived from hemoglobin. The main function of hemoglobin is to bind and release oxygen and carbon dioxide. *Plasmodium* spp. in the intraerythrocytic stage will digest the host cell cytosol which has a large amount of hemoglobin. This hemoglobin is the main generator of ROS.[25] The functional role of red blood cells (RBCs) is the transport of oxygen from the lungs to the tissues providing the oxygen required by all cells and tissues. Hemoglobin (Hb) accounts for 95–97% of the cytosolic proteins inside the RBC, and bind to oxygen reversibly. Extracellular hemoglobin can be a major source of oxidative stress. Under normal conditions, this potential source of oxidative stress is minimized by haptoglobin and hemopexin, which bind Hb and free heme, respectively. They inhibit the oxidative reactions of Hb and heme and facilitate their removal from circulation. Elevated levels of free extracellular Hb and heme are caused by the failure of neutralized reaction by hap- to globin and hemopexin.[36]

Hemoglobin is a source of energy for *Plasmodium* spp., whereas as long as blood transfusions are stored,

there is no formation of hemoglobin even though anticoagulants have been given. Hemolysis that occurs during storage causes the release of hemoglobin directly into the liquid through lipid-containing micro-vesicles, resulting in lost hemoglobin from intact RBCs entering the plasma supernatant.[26] In this study, hemoglobin levels from transfused blood containing *Plasmodium* spp. decreased from the first day, although the results of data analysis did not show differences between hemoglobin levels from transfused blood containing *Plasmodium* spp. and those from transfused blood that did not contain *Plasmodium* spp.

Hematocrit is the ratio of the number of RBCs to blood volume in percent units that shows the density of the blood with the amount of oxygen it carries. A decrease in hematocrit levels indicates that the invasion and rupture of red blood cells are more influential than the hemoglobin value as indicated by an increase in MCHC levels.[37] The results showed that there was a significant difference between the storage time and blood temperature of the donors on the hematocrit levels, although there was no significant difference between the hematocrit levels in the blood of infected and non-infected donors.

From the data of Figures 7-9, it could be assumed that there was a change of osmotic fragility of RBCs from transfused blood containing *Plasmodium* spp. and that which did not contain *Plasmodium* spp. Osmotic fragility begins with changes in the size of RBCs which can be seen from the erythrocyte index, containing MCV levels, MCH levels, and MCHC levels.[38–42] This shows that RBCs damage starts from the ability of the RBCs membrane and its ligands. Blood cells that come out of the vascular system and undergo the storage process release phospholipids from the hematocrit RBCs, and cause osmotic damage to form schistocytes and speroecinocytes. Finally, most RBCs are increased osmotic fragility.[38–40]

This study requires further exploration to see the effect of *Plasmodium* invasion on the viability of red blood cells or vice versa. From this study, it was suspected that there was a *Plasmodium* defense mechanism to keep the survival of erythrocyte through redox balance. A research using *Plasmodium* subculture method and analyzing of redox status in infected erythrocytes would answer the question about both the mechanism of erythrocytes and parasite viability during transfusion storage.

Conclusions

From this study, there is no significant difference in the profiles of erythrocytes between donor blood that is infected

by *Plasmodium* spp. and that which is not infected. This observational study showed that there were trends in significantly reducing all parameters of erythrocyte profile in the bags of donor blood relevant to the duration of storage, as well as the presence of malaria parasites in the blood. The transfusion transmission of the malaria parasite will still potentially occur as long as the erythrocytes are still viable.

Abbreviations

2,3 DPG, 2,3 Diphosphoglycerides; AIDS, Acquired Immune Deficiency Syndrome; ATP, Adenosine Tri Phosphate; CPDA 1, Citrate Phosphate Dextrose Adenine 1; Hb, Hemoglobin; HIV, Human Immunodeficiency Virus; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; NO, nitric oxide; PVM, parasitophorous vacuole membrane; RDT, Rapid Diagnostic Test; PCR, Polymerase Chain Reaction; ROS, Reactive oxidant species; RBCs, Red Blood Cells; TTM, Transfusion Transmitted Malaria; TVM, tubulo-vesicular membrane network; VDRL, Venereal Disease Research Laboratory; WHO, World Health Organization.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors declare that they have no conflict of interests for this work.

References

1. Getaneh A, Mohammedaman M. Asymptomatic malaria infection and associated factors among blood donors attending Arba Minch blood bank, Southwest Ethiopia. *Ethiop J Health Sci*. 2017;28 (3):315–335. doi:10.4314/ejhs.v28i3.9
2. O'Brien SF, Delage G, Seed CR, et al. The epidemiology of imported malaria and transfusion policy in 5 non-endemic countries. *Transfus Med Rev*. 2015;29(3):162–171. doi:10.1016/j.tmrv.2015.03.004
3. Indonesian Government Regulation No.7 of 2011 about Blood Services
4. Queen E, Ifeanyi OE, Chinedum OK. The effect of storage on full blood count in different anticoagulants. *IOSR J Dental Med Sci*. 2014;13(9):128–131.
5. Antonelou MH, Kriebardis AG, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol. *Transfusion*. 2010;50(2):376–389. doi:10.1111/j.1537-2995.2009.02449
6. Paul AS, Egan ES, Duraisingh MT. Host-parasite interactions that guide red blood cell invasion by malaria parasites. *Curr Opin Hematol*. 2015;22:220–226.
7. Nubila T, Ukaejiofo EO, Nubila NI, et al. Hematological profile of apparently healthy blood donors at a tertiary hospital in Enugu, southeast Nigeria: a pilot study. *Niger J Exp Clin Biosci*. 2014;2:33–36.
8. Kementerian Kesehatan Republik Indonesia. *Pertemuan Monitoring Dan Evaluasi Eliminasi*. Subdit Malaria – Ditjen: P2P; 2017.
9. Sons JW. Bio-preservation of RBCs for in vitro *Plasmodium falciparum* culture. *Br J Haematol*. 2016;175(4):733–747. doi:10.1111/bjh.13882
10. Chattopadhyay R, Majam VF, Kumar S. Survival of *Plasmodium falciparum* in human blood during refrigeration. *Transfusion*. 2011;51:630–635.
11. Gregory B, Dan A, Leonid L, Alexander G. Is it possible to reverse the storage-induced lesion of red blood cells? *Front Physiol*. 2018;9:914. doi:10.3389/fphys.2018.00914
12. Remy KE, Sun J, Wang D, et al. Transfusion of recently donated (fresh) red blood cells (RBCs) does not improve survival in comparison with current practice, while safety of the oldest stored units is yet to be established: a meta-analysis. *Vox Sang*. 2016;111(1):43–54. doi:10.1002/14651858.CD009330.pub2
13. Pawan KM, Prabhanshu K, Pranjal C. Biomarkers of oxidative stress in erythrocytes as a function of human age. *World J Methodol*. 2015;5 (4):216–222. doi:10.5662/wjm.v5.i4.216
14. Francesca R. The potential adverse effects of hemolysis. *Blood Transfus*. 2017;15(3):218–221.
15. Marion K, Jake B. The mechanics of malaria parasite invasion of the human erythrocyte – towards a reassessment of the host cell contribution. *Cell Microbiol*. 2016;18(3):319–329.
16. Antwi-Baffour S, Adjei JK, Agyemang-Yeboah F, et al. Proteomic analysis of micro-particles isolated from malaria positive blood samples. *Proteome Sci*. 2016;15:5.
17. Cora MC, King D, Betz LJ, Wilson R, Travios GS. Art factual changes in Sprague-Dawley Rat hematologic parameters after storage of samples at 3°C and 21°C. *Am Assoc Lab Anim Sci*. 2012;51 (5):616–621.
18. Lindstrom NM, Moore DM, Zimmerman K, Smith SA. Hematologic assessment in pet rats, mice, hamsters, and gerbils: blood sample collection and blood cell identification. *Vet Clin North Am Exot Anim Pract*. 2015;18(1):21–32.
19. Kissling S, Legallais C, Pruijm M, et al. A new prescription model for regional citrate anticoagulation in therapeutic plasma exchanges. *BMJ Nephrol*. 2017;18(1):81. doi:10.1186/s12882-017-0494-9
20. Verma M, Dahiya K, Malik M, Sehgal PK, Devi R. Soni A and Ghalaut VG. Effect of blood storage on complete biochemistry. *J Blood Disord Transfus*. 2015;6:6. doi:10.4172/2155-9864.1000359

21. Erman H, Aksu U, Belce A, et al. Aydın S and Çakatay U. Gender and chronological age affect erythrocyte membrane oxidative indices in citrate phosphate dextrose adenine-formula 1 (CPDA-1) blood bank storage condition. *Gen Physiol Biophys*. 2016;35:343–351. doi:10.4149/gpb_2016001

22. Rahbari M, Diederich K, Becker K, Krauth-Siegel RL, Jortzik E. Detection of thiol-based redox switch processes in parasites—Facts and future. *Biol Chem*. 2015;396(5):445–463. doi:10.1515/hsz-2014-0279

23. Amna MA, Sven CV, Afnan SA, et al. Whole blood storage in CPDA1 blood bag alters erythrocyte membrane proteome. *Oxid Med Cell Longev*. 2018;1–12. doi:10.1155/2018/6375379

24. Tatsuro Y, Michel P, Angelo D. Red blood cell storage lesion: causes and potential clinical consequences. *Blood Transfus*. 2019;17 (1):26–52. doi:10.2450/2019.0217-18

25. Flatt JV, Bawazir WM, Bruce LJ. The involvement of cation leaks in the storage lesion of red blood cell. *Front Physiol*. 2014;214(5):1–10. doi:10.3389/fphys.2014.00214

26. Joseph MR, Joy GM, Enika N. The pathophysiology of extracellular hemoglobin associated with enhanced oxidative reactions. *Front Physiol*. 2015;5:500. doi:10.3389/fphys.2014.00500

27. Birgit V, Thibault G, Don AL, et al. In silico mining for antimalarial structure-activity knowledge and discovery of novel antimalarial curcuminoids. *Molecules*. 2016;21(7):853. doi:10.3390/molecules 21070853

28. Sparrow RL. Time to revisit red blood cell additive solutions and storage conditions a role for "omics" analyses. *Blood Transfus*. 2012;10(Suppl2):s7–s11. doi:10.2450/2012.003S

29. Ginsburg H, Atamna H. The redox status of malaria-infected erythrocytes: an overview with an emphasis on unresolved Problems. 1997;1 (1):5–13.

30. Egée S, Bouyer G, Thomas SLY. Permeabilization of host cell membrane. *Encyclopedia Malaria*. 2015;1–14. doi:10.1007/978-1-4614-8757-9_38-1

31. Kirk K. Channels and transporters as drug targets in the *Plasmodium*-infected erythrocyte. *Acta Trop*. 2004;89:285–298.

32. Dickerman BK, Elsworth B, Cobbold SA, et al. Identification of inhibitors that dually target the new permeability pathway and dihydroorotate dehydrogenase in the blood stage of *Plasmodium falciparum*. *Sci Rep*. 2016;6:37502. doi:10.1038/srep37502

33. Adamu J, Jigam AA. Effects of malaria infection on some haematological and biochemical parameters in the general population and pregnant malaria patients attending two district hospitals in Niger State, Nigeria. *Glob J Infect Dis Clin Res*. 2019.

34. Desai SA, Krogstad DJ, McCleskey EW. A nutrient-permeable channel on the intraerythrocytic malaria parasite. *Nature*. 1993;362: 643–645.

35. Goldberg DE, Zimmerberg J. Hardly Vacuous: the Parasitophorous Vacuolar Membrane of Malaria Parasites. *Trends Parasitol*. 2020;36.2:138–146. doi:10.1016/j.pt.2019.11.006

36. Kanti BP, Syed IR. Biomarkers of oxidative stress in red blood cells. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2011;155(2):131–136.

37. Lauer SA, Rathod PK, Ghori N, Haidar K. A membrane network for nutrient import in red cells infected with the malaria parasite. *Science*. 1997;1122-1125.

38. María G, María C, Vicente-Ayuso AMB, et al. Red blood cell storage time and transfusion: current practice, concerns and future perspectives. *Blood Transfus*. 2017;15(3):222–231. doi:10.2450/ 2017.0345-16

39. Canavese M, Spaccapelo R. Protective or pathogenic effect of vascular endothelial growth factor (VEGF) as potential biomarker in cerebral malaria. *Pathog Glob Health*. 2014;108(2):67–75. doi:10.1179/2047773214Y.0000000130

40. Dunst J, Kamena F, Matuscewski K. Cytokine and chemokine in cerebral malaria pathogenesis. *Front Cell Infect Microbiol*. 2017;7:324.

41. Chaudhary R, Katharia R. Oxidative injury as contributory factor for red cells storage lesion during twenty eight days os storage. *Blood Transfus*. 2012;10(1):59–62. doi:10.2450/2011.0107-10

42. Keohane EM. Intrinsic Defects Leading to Increased Erythrocyte Destruction. In: Keohane EM, Smith LJ, Walenga JM, editors. *Rodak's Hematology: Clinical Principles and Applications*. 5th ed. Missouri: Elsevier Saunders; 2016:367–389.

Management of Sickle Cell Disease Complications Beyond Acute Chest Syndrome

Ugochi O Ogu, Nnenna U Badamosi, Pamela E Camacho, Amado X Freire & Patricia Adams-Graves

REVIEW

Management of Sickle Cell Disease Complications Beyond Acute Chest Syndrome

Ugochi O Ogu [iD][1]
Nnenna U Badamosi[2]
Pamela E Camacho[3]
Amado X Freire[4]
Patricia Adams-Graves[1]

[1]Center for Sickle Cell Disease, University of Tennessee Health Science Center, Memphis, TN, USA; [2]Division of Pediatric Hematology and Oncology, Medical College of Georgia, Augusta, GA, USA; [3]Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA; [4]Division of Pulmonary, Critical Care, and Sleep Medicine, University of Tennessee Health Science Center, Memphis, TN, USA

Abstract: Sickle cell disease results in numerous complications that can lead to significant morbidity and mortality. Amongst them, acute chest syndrome is the leading cause of mortality. As a result, most providers are in tune with this complication and well versed with management. As sickle cell patients now live longer, they face a multitude of other complications that if left unattended, can lead to significant morbidity and mortality as well. It is critical to look beyond acute chest syndrome and adopt a more comprehensive approach to the management of the sickle cell patient.
Keywords: sickle cell disease, acute chest syndrome, complications

Introduction

Sickle cell disease (SCD), an inheritable blood disorder due to a point mutation in the beta-globin gene resulting in the substitution of valine for glutamic acid at the 6th amino acid, was first described over 100 years ago.[1–3] Since then, the complex pathophysiology has been elucidated from simply the red blood cell to a multicellular event to include the blood vessel itself.[3–5] This hemoglobin (Hb) gene defect is responsible for serious and life-threatening complications of hemolytic anemia, inflammation, an impaired immunity to encapsulated organisms and vascular occlusion (Figure 1).[6] Secondary complications to these include stroke, skin ulceration, priapism, acute and chronic organ damage, and a shortened lifespan.[7] SCD complications are elusive due to the underestimation of the impact of social barriers, the inability to measure pain, and the failure to look beyond the underlying pathology during painful events. In fact, 22% of deaths associated with SCD complications are preceded by a painful crisis.[8] More specifically, acute chest syndrome (ACS) has been one of the most devastating SCD complications with a high mortality rate if there is a delay in diagnosis or mismanagement across all age groups. As such, there is a heightened awareness of ACS among healthcare providers, specifically those in the acute care/emergency setting. We want to help providers shift beyond ACS to a more comprehensive management approach by highlighting the social barriers to quality of care and the other complex, yet elusive, severe and potentially life-threatening complications of SCD.

The management of SCD is finally transforming into a more comprehensive and diverse approach. Blood therapy and pain treatment were the mainstay of support therapy until 1998 when Hydroxyurea (HU) became the only Food and Drug Administration (FDA) approved drug that targeted the molecular pathogenesis.

Correspondence: Patricia Adams-Graves
Tel +1 901-545-8538
Fax +1 901-545-6454
Email padamsgraves@uthsc.edu

101
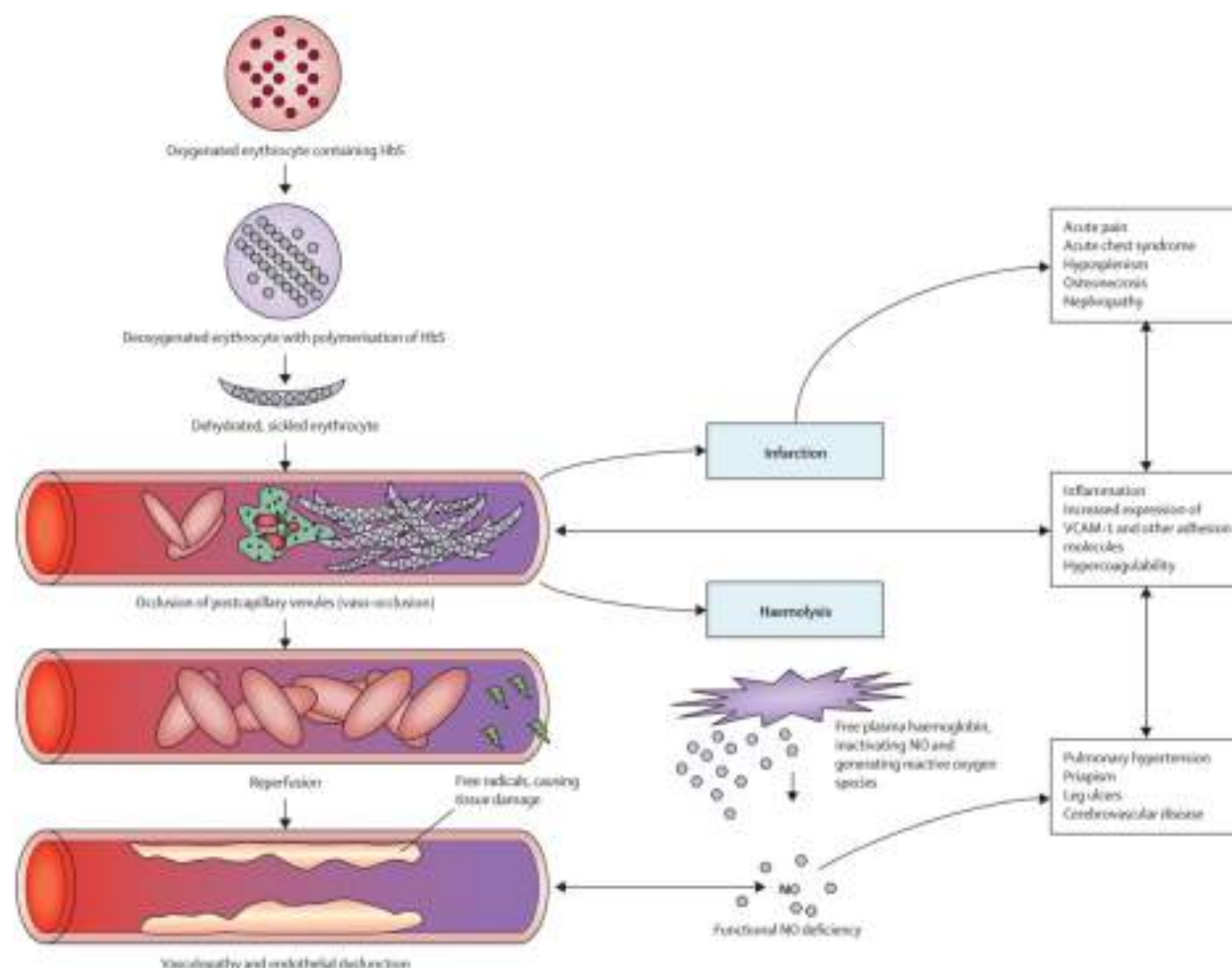
Figure 1 Pathophysiology of sickle cell disease.
Notes: Reprinted from Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet. 2010;376(9757):2018–2031, Copyright © 2010 Elsevier Ltd. Licensed under CC BY-NC-ND.[6]
Abbreviations: HbS, sickle hemoglobin; NO, nitric oxide; VCAM, vascular cell-adhesion molecule.

Elucidation of new pathophysiology has resulted in the evolution of new targeted drugs for SCD management.[1,2] There is now a transition from having one FDA approved drug over the past 110 years to a robust new arsenal of targeted drug development. Currently, there are over 30 novel pharmaceutical agents being studied and 3 have recently been FDA approved.[1,3,9–11] New targets for drug development in SCD include adhesion, antioxidation, inflammation, fetal Hb induction beyond HU, anti-sickling, anticoagulation, and opiate sparing drugs. In addition, we have expanded our approach from supportive care to curative procedures such as bone marrow transplant, gene therapy, and gene editing.

Until recently, the management of SCD has focused on the pathophysiology of the disease with less emphasis on addressing the complex social hardships which ultimately exists as barriers to quality care and management. Individuals with sickle cell disease navigate their lives through the hardships of associated complications such as acute chest syndrome and painful vaso-occlusive events while also facing a barrage of social barriers to quality care such as the transition from pediatric to adult care and health disparities. Now is the time to recognize that there is a need for a national movement to eliminate these barriers in order to truly ensure that the unmet social and medical needs are being addressed in the sickle cell population. Our goal is to review the management of the systemic complications of SCD beyond acute chest syndrome, pipeline novel agents, and health disparity gaps in order to help the health care system have a more heightened awareness of the underlying and elusive complications that if remain overlooked in the shadows of ACS may cause harm to individuals living with sickle cell disease.

Health Disparities

This is a time in which America is becoming more socially and consciously aware of the apparent unresolved consequences of systemic racism. A recent article in the New England Journal of Medicine (NEJM) highlighted racism and solutions in the US and its effect on SCD.[12] In the United States (US), the majority of people with sickle cell disease have a 30-year gap in life expectancy compared to the general population and are of African heritage, while on the other hand, there is a lower incidence in people of Hispanic, South Asian, South European, and Middle Eastern descent who are also affected.[7] These statistics play an important factor in the way those living with SCD experience real-life consequences of the health disparities that exist in our healthcare system.

The lack of national awareness, funding for research, therapeutic development, and social services for SCD in comparison to other conditions that are prevalent to a more universal racial demographic such as asthma, heart disease, breast cancer, colon cancer, and diabetes supports systemic racism as an underlying issue.[13] In fact, even among rare diseases this is true. The genetically acquired, life-threatening impact that SCD has on African Americans is similar to the impact that Cystic Fibrosis (CF) has on Caucasian Americans. Although SCD is more common than CF as it affects three times more Americans, it has been in its shadows with approximately ten times less funding, and has only one quarter of the number of FDA approved therapeutic agents.[14–16] Those living with SCD suffer barriers to quality care as there are profound healthcare gaps of socioeconomic status, racial discrimination, lack of sufficient federal funding, lack of consistent disease-specific access to quality care, and provider discord especially for pain management.[17–19] The long-standing history of stereotyping as opioid drug seeking fakers of true pain, mistreatment, neglect, and lack of quality care of individuals living with SCD, without a doubt illuminates how racial biases fundamentally obstruct justice in the form of human rights, the rights to proper healthcare, and quality of life.[12]

We must work together to put in place realistic policies and training to ensure that racial discrimination within the healthcare system, especially as it relates to the barriers to adequate healthcare for those living with SCD, is considered a reportable adverse event and given as much priority to prevent, as medication or surgical errors are given.[20–22] Our hope is to see all healthcare settings transform from a place of injustice, fear, and suffering into a place of holistic disease-specific, patient-centered care that provides safety, hope, and healing for those living with SCD.

Approach to Children and Transition to Adult Care
Central Nervous System (CNS) Complications

In children, cerebral vasculopathy is the most common CNS complication resulting from progressive inflammation and oxidative endothelial damage within intracranial vessels, leading to increased risk of transient ischemic events and infarctive strokes.[23,24] Long-term sequelae can range from mild cognitive or behavioral impairment to devastating neurologic effects or death. Screening for vasculopathy beginning at age two is recommended,[25] as well as early initiation of disease modifying therapy to mitigate anemia and endothelial damage.

Another less-common complication that can present with acute neurologic symptoms is acute cranial bony infarction associated with intracranial hemorrhage and/or thrombosis. Cranial bony infarcts are believed to result from diploic vascular disruption due to extramedullary hemopoiesis.[26,27] In addition, periosteal elevation following an infarctive episode may result in bleeding and hematoma formation. This constellation of complications is uncommon but should remain on the differential even when an acute stroke is suspected, as acute neurosurgical intervention may be required to prevent significant morbidity or mortality.

Cardiovascular Complications

Many children may present with clinical murmurs due to hyperdynamic blood flow and mild to moderate left ventricular hypertrophy. Pulmonary hypertension and cardiomyopathy due to iron overload may not be clinically apparent until adulthood. Screening echocardiograms are not routinely recommended in children unless new symptoms or new clinical findings are noted on exam.[28]

Aplastic Crisis

Parvovirus –B19 is an erythrotropic virus that selectively targets human red blood cells and their precursors.[29] In patients with SCD, this can lead to life-threatening anemia due to abrupt cessation of erythropoiesis. Features are new or worsening signs of anemia, with an acute drop in hemoglobin and reticulocytopenia in the absence of

blood loss or sequestration. Urgent recognition and transfusion are necessary to prevent circulatory failure and death.

Splenic Complications

Splenic complications in children can include impaired immunity to encapsulated organisms such as *Streptococcus pneumoniae, Neisseria meningitides* and *Salmonella* species, leaving patients prone to life-threatening infections such as pneumonia, meningitis, osteomyelitis and sepsis. As a result, all febrile illnesses are considered medical emergencies until proven otherwise in infants and under-immunized children. Routine childhood vaccines are monitored and usually administered through primary care providers, with the addition of extended pneumococcal and meningococcal vaccines as standard of care. The initiation of prophylactic antibiotics in early infancy has significantly decreased the morbidity and mortality historically associated with these infections.

Acute splenic sequestration of red cells and/or platelets is another potentially fatal complication in children. Autosplenectomy is less common in children who are initiated on disease-modifying therapy early in life, and therefore this complication may be seen in older children and teenagers. Reviewing splenic palpation and signs of anemia are an important component of anticipatory guidance for parents, with the need for urgent red cell transfusion if signs of circulatory failure.

Gastrointestinal Complications

Vaso-occlusion can occur in intra-abdominal vessels leading to acute abdominal pain as a presenting sign. A thorough evaluation for other causes of acute abdomen may be unrevealing in these patients. These vaso-occlusive episodes are managed appropriately with pain control and hydration.

Sickle hepatopathy is a broad term for a range of hepatic complications from mild liver dysfunction to chronic liver failure and cirrhosis. Acute vaso-occlusive events within hepatic parenchyma may present with fever, jaundice, transaminitis and intense abdominal pain similar to the acute abdominal presentation above.[30] Management is supportive with hydration and pain control. Intrahepatic sequestration, or trapping of red cells within the liver, can also lead to intense pain and transaminitis. A large, tender liver may be felt on examination or noted on ultrasound, in addition to an acute drop in hemoglobin. Management is also supportive with transfusion as needed for symptomatic anemia.

Secondary hemochromatosis may be seen in transfused patients with poorly managed transfusional iron overload with occasional transaminitis noted. However, chronic hepatic or cirrhotic changes are an uncommon presentation in childhood.

Other differentials for acute abdominal pain and jaundice with or without fever in SCD are cholelithiasis and/or cholecystitis resulting from pigmented gall stones due to chronic hemolysis leading to obstruction of hepatobiliary bile flow.[30] Obstructive gall stones may also lead to intrahepatic cholestasis or extra-hepatic obstruction and acute pancreatitis. Elevated inflammatory markers, transaminases and bilirubin beyond baseline are suggestive of this diagnosis. While a focused abdominal ultrasound may identify inflammation and/or stones, magnetic resonance or endoscopic cholangiopancreatography are more sensitive in making a diagnosis. Urgent evaluations by gastrointestinal and surgical teams will help determine the need for urgent cholecystectomy vs antibiotics and conservative management with endoscopic stone removal.

Peptic ulcers are not uncommon in children with SCD, especially given frequency of non-steroidal anti-inflammatory drug use. Chronic central to upper abdominal pain that may be relieved by antacids or food, may require evaluation and treatment by a gastroenterologist.

Constipation is another frequent complaint in children, usually as a side effect of narcotic pain medication use. An age-appropriate bowel regimen is recommended for use at home and inpatient, although an aggressive bowel cleanout may occasionally be required.

Genitourinary Complications

Sickle nephropathy results from hypoxia and ischemia within renal medullary vasculature, resulting in micro-infarction and papillary necrosis. In addition, intravascular hemolysis produces free hemoglobin which can lead to oxidative damage in renal tubular cells. Nephropathy can vary in presentation from microalbuminuria and proteinuria to significant renal dysfunction and renal failure. Patients at higher risk are those with significant anemia and hemolysis. Annual screening starting at age 10 can identify microalbuminuria as an early sign of nephropathy. The use of angiotensin-converting enzyme (ACE) inhibitors, in addition to SCD-modifying therapy has been shown to minimize progression of nephropathy.[28]

In male patients, priapism represents an unwanted persistent painful erection that can present at any age. Conservative measures to redirect penile blood flow such as exercise, distraction, or warm baths are recommended as first line. Pseudoephedrine is an α-adrenergic agonist that may be used

as needed at home for intermittent episodes. Painful episodes lasting more than 4 hours are considered an urologic emergency.[31]

Musculoskeletal

Osteonecrosis can occur in anywhere including cranial bones as described above but are frequently found at the ends of long bones in patients with SCD. These may present as persistent localized pain beyond a typical vaso-occlusive episode with or without fever. Magnetic resonance imaging can aid in the diagnosis, although necrotic bone may be difficult to distinguish from osteomyelitis. Conversely, necrotic bone can serve as a nidus for bacterial overgrowth and osteomyelitis. Management is usually conservative with pain medications, unless associated with osteomyelitis or bone abscess requiring debridement.

Avascular necrosis (AVN) is most often located within the femoral head and can be a source of chronic debilitating pain in teenagers and young adults. Management is usually conservative in growing patients, although this complication continues to be a reason for frequent emergency room visits, impaired mobility and diminished quality of life. Long-term pain management is individualized, and often involves a combination of narcotics, non-steroidal anti-inflammatories and other adjunct medications. Unfortunately, optimal pain control may be difficult to achieve in some patients. Conservative surgical techniques such as core decompression and stem cell injection into the joints have had variable results, and many of these patients inevitably require hip-replacement surgery.[32]

Transition to Adult Care

There are many significant challenges that are unique to the teenage and young adult population as they transition from pediatric to adult care models. Transition in itself is a complication of SCD that requires specific attention and should not be overlooked. It represents yet another example of an important social component that is not only in the shadows of ACS but also exists as a barrier to quality care. Individuals living with SCD are vulnerable to the concept of "falling through the cracks" within the healthcare system, as they transition from pediatric to adult care. Young adults (aged 18–30 years) account for the highest healthcare utilization compared to other age groups (greater than twice the amount of emergency room visits per year, higher inpatient stays and highest frequency of acute care visits).[33]

Many recurrent childhood complications as described above will persist through the transition years. In addition, early presentations of significant organ damage may start to manifest such as cardiomegaly, pulmonary hypertension, sickle nephropathy and proteinuria, avascular necrosis and chronic pain syndromes. In addition, young adults with a history of strokes in childhood, may present with cognitive deficits that become more notable as the need for health independence and self-efficacy increase. Young patients on chronic transfusions may have difficulty with continuation of transfusion as they transition, if significant iron overload or red cell alloimmunization have developed. Other undesirable outcomes of an unsuccessful transition to adult care include loss of health insurance coverage, limited availability of adult hematologists experienced in SCD, and inability to attend appointments due to transportation issues. The management of SCD beyond ACS must include transition to adult care in order to achieve a truly comprehensive approach to caring for those living with SCD.

Approach to Adults

Sickle cell disease is no longer a disease of childhood, as patients now live well into adulthood. As they progress into adulthood, they are faced with more challenges – increasing rate of comorbidities in the setting of a paucity of skilled and willing adult providers to care for them.[34] Acute complications in adulthood include vaso-occlusive crisis (VOC), acute chest syndrome, acute splenic sequestration, aplastic crisis, acute osteomyelitis, cerebrovascular disorders, hepatobiliary issues, acute kidney injury (AKI), priapism, venous thromboembolism (VTE) and multi-system organ failure. Chronic complications include avascular necrosis (AVN), chronic pain, leg ulcers, ocular issues, renal complications and cardiac complications (pulmonary hypertension, diastolic dysfunction). It is important to note that the aging sickle cell population may also experience the typical complications of adulthood and aging (mental health issues, diabetes, gout, hypertension, degenerative joint disease, autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus). These are managed similar to the general population.

Acute chest syndrome is a life-threatening complication in patients suffering from SCD, and the leading cause of mortality. It is usually present at a higher incidence in patients with homozygous SCD, triggers hospital admissions and can have catastrophic consequences.[35] Defined by an expert panel as an acute illness characterized by fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on chest x-ray, it could also present with multiple infiltrates, hypoxemia

conducive to respiratory failure with or without pain syndrome and associated organ failures due to hyper hemolysis, sequestration and/or microvascular occlusions.[36] Pneumonia or systemic infections, fat embolism and pulmonary infarction represent the most common mechanisms, with atypical bacterial or viral infections accounting for most cases.[37]

The standard management for ACS is the use of broad-spectrum antibiotics as well as supportive care such as adequate oxygenation, incentive spirometry, pain control and reduction of abnormal hemoglobin concentration. Decreased concentration can be achieved with transfusions (simple) or with emergent red cell exchange for severe cases. Oxygenation should satisfy patient's needs and provide symptoms relief with adequate oxygen saturation. Progressive delivery should be started with nasal canula, venturi mask or High Flow Oxygen vs noninvasive ventilation systems. If these options do not achieve desirable effects medical care should proceed to mechanical ventilation with endotracheal tube intubation for support. Patients requiring mechanical ventilation have usually bilateral alveolar infiltrates consistent with pulmonary edema which can be cardiogenic; related to fluid overload consequence of the aggressive hydration to control hemoglobin S concentration; or non-cardiogenic with Acute Respiratory Distress Syndrome (ARDS) physiology secondary to sepsis or Transfusion Associated Lung Injury (TRALI).

Acute pain/VOC is the hallmark of SCD and the most common reason that the sickle cell patient seeks medical attention. Triggers include hypoxia, dehydration, extremes of temperature, acidosis and infection. It is largely managed with pain medications and supportive care.

Pain can also be chronic which is usually multifactorial, ranging from actual tissue/organ damage (due to AVN, leg ulcers) to neuropathic and idiopathic pain. Chronic pain is defined as ongoing pain that is present on most days for over 6 months.[38] Opioids are most commonly used. However, use of non-opioid and non-pharmacological modalities such as meditation, massage, acupuncture, etc are garnering more attention.[39,40]

VTE defined as a deep vein thrombosis or pulmonary embolism (PE), has a high incidence in SCD patients. Up to 12% of patients have a VTE by age 40 years.[41] Diagnosis and treatment are as in the general population.

Multisystem organ failure is an acute, fatal complication of SCD, defined as acute decompensation of 2 or more of the following organs: lungs, kidney, liver. It is usually associated with VOC, fever, and an acute drop in Hb and/or platelets. An urgent exchange transfusion is indicated, as well as supportive care such as supplemental oxygen/mechanical ventilation and renal replacement therapy if warranted.

Leg ulcers are an uncommon complication in children and rare under 10 years of age.[42,43] However, they pose a significant source of pain and distress in the adult with SCD. Risk factors include hemoglobin SS genotype, severe anemia and increased hemolysis. Leg ulcers usually present initially in the second decade of life and may be present for several years.[44] They may arise following a traumatic event to the skin, or spontaneously.[45] The most common site is around the medial and lateral malleoli of the ankle. Management is via a multidisciplinary approach which includes wound debridement and dressings, pain management and infection control.[44]

Ocular manifestations in SCD include proliferative and non-proliferative sickle retinopathy, hyphema, vitreous hemorrhage and central retinal artery occlusion (CRAO). Retinopathy screening is conducted by annual dilated eye exams from age 10 years. Management of proliferative retinopathy involves laser photocoagulation. CRAO is a medical emergency and requires an urgent exchange transfusion.

Splenic complications, aplastic crisis, acute osteomyelitis, acute cerebrovascular events, hepatobiliary issues, renal complications, priapism, AVN, and cardiac complications are covered in detail in a previous section.

Reproductive Health and Pregnancy in Sickle Cell Disease

In men with SCD, reproductive issues include sperm abnormalities, hypogonadism and erectile dysfunction (ED). Up to 91% of males with SCD experience sperm abnormalities manifesting as low sperm count and density, poor motility, and increased abnormal morphology.[46] Puberty in general is delayed in SCD. Hypogonadism is present in up to 24% of males with SCD and may manifest as poor testosterone production, infertility, ED and poor libido.[47] There remains a debate as to whether it is of primary or secondary etiology. Current therapies include testosterone injections and clomiphene.

ED may result from repeated episodes of priapism. One study demonstrated a 21.4% prevalence of priapism, and 22.2% prevalence of ED within that cohort, 92.3% of the cohort experiencing repeated episodes of priapism.[48] Management options include penile implants/prostheses.

In women with SCD, reproductive issues include delayed puberty, choice of contraception and pregnancy-related complications. Puberty in SCD females is delayed by up to 2.4

years compared to the normal population.[49] The menstrual cycle is also associated with increase in pain. Choice of contraception remains a source of debate. Due to the theoretical risk of clot predisposition with the estrogen-containing products, providers usually opt for progestin only pills/injectable and IUD. Depo-Provera which also decreases the number of cycles has been helpful for those patients who experience a crisis with their cycles.

Pregnancy in SCD is associated with increased risk of VTE, pre-eclampsia/eclampsia, increased pain crisis, preterm labor, prematurity, intra-uterine growth retardation (IUGR), small for gestational age (SGA) babies and fetal demise.[50] Pregnancy in SCD is considered high risk and a multidisciplinary approach inclusive of a sickle cell expert and a knowledgeable maternal fetal medicine provider is recommended for close monitoring during this delicate period. The decision to prophylactically transfuse or not is usually provider dependent. Management of acute and chronic pain also poses a challenge with pregnancy in SCD. A retrospective study demonstrated that compared to non-SCD pregnant mothers who are on methadone for opioid dependence, neonatal abstinence syndrome (NAS) occurs at a similar rate in SCD pregnant mothers on daily opioids, and at a significantly lower rate in those treated episodically with opioids.[51] However, opioids must be used judiciously in pregnancy to mitigate these adverse effects on the fetus/newborn.

Sickle Cell Disease in the Age of COVID-19

Severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), also known as COVID-19 has now affected over 38 million patients worldwide.[52] Our knowledge base is rapidly evolving, but this virus poses a significant concern in our patient population. Sickle cell disease is an immunocompromised condition which puts patients at risk of complications from respiratory infections. Our experience of this particular complication is currently limited and a standard of care has not been established; however, there have been several collaborative groups that have aimed to identify patterns and suggest management options for our patients.[53,54]

Early publications have suggested increased morbidity in SCD patients. A recent French case series described outcomes of eighty-three inpatient individuals.[55] The experience reported an ICU admission rate of 20%, fifty-three percent of which required mechanical ventilation, including two patients which required extracorporeal membrane oxygenation, and two patients who died in the ICU with COVID-19

pneumopathy. This underscores the importance of early identification and intervention in our patients. The challenge is that there is significant overlap in presenting signs and symptoms of patients with ACS and COVID-19 infection. Fever, shortness of breath, cough, and myalgias are all overlapping symptoms of ACS, pulmonary embolus, vaso-occlusive crisis, and SARS-CoV-2.

The Medical College of Wisconsin has developed a voluntary international registry of patients with SCD and COVID-19 infection in the hopes to better understand its pathophysiology.[54] Interestingly, the most common presenting sign, in the approximately 350 patients registered, is pain, with less than 30% of patients presenting with pneumonia. This highlights the importance of looking beyond ACS and becoming hypervigilant of possible COVID-19 infection in patients without respiratory complaints. Recent case reports also support VOC and fever as the most common reported symptoms in patients with SCD and COVID-19 infection.[56] Other respiratory viral infections often trigger "sickle cell crisis", and COVID-19 appears to have a similar effect. It is imperative that we obtain SARS-CoV-2 PCR testing in any patient with SCD presenting with ACS and/or VOC symptoms.

The lack of large published studies investigating this disease also poses a challenge in the management of known COVID-19 infected patients. A recent review of the literature of nineteen SCD patients with COVID-19 reported from December 2019 to May 2020 described a varied combination of management all similar to those used in the treatment of ACS.[57] Their approach included supportive care with hydration, analgesics, empirical broad-spectrum antibiotics, red blood cell exchange, and simple blood transfusions. Oxygen-support ranged from low flow (2 L/min) to high flow, non-invasive and mechanical ventilation in critically ill patients. A single dose of tocilizumab (8 mg/kg) was reported in 2 cases (adult and pediatric) with success.

The American Society of Hematology has provided community resources as guidance for the management of suspected COVID-19 in patients with SCD.[58] A useful checklist has been developed in collaboration with ED physicians. Suggested interventions include supplemental oxygen to raise pO2 >94%, judicious fluid replacement and avoidance of fluid bolus as this may exacerbate pulmonary edema, broad-spectrum antibiotics after blood cultures if febrile, macrolide in addition to a third-generation cephalosporin in the event of pneumonia, vancomycin if there is concern for line or skin infection. Any patient with

COVID-19 who develops respiratory symptoms should have a type and cross available. Transfusion recommendations include simple transfusions if hemoglobin drops by more than 2 g/dL from baseline, or for patients with respiratory compromise with a goal hemoglobin of approximately 10 g/dL. If the hemoglobin is greater than 10 g/dl admissions should be transferred to an institution where exchange transfusion is available due to the risk of hyperviscosity with further simple transfusions. If patients are discharged home, close follow up is needed with telemedicine or in person visits within 24 hours. Patients should have a low threshold to return to the ED, especially if dyspnea worsens and should be provided with a pulse oximeter if possible.

Further management to consider during the age of COVID-19 includes the use of bronchodilators. The most common comorbidity reported in patients with SCD and COVID-19 is asthma.[54] Many institutions have suspended the use of nebulizers due to the risk of aerosolizing virus particles, however metered-dose inhalers should be used as replacement when appropriate. Additional management should also follow institutional standards of care for managing SCD and fever. These may include evaluation for typical seasonal viral infections and empiric oseltamivir, until influenza is ruled out; adequate pulmonary toileting, which includes ambulation as tolerated as well as incentive spirometry. Good pain control is also especially important to reduce atelectasis.

Lastly, it is of particular importance to address COVID-19-associated coagulopathy. Patients with sickle cell disease exhibit a baseline hypercoagulable state and are at an increased risk for venous thrombosis and pulmonary embolism. Some symptomatic patients may benefit from a PE protocol spiral computed tomography (CT) scan during work up. All children and adults hospitalized with SCD and COVID-19 should receive prophylactic anticoagulation dosing or "intermediate intensity" dosing (enoxaparin 0.5mg/kg twice daily), unless the risk of bleeding outweighs the risk of thrombosis. Inpatients should also have close monitoring of disseminated intravascular coagulation (DIC) markers (PT, aPTT, PTT, hepzyme, thrombin time, fibrinogen, D dimer) and inflammatory markers (CRP, ferritin, fibrinogen, IL-6, factor 8) to address thromboembolic risk throughout hospitalization.[58]

Sickle cell disease is a chronic medical condition requiring a multidisciplinary approach with continued "maintenance care" and constant education. It is of utmost importance that these interactions and collaboration continue during this global pandemic. Telemedicine has become an excellent tool to continue chronic sickle cell maintenance while aiming to avoid exposure. It is important to provide virtual appointments when possible rather than canceling regular maintenance appointments. It is also imperative to educate our patients on the importance of physical distancing, masking, and proper handwashing for the prevention of further spread.

Old and New Therapies
Blood Transfusion

Blood transfusion was the first therapy used in SCD, even at a time when the pathophysiology was still poorly understood.[59] Transfusions (simple and exchange transfusions) have remained a critical therapeutic and prophylactic intervention in sickle cell disease to date. Transfusion exerts its effect by dilution of Hb-S containing red blood cells (thereby decreasing the percentage of the abnormal Hb S), and increasing the oxygen carrying capacity of blood.[60] Therapeutic blood transfusions have been used for several indications such as: ACS, stroke, acute symptomatic anemia, aplastic crisis, splenic and hepatic sequestration, sickle hepatopathy, central retinal artery occlusion, and multisystem organ failure. Several randomized clinical trials have demonstrated the efficacy of prophylactic/chronic blood transfusions in primary and secondary stroke prevention, pre-operative management, and pregnancy.[61–67]

In addition to the above indications, providers have also utilized blood transfusions to alleviate other sickle cell disease complications, though not supported by concrete evidence. Before embarking on a chronic blood transfusion regimen with a patient, the potential benefits must be weighed against risks such as iron overload, red cell alloimmunization, transfusion reactions, and blood-borne viral infections.

FDA Approved Drugs

There are currently 4 FDA approved drugs for sickle cell disease, most gaining approval since 2017.

Hydroxyurea

Hydroxyurea, approved in 1998, was the first FDA-approved drug for sickle cell disease. It gained approval based on the randomized clinical trial that demonstrated decreased rates of vaso-occlusive crises, increased median time to first and second crises, and decreased rates of acute chest syndrome.[68] Initially approved for adults >18 years with sickle cell anemia, in 2017 it gained approval for children >2 years of age, based on an open-label trial conducted with pediatric patients.[69]

Hydroxyurea is a ribonucleotide reductase inhibitor that inhibits DNA replication. Its mechanism of action is not completely understood, but it is known to induce fetal hemoglobin (Hb F), which in turn inhibits intracellular Hb S polymerization and prevents sickling within the red blood cells.[70] In addition to increased Hb F synthesis and decreased Hb S polymerization, effects include increased Hb synthesis, decreased neutrophil count, hemolysis, RBC membrane damage, endothelial cell activation and adhesion, to mention a few.[71]

Endari (l-Glutamine)

L-glutamine is a conditionally essential amino acid; during periods of stress and severe illness, the body's production becomes insufficient. It is required for the synthesis of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), and indirectly regulates the metabolism of glutathione. NAD and its reduced form, NADH are involved in maintaining redox balance and sickle red cells have a low redox ratio compared to normal red cells.[72] The mechanism of action of l-glutamine in SCD is not fully understood but it has been shown to increase NADH in sickle RBCs, which is believed to reduce oxidative stress.[72] L-glutamine gained FDA approval in 2017 based on a randomized trial that demonstrated significantly reduced pain crises and hospitalizations in the drug group.[72] It is approved for sickle cell patients of all genotypes, age 5 years and above. This was hailed as the first SCD drug approval in almost 20 years since Hydroxyurea. However, concerns about patient accessibility and adherence have been reported.[73]

Adakveo (Crizanlizumab)

Crizanlizumab is a monoclonal antibody against the adhesion molecule P-selectin. P-selectin, expressed on the surface of endothelial cells mediates abnormal adhesion of sickle RBCs to the endothelium, and this process is implicated in the painful vaso-occlusion in SCD.[74] A randomized trial demonstrated significantly decreased rate of vaso-occlusive crises, and prolonged time to first and second crises on Crizanlizumab.[75] This led to its FDA approval in 2019 for sickle cell patients of all genotypes, age 16 years and above.

Oxbryta (Voxelotor)

Voxelotor is a small molecule that binds to the alpha chain of hemoglobin, increasing Hb S affinity for oxygen, delaying Hb S polymerization and preventing RBC sickling.[76] In essence, it is an HbS polymerization inhibitor.[77] A randomized trial demonstrated significantly higher percentage of participants with a Hb response (>1 g/dl from baseline), fewer instances of worsening anemia and significant reduction in baseline markers of hemolysis with the drug.[77] This led to its FDA approval in 2019 for sickle cell patients of all genotypes, age 12 years and above.

Curative Modalities
Bone Marrow Transplant
Bone marrow transplant (BMT) works by replacing the faulty "machinery" in the sickle cell patient: the marrow which produces abnormal sickle RBCs is replaced with a functioning marrow that produces normal RBCs that do not sickle. The first successful BMT as a cure for SCD was serendipitous. A young pediatric patient with both leukemia and sickle cell anemia underwent transplant as treatment for her leukemia, with resultant cure of her sickle cell disease as well.[78] This was followed by a quick succession of other institutional reports of their experience with BMT as a cure for SCD.[79–83] Outcomes have been impressive with overall and event free survival well above 90% with HLA-matched sibling donors.[84,85] Expanded donor pools such as matched unrelated donors and haploidentical donors have been explored, albeit at an increased risk of graft rejection and increased mortality.[86]

Gene Therapy
Gene therapy portends a potential for cure for SCD but is still in its nascent stages. Viral vectors are employed as vehicles to introduce a therapeutic anti-sickling coding gene (gene addition), to induce Hb F via silencing of repressors of the gamma-globin gene (Hb F induction), or to correct the sickle mutation via genomic engineering tools such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (gene correction).[87] The first successful gene therapy for SCD was reported in 2017, using a lentiviral vector-mediated addition of an anti-sickling beta-globin gene into the subject's hematopoietic stem cells.[88] Currently, multiple other clinical trials are in progress to investigate the efficacy of gene therapy in SCD. Anecdotal reports of successful gene therapy have been highlighted.[88–90] However, caution must be exercised, because as with every thorough clinical research, the ongoing trials need to be concluded to determine the efficacy of the intervention.

Pipeline Agents
In contrast to a few decades ago, there now exists numerous ongoing clinical trials with novel agents for the treatment of sickle cell disease in the pipeline.[91] Table 1

Table 1 Ongoing Clinical Trials of Novel Agents in Sickle Cell Disease

Drug	Mechanism	NCT Number (Study Acronym)	Clinical Trial Phase
Anti-sickling agents			
Nicotinamide with oral THU and Decitabine	Hb F induction	NCT04055818	Phase I
Panobinostat		NCT01245179	Phase I
Metformin		NCT02981329	Phase I
Gum Arabic		NCT04191213	Phase II/III
Voxelotor (formerly GBT-440)	Allosteric modifier (to the R-state)	NCT04247594	Phase II
		NCT04335721	Phase I/II
		NCT04188509	Phase III
		NCT03573882	Phase III
		NCT04400487	Phase IV
		NCT02850406 (HOPE Kids)	Phase II
		NCT04218084 (HOPE Kids 2)	Phase III
AG-348 (Mitapivat sulfate)	Allosteric activator of RBC pyruvate kinase-R	NCT04000165	Phase I
FT-4202		NCT03815695	Phase I
SCD-101	RBC ion transport channels	NCT02380079	Phase Ib
Memantine (NMDAR antagonist)		NCT03247218	Phase IIa/IIb
Anti-adhesion agents			
Crizanlizumab (formerly SEG-101)	P-selectin antagonist	NCT03814746 (STAND)	Phase III
		NCT03938454 (SPARTAN)	Phase II
		NCT04053764 (STEADFAST)	Phase II
		NCT03264989	Phase II
		NCT03474965	Phase II
Isoquercetin		NCT04474626	Phase II
IVIG	Blockade of fcγrIII receptors	NCT01757418	Phase I/II
Imatinib	Tyrosine kinase inhibitor	NCT03997903 (IMPACT)	Phase I
SHP655 (recombinant ADAMTS13)	ADAMTS13 protein replacement	NCT03997760 (RAISE-UP)	Phase I/II
CSL889	Hemopexin replacement	NCT04285827	Phase I
Anti-inflammatory agents and nitric oxide-related drugs			
Defibrotide	Antithrombotic, anti-inflammatory	NCT03805581	Phase II
Mometasone	Anti-inflammatory	NCT03758950 (IMPROVE2)	Phase II
Docosahexaenoic acid (SC411)		NCT02973360 (SCOT)	Phase II

(*Continued*)

Table 1 (Continued).

Drug	Mechanism	NCT Number (Study Acronym)	Clinical Trial Phase
Arginine	Increased NO production	NCT02447874	Phase I/II
		NCT02536170	Phase II
Sodium Nitrite (topical)		NCT02863068	Phase II
Riociguat (soluble guanylate cyclase stimulator)	Vasodilator	NCT02633397	Phase II
IMR-687 (selective phosphodiesterase-9 inhibitor)		NCT03401112	Phase II
		NCT04474314	Phase II
		NCT04053803	Phase II

Notes: Adapted from Rai P and Ataga KI. Drug Therapies for the Management of Sickle Cell Disease [version 1; peer review: 2 approved]. F1000Research 2020, 9(F1000 Faculty Rev):592 (https://doi.org/10.12688/f1000research.22433.1), licensed under CC-BY 4.0 (https://creativecommons.org/licenses/by/4.0/).[91]
Abbreviations: THU, tetrahydrouridine; NMDAR, N-methyl-D-aspartate receptor; IVIG, intravenous gammaglobulin; ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13.

reviews this information. Completed or terminated trials, as well as trials in gene therapy are omitted.

Conclusion

The complications and comorbidities of sickle cell disease are numerous. Acute chest syndrome remains the leading cause of mortality and must be taken keenly. However, various other sickle cell complications deserve attention, in order to ensure the wellbeing of the patient as a whole and improve their quality of life. Other complications that may lead to long-term comorbidities should not be overlooked. Whether it is acute chest syndrome, pain, or a social challenge like reduced quality of care due to racial discrimination, there is a need to look beyond the usual, and shift focus to a more comprehensive approach to the patient.

Multidisciplinary management as with management of leg ulcers or the pregnant sickle cell patient should be emphasized, so as to attain more favorable outcomes. Reproductive counseling can generate a profound impact on the burden of sickle cell disease. Interventions as simple as a routine annual ophthalmologic exam to rule out sickle cell retinopathy can have grave consequences if neglected in a subgroup of patients. Cerebrovascular events should be promptly followed up with intense rehabilitation to preserve residual gross and fine motor functioning. Updated vaccinations, to provide added protection against encapsulated organisms should be ensured. Routine health maintenance as in the general population, such as yearly flu vaccines, colonoscopies, and mammograms should be highly encouraged as well.

It is refreshing that several novel agents are in the pipeline, to deal with the underlying cause of sickle cell disease. Experimental gene therapy also promises a potential cure, if successful. However, true success in the clinical trials realm will entail successful implementation of their significant findings beyond the developed world – in low- and middle-income countries, where most of the world's sickle cell population reside. As discussed with the complications of sickle cell disease, the race to the cure must be attained on a global level.

Finally, sickle cell disease is a clear example of the fact that systemic racism is an unfortunate truth in American society. Therefore, we as healthcare providers should serve as role models to the rest of the country by dedicating ourselves to eradicating racially motivated healthcare disparities such as inadequate access to healthcare, suboptimal patient care, limited funding for research and therapy development, and poor quality of life in order to truly go beyond to new heights in the management of SCD.

Disclosure

Dr Ugochi O Ogu received Consultancy fees from Vertex Pharmaceuticals, outside the submitted work. Dr Patricia Adams-Graves is Consultant and speaker for Novartis and GBT, outside the submitted work. The authors report no other conflicts of interest in this work.

References

1. Fernandes Q. Therapeutic strategies in sickle cell anemia: the past present and future. *Life Sci.* 2017;178:100–108. doi:10.1016/j.lfs.2017.03.025

2. Vekilov PG. Sickle-cell haemoglobin polymerization: is it the primary pathogenic event of sickle-cell anaemia? *Br J Haematol.* 2007;139(2):173–184. doi:10.1111/j.1365-2141.2007.06794.x

3. Moerdler S, Manwani D. New insights into the pathophysiology and development of novel therapies for sickle cell disease. *Hematology Am Soc Hematol Educ Program.* 2018;2018(1):493–506. doi:10.1182/asheducation-2018.1.493

4. Piccin A, Murphy C, Eakins E, et al. Insight into the complex pathophysiology of sickle cell anaemia and possible treatment. *Eur J Haematol.* 2019;102(4):319–330. doi:10.1111/ejh.13212

5. Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood.* 2016;127(7):801–809. doi:10.1182/blood-2015-09-618538

6. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet.* 2010;376(9757):2018–2031. doi:10.1016/S0140-6736(10)61029-X

7. Thein MS, Igbineweka NE, Thein SL. Sickle cell disease in the older adult. *Pathology.* 2017;49(1):1–9. doi:10.1016/j.pathol.2016.10.002

8. Payne R. Sickle cell anemia and pain: will data prevail over beliefs? *Ann Emerg Med.* 2009;53(5):596–597. doi:10.1016/j.annemergmed.2008.10.022

9. Shah F, Dwivedi M. Pathophysiology and recent therapeutic insights of sickle cell disease. *Ann Hematol.* 2020;99(5):925–935. doi:10.1007/s00277-020-03977-9

10. Adebiyi MG, Manalo JM, Xia Y. Metabolomic and molecular insights into sickle cell disease and innovative therapies. *Blood Adv.* 2019;3(8):1347–1355. doi:10.1182/bloodadvances.2018030619

11. Singh PC, Ballas SK. Emerging drugs for sickle cell anemia. *Expert Opin Emerg Drugs.* 2015;20(1):47–61. doi:10.1517/14728214.2015.985587

12. Power-Hays A, McGann PT. When actions speak louder than words - racism and sickle cell disease. *N Engl J Med.* 2020;383:1902–1903. doi:10.1056/NEJMp2022125

13. Bundy DG, Strouse JJ, Casella JF, Miller MR. Urgency of emergency department visits by children with sickle cell disease: a comparison of 3 chronic conditions. *Acad Pediatr.* 2011;11(4):333–341. doi:10.1016/j.acap.2011.04.006

14. Centers for Disease Control and Prevention. Data and statistics: sickle cell disease; 2019. Available from: https://www.cdc.gov/ncbddd/sicklecell/data.html. Accessed November 2, 2020.

15. National Organization for Rare Disorders. Cystic Fibrosis; 2017. Available from: https://rarediseases.org/rare-diseases/cystic-fibrosis/. Accessed November 2, 2020.

16. Farooq F, Mogayzel PJ, Lanzkron S, Haywood C, Strouse JJ. Comparison of US federal and foundation funding of research for sickle cell disease and cystic fibrosis and factors associated with research productivity. *JAMA Netw Open.* 2020;3(3):e201737. doi:10.1001/jamanetworkopen.2020.1737

17. Haywood C, Lanzkron S, Bediako S, et al. Perceived discrimination, patient trust, and adherence to medical recommendations among persons with sickle cell disease. *J Gen Intern Med.* 2014;29(12):1657–1662. doi:10.1007/s11606-014-2986-7

18. Lee L, Smith-Whitley K, Banks S, Puckrein G. Reducing health care disparities in sickle cell disease: a review. *Public Health Rep.* 2019;134(6):599–607. doi:10.1177/0033354919881438

19. Pulte D, Lovett PB, Axelrod D, Crawford A, McAna J, Powell R. Comparison of emergency department wait times in adults with sickle cell disease versus other painful etiologies. *Hemoglobin.* 2016;40(5):330–334. doi:10.1080/03630269.2016.1232272

20. Hsu LL, Green NS, Donnell Ivy E, et al. Community health workers as support for sickle cell care. *Am J Prev Med.* 2016;51(1 Suppl 1):S87–S98. doi:10.1016/j.amepre.2016.01.016

21. Adams-Graves P, Bronte-Jordan L. Recent treatment guidelines for managing adult patients with sickle cell disease: challenges in access to care, social issues, and adherence. *Expert Rev Hematol.* 2016;9(6):541–552. doi:10.1080/17474086.2016.1180242

22. Whiteman LN, Lanzkron S, Stewart RW, Haywood C, Strouse JJ, Feldman L. Quality improvement process in a sickle cell infusion center. *Am J Med.* 2015;128(5):541–544. doi:10.1016/j.amjmed.2014.11.020

23. Corvest V, Blais S, Dahmani B, et al. [Cerebral vasculopathy in children with sickle cell disease: key issues and the latest data]. *Arch Pediatr.* 2018;25(1):63–71. French. doi:10.1016/j.arcped.2017.11.015

24. Kassim AA, Galadanci NA, Pruthi S, DeBaun MR. How I treat and manage strokes in sickle cell disease. *Blood.* 2015;125(22):3401–3410. doi:10.1182/blood-2014-09-551564

25. Adams RJ. TCD in sickle cell disease: an important and useful test. *Pediatr Radiol.* 2005;35(3):229–234. doi:10.1007/s00247-005-1409-7

26. Medepalli V, Bajaj S, Bajaj M, Badamosi N Recurrent multifocal calvarial bony infarcts and intracranial hematomas in a patient with sickle cell anemia; 2020. Available from: https://www.eurorad.org/case/16842. Accessed November 2, 2020.

27. Hamm J, Rathore N, Lee P, et al. Cranial epidural hematomas: a case series and literature review of this rare complication associated with sickle cell disease. *Pediatr Blood Cancer.* 2017;64(3):e26237. doi:10.1002/pbc.26237

28. Liem RI, Lanzkron S, Coates T, et al. American Society of Hematology 2019 guidelines for sickle cell disease: cardiopulmonary and kidney disease. *Blood Adv.* 2019;3(23):3867–3897. doi:10.1182/bloodadvances.2019000916

29. Wyrick-Glatzel J, Conway-Klaassen J. Clinical utility of the IRF: assessment of erythroid regeneration following parvo B19 infection. *Clin Lab Sci.* 2002;15(4):208–212.

30. Shah R, Taborda C, Chawla S. Acute and chronic hepatobiliary manifestations of sickle cell disease: a review. *World J Gastrointest Pathophysiol.* 2017;8(3):108–116. doi:10.4291/wjgp.v8.i3.108

31. Anele UA, Le BV, Resar LMS, Burnett AL. How I treat priapism. *Blood.* 2015;125(23):3551–3558. doi:10.1182/blood-2014-09-551887

32. Martí-Carvajal AJ, Solà I, Agreda-Pérez LH. Treatment for avascular necrosis of bone in people with sickle cell disease. *Cochrane Database Syst Rev.* 2016;8.

33. Lebensburger JD, Bemrich-Stolz CJ, Howard TH. Barriers in transition from pediatrics to adult medicine in sickle cell anemia. *J Blood Med.* 2012;3:105–112. doi:10.2147/JBM.S32588

34. Ogu UO, Billett HH. Comorbidities in sickle cell disease: adult providers needed! *Indian J Med Res.* 2018;147(6):527–529. doi:10.4103/ijmr.IJMR_1019_18

35. Jain S, Bakshi N, Krishnamurti L. Acute chest syndrome in children with sickle cell disease. *Pediatr Allergy Immunol Pulmonol.* 2017;30(4):191–201. doi:10.1089/ped.2017.0814

36. Ballas SK, Lieff S, Benjamin LJ, et al. Definitions of the phenotypic manifestations of sickle cell disease. *Am J Hematol.* 2010;85(1):6–13. doi:10.1002/ajh.21550

37. Vichinsky EP, Neumayr LD, Earles AN, et al. Causes and outcomes of the acute chest syndrome in sickle cell disease. *N Engl J Med.* 2000;342(25):1855–1865. doi:10.1056/NEJM200006223422502

38. Dampier C, Palermo TM, Darbari DS, Hassell K, Smith W, Zempsky W. AAPT diagnostic criteria for chronic sickle cell disease pain. *J Pain.* 2017;18(5):490–498. doi:10.1016/j.jpain.2016.12.016

39. Edwards LY, Edwards CL. Psychosocial treatments in pain management of sickle cell disease. *J Natl Med Assoc.* 2010;102(11):1084–1094. doi:10.1016/S0027-9684(15)30737-9

40. Williams H, Tanabe P. Sickle cell disease: a review of nonpharmacological approaches for pain. *J Pain Symptom Manage.* 2016;51(2):163–177. doi:10.1016/j.jpainsymman.2015.10.017

41. Shet AS, Wun T. How I diagnose and treat venous thromboembolism in sickle cell disease. *Blood*. 2018;132(17):1761–1769. doi:10.1182/blood-2018-03-822593

42. Olatunya OS, Albuquerque DM, Adekile AD, Costa FF. Evaluation of sociodemographic, clinical, and laboratory markers of sickle leg ulcers among young Nigerians at a tertiary health institution. *Niger J Clin Pract*. 2018;21(7):882–887. doi:10.4103/njcp.njcp_4_18

43. Serjeant GR, Serjeant BE, Mohan JS, Clare A. Leg ulceration in sickle cell disease: medieval medicine in a modern world. *Hematol Oncol Clin North Am*. 2005;19(5):943–ix. doi:10.1016/j.hoc.2005.08.005

44. Minniti CP, Kato GJ. Critical reviews: how we treat sickle cell patients with leg ulcers. *Am J Hematol*. 2016;91(1):22–30. doi:10.1002/ajh.24134

45. Vasconcelos A, Prior AR, Ferrão A, Morais A. An adolescent with sickle cell anaemia experiencing disease-related complications: priapism and leg ulcer–a management challenge. *BMJ Case Rep*. 2012;2012:bcr1120115146–bcr1120115146. doi:10.1136/bcr.11.2011.5146

46. Smith-Whitley K. Reproductive issues in sickle cell disease. *Blood*. 2014;124(24):3538–3543. doi:10.1182/blood-2014-07-577619

47. Taddesse A, Woldie IL, Khana P, et al. Hypogonadism in patients with sickle cell disease: central or peripheral? *Acta Haematol*. 2012;128(2):65–68. doi:10.1159/000337344

48. Madu AJ, Ubesie A, Ocheni S, et al. Priapism in homozygous sickle cell patients: important clinical and laboratory associations. *Med Princ Pract*. 2014;23(3):259–263. doi:10.1159/000360608

49. Serjeant GR, Singhal A, Hambleton IR. Sickle cell disease and age at menarche in Jamaican girls: observations from a cohort study. *Arch Dis Child*. 2001;85(5):375–378. doi:10.1136/adc.85.5.375

50. Smith-Whitley K. Complications in pregnant women with sickle cell disease. *Hematology Am Soc Hematol Educ Program*. 2019;2019 (1):359–366. doi:10.1182/hematology.2019000039

51. Nnoli A, Seligman NS, Dysart K, Baxter JK, Ballas SK. Opioid utilization by pregnant women with sickle cell disease and the risk of neonatal abstinence syndrome. *J Natl Med Assoc*. 2018;110 (2):163–168. doi:10.1016/j.jnma.2017.04.002

52. Johns Hopkins University & Medicine. COVID-19 map; 2020. Available from: https://coronavirus.jhu.edu/map.html. Accessed November 2, 2020.

53. Sickle Cell Society. Coronavirus (COVID-19) & sickle cell disorder; 2020. Available from: https://www.sicklecellsociety.org/coronavirus-and-scd/. Accessed November 2, 2020.

54. Medical College of Wisconsin. SECURE-SCD registry; 2020. Available from: https://covidsicklecell.org/updates-data/. Accessed November 2, 2020.

55. Arlet JB, de Luna G, Khimoud D, et al. Prognosis of patients with sickle cell disease and COVID-19: a French experience. *Lancet Haematol*. 2020;7(9):e632–e634. doi:10.1016/S2352-3026(20)30204-0

56. Hussain FA, Njoku FU, Saraf SL, Molokie RE, Gordeuk VR, Han J. COVID-19 infection in patients with sickle cell disease. *Br J Haematol*. 2020;189(5):851–852. doi:10.1111/bjh.16734

57. Sahu KK, Siddiqui AD, Cerny J. Managing sickle cell patients with COVID-19 infection: the need to pool our collective experience. *Br J Haematol*. 2020;190(2):e86–e89. doi:10.1111/bjh.16880

58. American Society of Hematology. COVID-19; 2020. Available from: https://www.hematology.org/covid-19. Accessed November 2, 2020.

59. Baker JP. Sickle Cell Anemia. *Trans Am Clin Climatol Assoc*. 1941;57:203–212.

60. Howard J. Sickle cell disease: when and how to transfuse. *Hematology Am Soc Hematol Educ Program*. 2016;2016 (1):625–631. doi:10.1182/asheducation-2016.1.625

61. Adams RJ, McKie VC, Hsu L, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. *N Engl J Med*. 1998;339 (1):5–11. doi:10.1056/NEJM199807023390102

62. Adams RJ, Brambilla D. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. *N Engl J Med*. 2005;353(26):2769–2778.

63. Ware RE, Helms RW. Stroke with transfusions changing to hydroxyurea (SWiTCH). *Blood*. 2012;119(17):3925–3932. doi:10.1182/blood-2011-11-392340

64. DeBaun MR, Gordon M, McKinstry RC, et al. Controlled trial of transfusions for silent cerebral infarcts in sickle cell anemia. *N Engl J Med*. 2014;371(8):699–710. doi:10.1056/NEJMoa1401731

65. Ware RE, Davis BR, Schultz WH, et al. Hydroxycarbamide versus chronic transfusion for maintenance of transcranial doppler flow velocities in children with sickle cell anaemia-TCD With Transfusions Changing to Hydroxyurea (TWiTCH): a multicentre, open-label, Phase 3, non-inferiority trial. *Lancet*. 2016;387 (10019):661–670. doi:10.1016/S0140-6736(15)01041-7

66. Howard J, Malfroy M, Llewelyn C, et al. The Transfusion Alternatives Preoperatively in Sickle Cell Disease (TAPS) study: a randomised, controlled, multicentre clinical trial. *Lancet*. 2013;381(9870):930–938. doi:10.1016/S0140-6736(12)61726-7

67. Koshy M, Burd L, Wallace D, Moawad A, Baron J. Prophylactic red-cell transfusions in pregnant patients with sickle cell disease. A randomized cooperative study. *N Engl J Med*. 1988;319 (22):1447–1452. doi:10.1056/NEJM198812013192204

68. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med*. 1995;332(20):1317–1322. doi:10.1056/NEJM199505183322001

69. U.S. Food and Drug Administration. FDA approves hydroxyurea for treatment of pediatric patients with sickle cell anemia; 2020. https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-hydroxyurea-treatment-pediatric-patients-sickle-cell-anemia. Accessed October 18, 2020.

70. McGann PT, Ware RE. Hydroxyurea therapy for sickle cell anemia. *Expert Opin Drug Saf*. 2015;14(11):1749–1758. doi:10.1517/14740338.2015.1088827

71. Verma HK, Lakkakula S, Lakkakula BVKS. Retrospection of the effect of hydroxyurea treatment in patients with sickle cell disease. *Acta Haematol Pol*. 2018;49(1):1. doi:10.2478/ahp-2018-0001

72. Niihara Y, Miller ST, Kanter J, et al. A phase 3 trial of l-glutamine in sickle cell disease. *N Engl J Med*. 2018;379(3):226–235. doi:10.1056/NEJMoa1715971

73. Ogu UO, Thomas M, Chan F, et al. L-glutamine use in adults with sickle cell disease: clinical trials where success meets reality. *Am J Hematol*. 2020.

74. Matsui NM, Borsig L, Rosen SD, Yaghmai M, Varki A, Embury SH. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. *Blood*. 2001;98(6):1955–1962. doi:10.1182/blood.V98.6.1955

75. Ataga KI, Kutlar A, Kanter J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*. 2017;376 (5):429–439. doi:10.1056/NEJMoa1611770

76. Oksenberg D, Dufu K, Patel MP, et al. GBT440 increases haemoglobin oxygen affinity, reduces sickling and prolongs RBC half-life in a murine model of sickle cell disease. *Br J Haematol*. 2016;175 (1):141–153. doi:10.1111/bjh.14214

77. Vichinsky E, Hoppe CC, Ataga KI, et al. A phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med*. 2019;381 (6):509–519. doi:10.1056/NEJMoa1903212

78. Johnson FL, Look AT, Gockerman J, Ruggiero MR, Dalla-Pozza L, Billings FT. Bone-marrow transplantation in a patient with sickle-cell anemia. *N Engl J Med*. 1984;311(12):780–783. doi:10.1056/NEJM198409203111207

79. Vermylen C, Fernandez Robles E, Ninane J, Cornu G. Bone marrow transplantation in five children with sickle cell anaemia. *Lancet*. 1988;331(8600):1427–1428. doi:10.1016/S0140-6736(88)92239-8

80. Ferster A, De Valck C, Azzi N, Fondu P, Toppet M, Sariban E. Bone marrow transplantation for severe sickle cell anaemia. *Br J Haematol*. 1992;80(1):102–105. doi:10.1111/j.1365-2141.1992. tb06407.x

81. Vermylen C, Cornu G. Bone marrow transplantation for sickle cell disease. The European experience. *Am J Pediatr Hematol Oncol*. 1994;16(1):18–21.

82. Johnson FL, Mentzer WC, Kalinyak KA, Sullivan KM, Abboud MR. Bone marrow transplantation for sickle cell disease. The United States experience. *Am J Pediatr Hematol Oncol*. 1994;16(1):22–26.

83. Walters MC, Patience M, Leisenring W, et al. Bone marrow transplantation for sickle cell disease. *N Engl J Med*. 1996;335 (6):369–376. doi:10.1056/NEJM199608083350601

84. Walters MC, De Castro LM, Sullivan KM, et al. Indications and results of HLA-identical sibling hematopoietic cell transplantation for sickle cell disease. *Biol Blood Marrow Transplant*. 2016;22 (2):207–211. doi:10.1016/j.bbmt.2015.10.017

85. Gluckman E, Cappelli B, Bernaudin F, et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. *Blood*. 2017;129(11):1548–1556. doi:10.1182/blood-2016-10-745711

86. Kato GJ, Piel FB, Reid CD, et al. Sickle cell disease. *Nat Rev Dis Primers*. 2018;4:18010.

87. Demirci S, Uchida N, Tisdale JF. Gene therapy for sickle cell disease: an update. *Cytotherapy*. 2018;20(7):899–910. doi:10.1016/j. jcyt.2018.04.003

88. Ribeil JA, Hacein-Bey-Abina S, Payen E, et al. Gene therapy in a patient with sickle cell disease. *N Engl J Med*. 2017;376 (9):848–855. doi:10.1056/NEJMoa1609677

89. Magrin E, Semeraro M, Magnani A, et al. *Results from the Completed Hgb-205 Trial of Lentiglobin for B-Thalassemia and Lentiglobin for Sickle Cell Disease Gene Therapy*. Washington, DC: American Society of Hematology; 2019.

90. Kanter J, Tisdale JF, Mapara MY, et al. *Resolution of Sickle Cell Disease Manifestations in Patients Treated with Lentiglobin Gene Therapy: Updated Results from the Phase 1/2 Hgb-206 Group C Study*. Washington, DC: American Society of Hematology; 2019.

91. Rai P, Ataga KI. Drug Therapies for the Management of Sickle Cell Disease. F1000Res. 2020; 9.

Efficacy of rFVIIIFc versus Emicizumab for the Treatment of Patients with Hemophilia A without Inhibitors: Matching-Adjusted Indirect Comparison of A-LONG and HAVEN Trials

Robert Klamroth, Piotr Wojciechowski, Samuel Aballéa, Françoise Diamand, Zalmai Hakimi, Jameel Nazir, Lydia Abad-Franch, Stefan Lethagen, Elena Santagostino & Michael D Tarantino

ORIGINAL RESEARCH

Efficacy of rFVIIIFc versus Emicizumab for the Treatment of Patients with Hemophilia A without Inhibitors: Matching-Adjusted Indirect Comparison of A-LONG and HAVEN Trials

Robert Klamroth [1]
Piotr Wojciechowski [2]
Samuel Aballéa [3]
Françoise Diamand [4]
Zalmai Hakimi [5]
Jameel Nazir [5]
Lydia Abad-Franch [6]
Stefan Lethagen [7]
Elena Santagostino [8]
Michael D Tarantino [9]

[1]Department of Internal Medicine, Hemophilia Treatment Centre, Vivantes Klinikum im Friedrichshain, Berlin, Germany; [2]Creativ-Ceutical, Krakow, Poland; [3]Creativ-Ceutical, Rotterdam, the Netherlands; [4]Creativ-Ceutical, Paris, France; [5]Health Economics and Outcomes Research (Global), Sobi, Stockholm, Sweden; [6]Global Medical Affairs Hematology, Sobi, Stockholm, Sweden; [7]Medical and Clinical Sciences, Sobi, Stockholm, Sweden; [8]Medical Affairs Hematology, Sobi, Stockholm, Sweden; [9]The Bleeding and Clotting Disorders Institute, University of Illinois College of Medicine-Peoria, Peoria, IL, USA

Correspondence: Robert Klamroth
Department of Internal Medicine – Angiology and Hemostaseology, Center for Vascular Medicine, Hämophiliezentrum/ Gerinnungssprechstunde, Vivantes Klinikum im Friedrichshain, Landsberger Allee 49, D, Berlin, 10249, Germany, Tel +49 (0)30 130 231575
Fax +49 (0)30 130 231313
Email robert.klamroth@vivantes.de

Purpose: Primary prophylaxis, using factor VIII replacement, is the recognized standard of care for severe hemophilia A. Recombinant factor VIII-Fc fusion protein (rFVIIIFc) and emicizumab, a humanized, bispecific antibody, are approved for routine prophylaxis of bleeding episodes in severe hemophilia A. These products have different mechanisms of action, methods of administration and treatment schedules. In the absence of head-to-head trials, indirect treatment comparisons can provide informative evidence on the relative efficacy of the two treatments. The aim of the study was to compare the approved dosing regimens for each product, rFVIIIFc individualized prophylaxis and emicizumab administered once every week (Q1W), every 2 weeks (Q2W) or every 4 weeks (Q4W), based on clinical trial evidence.

Patients and Methods: The comparison was conducted using matching-adjusted indirect comparison since clinical evidence did not form a connected network. Individual patient data for rFVIIIFc (A-LONG) were compared with data for emicizumab (HAVEN trial program) for mean annualized bleeding rate (ABR) and proportion of patients with zero bleeds. Safety data reported across the analyzed treatment arms were tabularized but not formally compared.

Results: After matching, no significant differences were observed between mean ABR for rFVIIIFc and emicizumab administered Q1W, Q2W or Q4W. The proportion of patients with zero bleeds was significantly higher with rFVIIIFc compared with emicizumab administered Q4W (51.2% versus 29.3%, respectively; odds ratio 2.53; 95% confidence interval 1.09–-5.89); no significant differences noted when rFVIIIFc was compared with emicizumab administered Q1W or Q2W. The mean number of adverse events expressed per participant was 1.9 for individualized prophylaxis with rFVIIIFc and 3.7–4.0, 4.1 and 3.6 for emicizumab administered Q1W, Q2W or Q4W, respectively.

Conclusion: This indirect treatment comparison suggests that rFVIIIFc individualized prophylaxis is more efficacious than emicizumab Q4W, and at least as effective as more frequent emicizumab regimens, for the management of hemophilia A.

Keywords: annualized bleeding rate, antibodies, bispecific, comparative effectiveness research, efmoroctocog alfa, factor VIII deficiency, treatment outcome

Introduction

Factor replacement products are the mainstay of treatment for individuals with hemophilia A,[1] a serious bleeding disorder characterized by frequent and spontaneous bleeding into joints and muscles.[2] Recurrent joint bleeding is a hallmark of

severe hemophilia and a major cause of morbidity, leading to progressive irreversible joint damage and the development of hemophilic arthropathy.[3]

Primary prophylaxis, using replacement factor VIII (FVIII), is the recognized standard of care for individuals with severe hemophilia A, and, initiated early in life, it can prevent joint damage and reduce the frequency of joint and other hemorrhages.[4,5] Significant heterogeneity exists between patients with hemophilia with respect to bleeding phenotype and response to treatment; therefore, to optimize outcomes, treatment schedules should be flexible and tailored to individual patient's needs.[6]

Recombinant factor VIII-Fc fusion protein (rFVIIIFc) is approved for on-demand treatment and control of bleeding episodes, routine prophylaxis to reduce the frequency of bleeding episodes, and perioperative management of bleeding in pediatric, adolescent and adult patients with hemophilia A.[7,8] The recommended dose for long-term prophylaxis according to the European label is 50 IU/kg every 3–5 days, which can be adjusted based on a patient's response in the range 25–65 IU/kg,[8] thus providing an opportunity for adjusting dosing to the requirements of each individual patient.

The safety and efficacy of rFVIIIFc was established in two Phase 3 studies of previously treated pediatric (Kids A-LONG) and adult/adolescent (A-LONG) patients with severe hemophilia A.[9,10] An individualized prophylaxis regimen, which aimed for trough levels of 1–3 IU/dL in adults/adolescents, provided clinically meaningful reductions in annualized bleeding rates (ABRs) compared with on-demand treatment.[9] These results were confirmed in an extension study (ASPIRE), with low ABRs and extended dosing intervals sustained for up to 5.9 years of treatment.[11]

Emicizumab is a recombinant humanized, bispecific, monoclonal antibody that mimics the function of activated FVIII by bridging activated factors IX and X to induce coagulation at the site of bleeding.[12] Emicizumab is approved for routine prophylaxis of bleeding episodes in patients with severe hemophilia A with or without FVIII inhibitors.[13,14] It is administered as a subcutaneous injection at the recommended dose of 3 mg/kg once weekly for the first 4 weeks (loading dose), followed by a maintenance dose of 1.5 mg/kg once weekly, 3 mg/kg every 2 weeks, or 6 mg/kg every 4 weeks. The safety and efficacy of emicizumab was investigated in the HAVEN clinical trial program.[15–18]

rFVIIIFc and emicizumab have different modes of action, methods of administration and treatment schedules, and despite the lack of head-to-head clinical trial evidence, a comparison of these therapeutic strategies would be beneficial. In this regard, the aim of this study was to compare the efficacy of rFVIIIFc individualized prophylaxis versus emicizumab for the treatment of patients with hemophilia A, based on clinical trial evidence.

Patients and Methods
Data Sources and Sample Selection

The pivotal trials, which provided efficacy and safety data for market authorization, were used as source data for comparison of the approved dosing regimens for each product (rFVIIIFc individualized prophylaxis;[7,8] and emicizumab administered once every week [Q1W], every 2 weeks [Q2W] or every 4 weeks [Q4W])[13,14], in the target population of adult/adolescents (≥12 years) with hemophilia A without inhibitors. Methodology and findings of these trials (A-LONG for rFVIIIFc; HAVEN clinical trial program for emicizumab) have been described previously.[9,16,17]

Briefly, A-LONG was a phase 3 open-label, multicenter, partially randomized study of rFVIIIFc in patients aged ≥12 years of age with severe hemophilia A.[9] Enrolled patients were assigned to one of the three treatment arms: individualized prophylaxis (25–65 IU/kg every 3–5 days; n=118), weekly prophylaxis (65 IU/kg; n=24), or episodic treatment (10–50 IU/kg; n=23). Prior to enrollment, patients in the individualized prophylaxis arm could have received FVIII as prophylaxis or on-demand, while those recruited to the other two arms could only have received on-demand treatment. In the individualized prophylaxis arm, to maintain good control of breakthrough bleeding, each patient's pharmacokinetic (PK) parameters were used to guide individual adjustments to dosing interval (down to 3 days or up to 5 days) and/or dose (up to 65 IU/kg) to target a steady-state FVIII trough level of 1–3 IU/dL. Adjustments were also made if a patient experienced two spontaneous bleeding episodes within an 8-week period.

For emicizumab, data were included from both HAVEN 3 and HAVEN 4. HAVEN 3 was a partially randomized study of emicizumab in patients aged ≥12 years of age with severe hemophilia A without current FVIII inhibitors (<0.6 Bethesda units per mL).[16] Participants receiving episodic therapy with FVIII were randomly assigned in a 2:2:1 ratio to receive emicizumab Q1W (1.5 mg/kg; group A; n=36) or Q2W (3.0 mg/kg

group B; n=35), or to continue on-demand therapy with FVIII (group C, n=18). An additional 63 patients who received FVIII prophylaxis before study entry were allocated to group D and received prophylaxis with emicizumab Q1W (1.5 mg/kg). HAVEN 4 was a non-randomized, single-arm study of emicizumab in patients aged ≥12 years of age with severe hemophilia A (n=41), which included five patients with hemophilia A with inhibitors undergoing treatment with FVIII concentrates or bypassing agents.[17] All patients received a subcutaneous loading dose of emicizumab of 3 mg/kg Q1W for the initial 4 weeks, followed by emicizumab prophylaxis Q4W (6.0 mg/kg) for at least 24 weeks. Data from all prophylaxis arms of both studies were used in the analysis, which included patients treated with 1.5 mg/kg Q1W, 3.0 mg/kg Q2W and 6.0 mg/kg Q4W.[16,17] HAVEN 1 and HAVEN 2 included patients with hemophilia A with inhibitors aged ≥12 years or <12 years of age, respectively, and were excluded from the analysis.[15,18]

Methodology of Indirect Comparisons

The pivotal trials included in the analysis had some similarities in their design. Both A-LONG and HAVEN 3 comprised randomized and non-randomized arms, while HAVEN 4 was a non-comparative, single-arm study. In both A-LONG and HAVEN 3, on-demand treatment was assessed as a reference regimen within the randomized arms of each study. Despite this, a network meta-analysis including the on-demand arms was considered unfeasible, since rFVIIIFc individualized prophylaxis was assessed within a separate, non-randomized arm of A-LONG and could not be assessed in relation to on-demand treatment. In the absence of head-to-head trials and/or a connected network of clinical evidence, the matching-adjusted indirect comparison (MAIC), which adjusts for differences in baseline characteristics between treatments, was selected as the most suitable method to compare rFVIIIFc with emicizumab for the prophylactic treatment of hemophilia and was performed according to guidelines developed by the National Institute for Health and Care Excellence Decision Support Unit.[19] Individual patient data (IPD), including baseline characteristics and effects observed in the individualized prophylaxis arm, were available for rFVIIIFc from the A-LONG trial. Data were anonymized and no information allowing individual patients to be identified was included. Each individual patient was assigned a weight calculated from the logistic regression model (see equation presented below), so that weighted mean baseline characteristics of the study population match the baseline characteristics reported for the comparator trial.[20]

$$\ln(w_{it}) = \alpha_0 + \alpha_1^T X_{it}$$

Where:

X_{it} = the covariate vector for the i-th individual receiving treatment t

W_{it} = weight assigned to the i-th individual receiving treatment t

The weights assigned to each patient individually can be interpreted as the estimated odds (relative propensity) of being in the comparator trials relative to the original study (A-LONG) and the weighted baseline characteristics of the A-LONG trial match the characteristics of the comparator population. The weights were used to recalculate the effect of the treatment in order to allow for the population-adjusted comparison with the estimates observed for the comparator.

Safety data reported across the analyzed treatment arms in the identified studies were tabularized but not formally compared using MAIC methodology since there was no unequivocal evidence for the interaction between baseline variables and the risk of adverse events (AEs).

Outcome Assessments

Efficacy outcomes assessed were mean ABR and proportion of patients with zero bleeds. These outcomes are clinically relevant and frequently reported treatment outcomes in clinical trials of hemophilia. The A-LONG protocol stipulated all bleeding events required administration of FVIII, regardless of severity; therefore, the estimates for the incidence of bleeding episodes reported for the A-LONG trial refer to all bleeding episodes. In the HAVEN 3 and HAVEN 4 trials, data were collected for all bleeds (treated and untreated) and treated bleeds; the clear algorithm defining which events qualified for FVIII treatment in the HAVEN program was not provided. For consistency with A-LONG, data for all bleeds were used in this analysis.

Data Analysis

IPD for rFVIIIFc from the A-LONG trial were weighted and matched to aggregated corresponding baseline characteristics for emicizumab in the comparator trials. Baseline variables included for adjustment were: age (mean, (standard deviation [SD]); target joint, including mean (SD) number of target joints (when available), or

proportion of patients with 1 or ≥2 target joint(s); proportion of patients with prior prophylaxis; ethnicity (proportion of white patients); and treatment duration (mean, SD). Outcomes were recalculated using assigned weights; mean ABR was estimated using weighted negative binomial regression model (using R software v.3.5.5 with MASS package), which was consistent with the analysis in HAVEN trials. Odds of zero bleeds were calculated by dividing the reweighted number of patients with and without bleeding episodes. Weighted outcomes from A-LONG were statistically compared with observed values for emicizumab.

For each adjustment, the matched baseline characteristics are presented with the corresponding estimates of effective sample size for patients receiving rFVIIIFc. Recalculated ABR and the odds of patients with zero bleeds were compared with the estimates related to emicizumab. The odds ratio (OR) was calculated using the standard formula.[21] Relative treatment effects are presented as incidence rate ratios (IRR) with 95% confidence intervals (CI) for ABR, and ORs with 95% CI for the proportion of patients with zero bleeds. The IRR, together with the associated 95% CI, was calculated as the exponent of the difference between the log values of ABRs for rFVIIIFc and emicizumab.[21] A difference in IRR or OR was considered statistically significant when the associated 95% CI did not include 1.0. Statistical comparisons were conducted in R (R v.3.5.5 [https://www.r-project.org/]).

Results
Baseline Characteristics Before Matching
The analysis included 117 patients who received rFVIIIFc individualized prophylaxis in A-LONG, and 99 and 41 patients from HAVEN 3 and HAVEN 4, respectively, who received emicizumab. Median age was 29 years in A-LONG and ranged from 36 to 41 years in the two HAVEN trials. Across the trials, the length of treatment varied; in A-LONG median duration of rFVIIIFc treatment in the individualized prophylaxis arm was 32.1 weeks; the median duration of the efficacy period ranged from 29.6 to 33.7 weeks in HAVEN 3 and was 25.6 weeks in HAVEN 4.

Prior to study entry, the treatment regimen was prophylaxis in 73.7% of patients in the individualized prophylaxis arm in A-LONG, and 41.4% and 73.0% in HAVEN 3 and HAVEN 4, respectively. At baseline, the proportion of patients with ≥1 target joint was 68.5% in

A-LONG. Overall, in HAVEN 3 and HAVEN 4, the proportion of patients with ≥1 target joint across the treatment arms was 41.3–94.4% and 61.0–86.0%, respectively. In A-LONG, the median number of bleeding events in the 12 months prior to study entry was 6.0 and 27.0 in patients receiving a prior prophylaxis or prior episodic regimen, respectively. In the HAVEN program, the proportion of bleeding events was reported for the prior 24 weeks only. In HAVEN 3 the proportion of patients with <9 bleeding events in the 24 weeks before trial entry was 25.0%, 14.3%, 22.2% and 84.1% in treatments arms A to D, respectively. The median number of bleeding events in the 24 weeks before study entry was 5.0 in HAVEN 4.

Matching of Baseline Characteristics
IPD for rFVIIIFc from the individualized prophylaxis arm of A-LONG were matched to the baseline characteristics of emicizumab Q1W (n=99; Supplementary Table 1), Q2W (n=35; Supplementary Table 2) and Q4W (n=41; Supplementary Table 3). The effective sample size (ESS) for rFVIIIFc for each comparison after matching was n=94 (Q1W), n=19 (Q2W) and n=36 (Q4W), respectively.

Annualized Bleeding Rate, All Bleeds
After matching, the mean ABR was 2.73 for individualized prophylaxis with rFVIIIFc and 2.93 for emicizumab administered Q1W. The difference in ABR between the two treatments was not statistically significant (IRR 0.93; 95% CI 0.63–1.39; Figure 1). Similarly, there was no statistically significant difference between mean ABR for individualized prophylaxis with rFVIIIFc and emicizumab administered Q2W (1.49 versus 2.60; IRR 0.57; 95% CI 0.28–1.17) and Q4W (2.75 versus 4.50; IRR 0.61; 95% CI 0.37–1.02).

Proportion of Patients With Zero Bleeds
The proportion of patients with zero bleeds was significantly higher with individualized prophylaxis with rFVIIIFc compared with emicizumab administered Q4W (51.2% versus 29.3%, respectively; OR 2.53; 95% CI 1.09–5.89; Figure 2). There were no statistically significant differences in the proportion of patients with zero bleeds between rFVIIIFc and emicizumab administered Q1W (47.6% versus 46.5%; OR 1.05; 95% CI 0.60–1.82) or Q2W (54.2% versus 40.0%; OR 1.78; 95% CI 0.62–5.11).

A

B
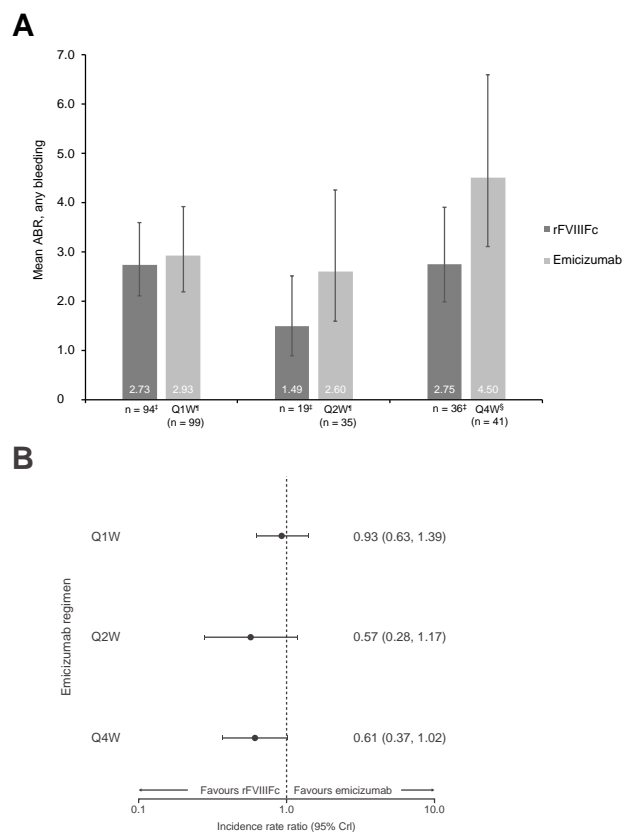
Figure 1 (**A** and **B**) Mean ABR, any bleeding, after matching for all selected baseline variables;[†]forest plot represents relative treatment effects presented as IRR with 95% CI.

Notes: [†]Age, number of target joints, proportion of patients with prior prophylaxis, ethnicity (proportion of white patients) and treatment duration (weeks); [‡]Effective sample size; [¶]Data from HAVEN 3; [§]Data from HAVEN 4

Abbreviations: ABR, annualized bleeding rate; CI, confidence interval; IRR, incidence rate ratio; Q1W, once a week; Q2W, once every 2 weeks; Q4W, once every 4 weeks.

A

B

Figure 2 (**A** and **B**) Proportion of patients with zero bleeds after matching for all selected baseline variables; [†]forest plot represents relative treatment effects presented as OR with 95% CI.

Notes: [†]Age, number of target joints, proportion of patients with prior prophylaxis, ethnicity (proportion of white people) and treatment duration (weeks); [‡]Effective sample size; [¶]Data from HAVEN 3; [§]Data from HAVEN 4.

Abbreviations: CI, confidence interval; OR, odds ratio; Q1W, once a week; Q2W, once every 2 weeks; Q4W, once every 4 weeks.

Safety

A summary of safety data from the individualized prophylaxis arm of A-LONG and all prophylaxis arms from HAVEN 3 and HAVEN 4 is presented in Supplementary Table 4. The mean number of AEs expressed per participant was 1.9 for individualized prophylaxis with rFVIIIFc, and 3.7–4.0, 4.1 and 3.6 for emicizumab administered Q1W, Q2W and Q4W, respectively. Injection site reactions were reported in 20–32% of patients receiving respective regimens of emicizumab prophylaxis, and thus were among the most frequently reported events. Injection site reactions were not reported among AEs in patients receiving individualized prophylaxis with rFVIIIFc in A-LONG. The proportions of patients reporting arthralgia, headache and upper respiratory tract infection were numerically lower with rFVIIIFc individualized prophylaxis than those observed in the respective emicizumab arms. There was no evidence of differences regarding serious AEs between treatments.

Discussion

The results of this MAIC analysis indicate that rFVIIIFc is more efficacious than emicizumab Q4W and at least as efficacious as more frequent emicizumab regimens, for the management of patients with hemophilia A. Individualized prophylaxis with rFVIIIFc, the approved dosing regimen,[7,8] was shown to be associated with a significantly greater proportion of patients with zero bleeds than emicizumab administered Q4W, while no statistically significant differences were observed in the proportion of patients with zero bleeds when rFVIIIFc was compared with emicizumab administered Q1W or Q2W. In addition, there were no statistically significant differences for mean ABR with

individualized prophylaxis with rFVIIIFc and emicizumab administered Q1W, Q2W or Q4W. Although five of the six comparisons of bleeding events did not achieve statistical significance, clear trends in favor of rFVIIIFc were observed.

In the A-LONG study, the prophylactic dosing regimen of rFVIIIFc was not designed to optimize the prevention of bleeds; the protocol prescribed a PK-tailored dosing regimen aimed at targeting FVIII trough levels between 1 and 3 IU/dL. This relatively modest treatment target may make a comparison with emicizumab unfavorable for rFVIIIFc, as emicizumab more than likely reached its ceiling limit for optimal prophylactic efficacy with the published dosing regimens.

The results of the current study contrast with the publication by Reyes et al, the results of which suggested the superiority of prophylaxis with emicizumab over FVIII prophylaxis (combined comparator of four different FVIII concentrates, including rFVIIIFc) in patients with hemophilia A without inhibitors.[22] Importantly, the Reyes analysis included data from the weekly prophylaxis arm of A-LONG, which is not aligned to the approved dosing regimens of rFVIIIFc, and may have led to the overestimation of ABRs for rFVIIIFc, thus boosting the relative efficacy of emicizumab in comparison. Detailed analysis of the Reyes study has been described elsewhere.[23]

For the current analysis, several methodologies were considered, including a network meta-analysis as utilized by Reyes et al,[22] a meta-analysis using the Bucher method and MAIC. Patients were not randomly assigned to the individualized prophylaxis arm with rFVIIIFc in A-LONG, and therefore, a connected network of evidence could not be formed between the rFVIIIFc individualized prophylaxis and emicizumab prophylaxis arms. As such, the standard meta- and network meta-analyses were considered not appropriate for these trial data. However, an indirect treatment comparison was still feasible, and it was important to adjust for differences in baseline characteristics between studies. MAIC is a validated method for the comparison of outcomes of interventions, which can overcome methodological limitations of indirect comparisons and network meta-analyses.[24] IPD from studies of one treatment are matched with aggregate data from published studies of another treatment, allowing treatment outcomes to be compared across balanced trial populations; thus, reducing observed cross-trial differences.[20] In the absence of head-to-head studies, we propose MAIC as the most robust method to compare treatments and the most appropriate for the current analysis.

Prophylaxis with FVIII replacement products is the standard of care for the management of hemophilia A; treatment has evolved and is focused on increasing protection by raising factor levels above previous targets. This has the potential to allow a more active lifestyle with improved outcomes, including prevention of bleeding and joint disease progression and thus, potentially a better quality of life.[25] The HAVEN trial program demonstrated the efficacy of emicizumab for the treatment of patients with hemophilia A both with and without inhibitors.[16,17] However, long-term data are not currently available and there are some safety concerns associated with a risk of thrombosis in combination with other procoagulant drugs.[1] Of note, during the HAVEN 4 study, 61% of patients received at least one concomitant dose of FVIII concentrates or bypassing agents and 39% received these treatments prior to activities that may lead to bleeding.[17] This indicates that the ABR with emicizumab prophylaxis alone is likely to be underestimated. A pre-specified sub-group analysis of HAVEN 4 concluded that emicizumab efficacy (6 mg/kg Q4W) was unaffected by FVIII inhibitor status, presence of target joints, or type of previous FVIII or bypassing agent treatment regimen (episodic versus prophylactic).[17] This reinforces the validity of the current analysis, as based on this conclusion, the ABRs in patients treated with emicizumab are not influenced by the presence of inhibitors, although the number of patients with inhibitors in HAVEN 4 is very low (expansion cohort, n=5).

The study has the following limitations. The outcomes assessed in our analysis were restricted to mean ABR and proportion of patients with zero bleeds. It would have been interesting to compare both interventions in terms of additional outcomes, such as FVIII utilisation to prevent bleeds before physical activity; however, relevant information was not provided in the HAVEN trials. In addition, there was a loss of sample size when comparing rFVIIIFc with emicizumab Q2W (ESS=19) and Q4W (ESS=36), which was largely due to the discrepancies in the proportion of patients with previous prophylaxis (73.5% versus 0%) and treatment duration (32.6 weeks versus 26.6 weeks), respectively. Furthermore, the adjustment for other confounding factors, eg weight, geographic region, FVIII genotype, which may also have an influence on the findings reported here, were considered; unfortunately, this was not possible as relevant information was not provided in the HAVEN trials. Safety outcomes reported across the

treatment arms included in this analysis are presented; the mean number of AEs reported per participant was numerically lower for rFVIIIFc than for emicizumab (1.9 versus 3.6–4.1). Emicizumab was also associated with frequent injection site reactions, which were not reported for patients receiving individualized prophylaxis with rFVIIIFc in A-LONG. The proportions of patients reporting arthralgia, headache and upper respiratory tract infection were also numerically lower with rFVIIIFc than for those observed in the respective emicizumab arms. These AEs can potentially increase the burden associated with long-term emicizumab prophylaxis. However, no formal statistical analysis was carried out and this should be considered when interpreting these data. In addition, safety outcomes from the long-term follow-up studies should be considered for the full characterization of safety profiles.

Conclusion

This indirect treatment comparison indicates that rFVIIIFc individualized prophylaxis is more efficacious than emicizumab administered Q4W for the proportion of patients with zero bleeds. Similar efficacy was observed for mean ABR with rFVIIIFc individualized prophylaxis compared with emicizumab administered Q1W, Q2W and Q4W, with trends in favor of rFVIIIFc.

Abbreviation

ABR, annualized bleeding rate; CI, confidence interval; ESS, effective sample size; FVIII, factor VIII; IPD, Individual patient data; IRR, incidence rate ratio; rFVIIIFc, Recombinant factor VIII-Fc fusion protein; MAIC, matching-adjusted indirect comparison; OR, odds ratio; PK, pharmacokinetic; Q1W, once every week; Q2W, every 2 weeks; Q4W, every 4 weeks; SD, standard deviation.

Author Contributions

PW, SA, ZH and JN collected and interpreted the data. RK, FD, LAF, SL, ES, MDT interpreted the data. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

RK reports research funding and honoraria for consulting and lectures from Bayer, Biomarin, Biotest, CSL Behring, Novo Nordisk, Octapharma, Pfizer, Roche, Takeda/Shire and Sobi. PW, SA and FD are employees of Creativ-Ceutical a consultancy company that received funding from Sobi for this research. ZH, JN, LAF, SL and ES are employees of Sobi. MDT reports speaking and consultancy fees from Amgen, HemaBiologics, Pfizer, Principia, Takeda, Grifols, Octapharma and Biomarin; consultancy fees from Novo Nordisk, Genetech and Roche; and trial investigator for Takeda and Spark Therapeutics. He has a private practice that offers in and out-patient consultation services and the CEO and CFO for Bleeding and Clotting Disorders Institute. The authors report no other conflicts of interest in this work.

References

1. Aledort L, Mannucci PM, Schramm W, Tarantino M. Factor VIII replacement is still the standard of care in haemophilia A. *Blood Transfus*. 2019;17(6):479–486. doi:10.2450/2019.0211-19
2. National Organization for Rare Disorders (NORD®). Haemophilia A. Available from: https://rarediseases.org/rare-diseases/hemophilia-a/. Accessed October 2020.
3. Valentino LA. Blood-induced joint disease: the pathophysiology of hemophilic arthropathy. *J Thromb Haemost*. 2010;8(9):1895–1902. doi:10.1111/j.1538-7836.2010.03962.x
4. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535–544. doi:10.1056/NEJMoa067659
5. Gringeri A, Lundin B, von Mackensen S, Mantovani L, Mannucci PM, Group ES. A randomized clinical trial of prophylaxis in children with hemophilia A (the ESPRIT Study). *J Thromb Haemost*. 2011;9(4):700–710. doi:10.1111/j.1538-7836.2011.04214.x
6. Morfini M, Benson G, Jimenez-Yuste V, et al. Tailoring care to haemophilia patients' needs: which specialty and when? *Blood Transfus*. 2015;13(4):644–650. doi:10.2450/2015.0302-14
7. Bioverativ Therapeutics Inc. ELOCTATE® Prescribing Information; 2019. Available from: https://www.fda.gov/media/88746/download. Accessed October 2020.
8. Swedish Orphan Biovitrum AB (publ). ELOCTA® summary of product characteristics; 2019. Available from: https://www.ema.europa.eu/en/documents/product-information/elocta-epar-product-information_en.pdf. Accessed October 2020.
9. Mahlangu J, Powell JS, Ragni MV, et al. Phase 3 study of recombinant factor VIII Fc fusion protein in severe hemophilia A. *Blood*. 2014;123(3):317–325. doi:10.1182/blood-2013-10-529974
10. Young G, Mahlangu J, Kulkarni R, et al. Recombinant factor VIII Fc fusion protein for the prevention and treatment of bleeding in children with severe hemophilia A. *J Thromb Haemost*. 2015;13 (6):967–977. doi:10.1111/jth.12911
11. Nolan B, Mahlangu J, Pabinger I, et al. Recombinant factor VIII Fc fusion protein for the treatment of severe haemophilia A: final results from the ASPIRE extension study. *Haemophilia*. 2020;26 (3):494–502. doi:10.1111/hae.13953
12. Franchini M, Marano G, Pati I, et al. Emicizumab for the treatment of haemophilia A: a narrative review. *Blood Transfus*. 2019;17 (3):223–228. doi:10.2450/2019.0026-19

13. Genetech Inc. Hemlibra® prescribing information; 2018. Available from: https://www.gene.com/download/pdf/hemlibra_prescribing.pdf. Accessed October 2020.

14. Roche Pharma AG Hemlibra® summary of product characteristics; 2019. Available from: https://www.ema.europa.eu/en/documents/product-information/hemlibra-epar-product-information_en.pdf. Accessed October 2020.

15. Oldenburg J, Mahlangu JN, Kim B, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377(9):809–818. doi:10.1056/NEJMoa1703068

16. Mahlangu J, Oldenburg J, Paz-Priel I, et al. Emicizumab prophylaxis in patients who have hemophilia a without inhibitors. *N Engl J Med*. 2018;379(9):811–822. doi:10.1056/NEJMoa1803550

17. Pipe SW, Shima M, Lehle M, et al. Efficacy, safety, and pharmacokinetics of emicizumab prophylaxis given every 4 weeks in people with haemophilia A (HAVEN 4): a multicentre, open-label, non-randomised phase 3 study. *Lancet Haematol*. 2019;6(6):e295–e305. doi:10.1016/S2352-3026(19)30054-7

18. Young G, Liesner R, Chang T, et al. A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors. *Blood*. 2019;134(24):2127–2138. doi:10.1182/blood.2019001869

19. Phillippo DM, Ades AE, Dias S, Palmer S, Abrams KR, Welton NJ. NICE DSU Technical support document 18: methods for population-adjusted indirect comparisons in submission to NICE; 2016. Available from: http://nicedsu.org.uk/wp-content/uploads/2017/05/Population-adjustment-TSD-FINAL.pdf. Accessed October 2020.

20. Signorovitch JE, Sikirica V, Erder MH, et al. Matching-adjusted indirect comparisons: a new tool for timely comparative effectiveness research. *Value Health*. 2012;15(6):940–947. doi:10.1016/j.jval.2012.05.004

21. Higgins JPT, Green S. Cochrane handbook for systematic reviews of interventions version 5.1.0 (updated March 2011). Chochrane; 2011. Available from: www.training.cochrane.org/handbook. Accessed October 2020.

22. Reyes A, Revil C, Niggli M, et al. Efficacy of emicizumab prophylaxis versus factor VIII prophylaxis for treatment of hemophilia A without inhibitors: network meta-analysis and sub-group analyses of the intra-patient comparison of the HAVEN 3 trial. *Curr Med Res Opin*. 2019;35(12):2079–2087. doi:10.1080/03007995.2019.1649378

23. Jain N, Lethagen S, Reyes A RE, et al. Efficacy of emicizumab prophylaxis versus factor VIII prophylaxis for treatment of hemophilia A without inhibitors: network meta-analysis and sub-group analyses of the intra-patient comparison of the HAVEN 3 trial. *Curr Med Res Opin*. 2020;36(7):1125–1127. doi:10.1080/03007995.2020.1744549

24. Batt K, Gao W, Ayyagari R, et al. Matching-adjusted indirect comparisons of annualized bleeding rate and utilization of BAY 94-9027 versus three recombinant factor VIII agents for prophylaxis in patients with severe hemophilia A. *J Blood Med*. 2019;10:147–159. doi:10.2147/JBM.S206806

25. Jimenez-Yuste V, Auerswald G, Benson G, et al. Achieving and maintaining an optimal trough level for prophylaxis in haemophilia: the past, the present and the future. *Blood Transfus*. 2014;12 (3):314–319. doi:10.2450/2014.0298-13

Determinants of Care-Seeking Practices for Children with Sickle Cell Disease in Ekiti, Southwest Nigeria

Oladele Simeon Olatunya, Adefunke Olarinre Babatola, Adewuyi Temidayo Adeniyi, Olubunmi Adeola Lawal, Alaba Olanrewaju Daramola, Tosin Anthony Agbesanwa, Temitope Olumuyiwa Ojo, Paul Oladapo Ajayi, Adeleke Ajayi Ibijola, Akinwumi Kolawole Komolafe & Adekunle Adekile

ORIGINAL RESEARCH

Determinants of Care-Seeking Practices for Children with Sickle Cell Disease in Ekiti, Southwest Nigeria

Oladele Simeon Olatunya [1,2]
Adefunke Olarinre Babatola [1,2]
Adewuyi Temidayo Adeniyi [1,2]
Olubunmi Adeola Lawal [3]
Alaba Olanrewaju Daramola [4]
Tosin Anthony Agbesanwa [5]
Temitope Olumuyiwa Ojo [6]
Paul Oladapo Ajayi [7]
Adeleke Ajayi Ibijola [8]
Akinwumi Kolawole Komolafe [2]
Adekunle Adekile [9]

[1]Department of Paediatrics, College of Medicine, Ekiti State University, Ado Ekiti, Nigeria; [2]Department of Paediatrics, Ekiti State University Teaching Hospital, Ado Ekiti, Nigeria; [3]Department of Paediatrics, Federal Teaching Hospital, Ido Ekiti, Ekiti State, Nigeria; [4]Department of Hematology and Blood Transfusion, Ekiti State University, Ado Ekiti, Ekiti State, Nigeria; [5]Department of Family Medicine, College of Medicine, Ekiti State University, Ado Ekiti, Ekiti State, Nigeria; [6]Department of Community Health, Obafemi Awolowo University Ile-Ife, Ile-Ife, Osun State, Nigeria; [7]Department of Community Medicine, College of Medicine, Ekiti State University, Ado Ekiti, Ekiti State, Nigeria; [8]Department of Hematology, Federal Teaching Hospital, Ido Ekiti/Afe Babaloa University, Ado Ekiti, Ekiti State, Nigeria; [9]Department of Pediatrics, Faculty of Medicine, Kuwait University, Kuwait City, Kuwait

Correspondence: Oladele Simeon Olatunya
Department of Paediatrics, College of Medicine, Ekiti State University, Ado Ekiti, Ekiti State, Nigeria
Tel +2348038617705
Email ladeletunya@yahoo.com

Background: Due to the chronic nature of sickle cell disease (SCD), affected individuals may seek help from diverse places thus raising the need to understand their health-seeking behavior (HSB) in order to design an appropriate management policy for them.

Aim: The aim of this study was to evaluate the HSB among pediatric SCD patients relative to their non-SCD counterparts attending a tertiary facility in Southwest Nigeria and identified predictors of poor HSB among SCD patients.

Methods: A total of 110 children with SCD were recruited and studied for their HSPs which were compared with 110 non-SCD patients with other chronic medical conditions. Questionnaires were used to obtain self-reported information on participants' socio-demographic data and HSB. Logistic regression was used to determine the predictors of poor HSB among the SCD cohort.

Results: More SCD patients received treatments at private hospitals, patent medicine stores and faith-based centers compared to their non-SCD counterparts (p=0.0052; 0.006; and 0.007), respectively. No difference was observed in the patronage of traditional care centres 10 (9.1%) vs 6 (5.5%). More SCD patients 61 (55.5%) vs 35 (31.8%) exhibited poor HSB (p=0.0004). SCD patients who were not enrolled on health insurance scheme were 18 times more likely to have poor HSB (OR=18.38, 95% CI (4.41–76.57), p value= <0.0001) while absence of VOC within the preceding year reduces the risk of poor HSB by 91.5% (OR=0.085, 95% CI (0.028–0.258), p value= <0.0001).

Conclusion: SCD patients in the study locality had poor HSB. This raises the need for their education on proper HSB. More enrollment into health insurance scheme and the prevention of VOC will lessen the burden of poor HSB. The high patronage of non-hospital care facilities in this study raises the need for stakeholders to monitor activities and train the operators at these informal care centres.

Keywords: sickle cell disease, health-seeking behavior, non-orthodox care, Nigeria

Introduction

Sickle cell disease (SCD) is an inherited blood disorder characterized by chronic anemia, acute painful episodes, and chronic organs damage.[1,2] It is associated with a significant reduction in life expectancy especially in resource-poor countries with low quality of health care and weak health systems.[1,2] Individuals homozygous for the sickle hemoglobin (HbS) have sickle cell anemia (SCA), which is the most severe form of the disease spectrum. It is the most common genetic disease in sub-Saharan Africa.[1–3] Globally, about 312,000 neonates are born with SCA annually, 75% of which occur in Africa.[3] Nigeria has the highest burden of the disease where between 1% and 2% of newborns are born annually

with the medical condition.[2] It is also an important cause of childhood morbidity and mortality in the country.[2]

The clinical manifestations of the disease are many and these include serious chronic and debilitating conditions.[3,4,5–7] Due to the genetic nature and complexities of its pathophysiology, the management of SCD is also still evolving and this includes supportive and potentially curative interventions.[4,5,7] However, in developing countries like Nigeria, the management and care available are still largely supportive and suboptimal.[2,8] Hence, individuals affected with SCD may be left to worry about how to get help for their medical condition.

Health-seeking behavior (HSB) is defined as an action undertaken by individuals who perceive themselves as having a health problem or to be ill, for the purpose of finding an appropriate remedy or simply put, the varied response of individuals to states of ill-health, depending on their knowledge and perceptions of health, socioeconomic constraints, adequacy of available health services and attitude of healthcare providers.[9,10]

Being sick or having ill health is a major life event that may disrupt basic activities of life[10] and what people do when they are ill has major implications for morbidity, progression and outcome of the illness.[10] These include places where people seek health care and the kind of treatments sought for some of which may be inimical and harmful. Furthermore, it has been noted that people take decisions based on their respective socio-cultural, economic, demographic circumstances, disease pattern, perceived severity of the illness, attitude of health provider and organization of the health-care system.[10–13]

Studies have reported the involvement of non-qualified and alternative caregivers in the management of chronic diseases especially in rural areas and resource-poor countries.[14–18] Similar patterns have been observed in the care of patients with SCD.[17,19–21] These observations reflect that many people in Nigeria and other developing countries have strong beliefs in religious institutions and non-orthodox care centres for healing and healthcare services as people in these communities sometimes refer to children suffering from chronic medical conditions like SCD as being afflicted by spirits/devils because of the nature and clinical presentation of these diseases and as such, believe that these children need spiritual and other non-orthodox care.[18,20] They may, therefore, seek miracles, and healing at some faith-based centers and other non-orthodox facilities. These findings raise the need to understand the health-seeking behavior of any target population before one can design effective and acceptable health-care services for the care of such population or group of patients.

This study examined the healthcare-seeking behavior and treatment practices of children with SCD in comparison with children with other chronic medical conditions that are not SCD attending a tertiary hospital in Southwestern Nigeria. Information from this study may assist policy makers in formulating rational policies to provide efficient, effective, acceptable, affordable and accessible healthcare services targeted at improving the health of the pediatrics sickle cell population.

Patients and Methods
Study Design and Setting
This study was conducted at the pediatric hematology unit and children out-patient clinics of the Ekiti State University Teaching Hospital (EKSUTH), Ado Ekiti which is a tertiary government-owned hospital in Nigeria between March and August 2018. EKSUTH is a tertiary referral hospital for all the Primary and Secondary Health facilities in Ekiti State and neighboring towns in other adjoining States. Children with SCD alongside their counterparts with other chronic medical disease conditions/illnesses were purposively recruited serially over a 6-month period. At the EKSUTH, the pediatric hematology unit is co-joined with the pediatric oncology unit and attends to all hemato-oncological-related illnesses in which pediatric SCD cases predominate while children with other illnesses are seen in the outpatients' and other specialist clinics. The pediatric hemato-oncology unit of the hospital is headed by a pediatrician (the lead researcher) and supported by the complements of resident doctors, nurses, other medical staffs and specialists. It runs a weekly specialist clinic where predominantly SCD patients and a few other pediatric hemato-oncology cases are referred and seen. The unit also admits and treats SCD patients presenting with complications/emergencies like bone pain crisis, severe anemia, infections, stroke, etc. The pediatric hematology unit offers routine malaria prophylaxis and multivitamins supplementation. It also offers hydroxyurea therapy and chronic blood transfusion to eligible SCD patients. However, compliances with the latter two protocols are poor. There is no routine penicillin prophylaxis or transcranial doppler ultrasound screening as these are done on a case-by-case basis and only when indicated.

Sampling Method

Purposive sampling was used to select the participants and the minimum sample size was arrived at for either group using the formula for calculating sample size for comparison of proportions n = (Zα/2+Zβ)2 * (p1(1-p1)+p2 (1-p2))/(p1-p2)2, where n = minimum sample size per group.[22] Given the 56.6% prevalence (P1) of use of non-orthodox care for children with epilepsy in Ibadan, Nigeria[23] and 36% prevalence (P2) of use of non-orthodox care for children SCD in Lagos, Nigeria[24] and β = 0.20, at 95% confidence level $Z_{α/2}$ is 1.96 for a two-tailed test, the estimated sample size was 88 per group. To compensate for non-response, assuming a 10% non-response, the sample size per group was further calculated using the following formula:

$$N_{nr} = n/1 - n_{nr}$$

n = calculated sample size for each group =88

n_{nr} = non-response rate =10%=0.1

N_{nr} = sample size per comparison group, compensated for non-response rate.[25]

$$N_{nr} = n/1 - 0.1$$

N_{nr} = n/0.9. = 88/0.9 = 97. Hence, the minimum sample size required per group was 97. However, 110 participants were studied per group.

Study Population

All consecutive SCD patients who attended the pediatric hematology SCD clinic and whose parents gave consent to participate in the study were recruited. The controls were non-SCD children whose parents gave consent to participate in the study and attended the children hemato-oncology unit, out-patient clinics and other specialist clinics of the hospital within the same period for follow up of their wards chronic illnesses (diabetes-3, asthma-25, hepatitis-2, chronic kidney diseases-5, tuberculosis-5, active HIV disease-5, recurrent seizures 51 (non-febrile -31, and febrile-20), Down syndrome-6, and childhood cancer-8). The Hb genotypes of the participants in both arms were determined by Hb electrophoresis.

However, the SCD patients also had earlier had their SCD status further confirmed by high-performance liquid chromatography (HPLC), Biorad, USA Variant II, using the Beta thalassemia short program and DNA Polymerase Chain Reaction (PCR).[26]

Study Procedure and Data Collection

The purpose of the study was explained to the children and their parents/caregivers after which the consents of the caregivers were obtained as appropriate. Also, patients' assents were obtained for participants aged 7 years and above as applicable. Information on each participant's health-seeking behavior was obtained with a pre-tested questionnaire. The study instrument was revised by community physicians after the pretest at the medical outpatients' clinic of the hospital to improve its quality and incorporate observations made during the preliminary study. The questionnaire was translated into Yoruba language which is the main spoken language in the locality by an expert and was administered with the assistant of resident doctors who were fluent in both the English language and the indigenous language. Information obtained through the questionnaire included the participants' socio-demographic characteristics, occupation of the parents, parents' highest level of formal education, history of previous treatments/interventions (both hospital and non-hospital based), where such treatments were given and this was used to ascertain their health-seeking behavior in the preceding 1 year prior to recruitment. For the purpose of this study, health-seeking behavior was defined as actions undertaken by caregivers/ parents for the purpose of finding a remedy to their wards' ill health or health problems.[9,10] The type of place/setting, ie whether health facility (private or public hospitals) which was considered as standard orthodox care or other non-standard or non-orthodox care centres where parents/ caregivers sought treatment(s) for their wards were noted. The health-seeking behavior was considered good when treatment was received at a hospital (private or public) and poor when treatment was received at non-orthodox, ie (non-hospital setting). In addition, the medical records of the SCD patients were retrieved manually and examined to establish the clinical evolution of the patients' disease condition vis a vis the occurrence of acute and chronic lifetime disease complications for which admissions were offered, the number of admissions, as well as other details of the treatment they received at the pediatric hematology unit within the preceding year prior recruitment. The clinical evolution record and typing of disease evolution/complications such as the occurrence of vaso-occlusive crisis (VOC), ie number of severe bone pain crises that disrupt daily activities, required hospital admission and use of opioids analgesics within the preceding 1 year prior to

recruitment, as well as other details of their disease evolution/complications were as earlier described.[26] The social classes of the parents were determined according to the Oyedeji classification system using their formal educational attainment and occupation as previously described.[27]

Ethical Consideration

This study was performed according to the Declaration of Helsinki on research involving human subjects. Ethical approval was obtained from the Ethics and Research Committee of the EKSUTH and written informed consent was obtained from the guardians/parents of the children (patients and controls) as well as patients' assents where applicable and they were assured of the confidentiality of their responses. Willingness to participate or not did not affect the care given to participants as all received the same standard care. Data were stored on a computer after personal identifiers had been removed and were only accessible to the research team with a password.

Data Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 25.0. Frequencies and proportions were used to summarize categorical variables. Quantitative variables were summarized using mean and standard deviation. Chi-square test and Fisher's Exact Test were used to compare health-seeking behavior between the SCD patients and their non-SCD counterparts. Furthermore, binary logistic regression was fitted to identify the determinants of poor health-seeking behavior among the SCD cohort. The determinants/predictors were selected based on the literature.[17,24,28] Both the Omnibus Tests of Model Coefficients and the Hosmer-Lemeshow goodness-of-fit test were used to assess the fitness of the final model and both tests indicate the model was well fitted with the Omnibus Tests of Model Coefficients being (χ2=35.3, p<0.0001) and the Hosmer-Lemeshow goodness-of-fit test at (χ2=5.74, p=0.57) respectively. The significance level was set at p-value < 0.05 for all tests.

Results

The study involved 110 SCD patients (107 hemoglobin SS and 3 hemoglobin SC) and 110 non-SCD (84 Hb AA and 26 Hb AS) patients age- and sex-matched controls. The mean ages of the SCD patients and their non-SCD counterparts were 8.39 ± 4.49 and 8.85 ± 3.22 years, respectively.

Sociodemographic Characteristics of the Study Participants

There is no difference between SCD patients and controls with regards to socioeconomic class (Table 1).

Pattern of Past Treatments/Care-Seeking Practices

More SCD patients compared to their non-SCD counterparts 66 (60.0%) vs 35 (31.8%) (p<0.0001) received treatments at some other places in addition to treatments given at our tertiary facility. Also, more SCD patients received care at private hospitals, patent medicine stores and faith-based centers than the controls (p<0.05) Table 2. In addition, more SCD patients compared to their non-SCD counterparts had received blood transfusion (p=0.0002). Although more SCD patients tended to patronize traditional centres and received diverse treatments 10 (9.1%) vs 6 (5.5%); p=0.43, both groups tended to have an equal rate of therapeutic scarifications at non-orthodox traditional places 8/10 (80%) vs 5/6 (83.3%). Nevertheless, the majority of the 13 participants 8/13 (62%) who had scarifications, were SCD patients. There were no differences with respect to other non-orthodox interventions received by the participants (Table 2).

In general, more SCD patients compared to their non-SCD counterparts 61 (55.5%) vs 35 (31.8%) (p=0.0004) exhibited poor health-seeking behavior.

Admission Pattern and Rate of the SCD Cohort

A total of 72 (65%) SCD patients were admitted for various comorbidities and complications of their disease in the preceding 1 year. The number of admissions ranged between 1

Table 1 Sociodemographic Characteristics of the Study Participants

Characteristics		SCD Patient N=110 n (%)	Control N=110 n (%)	P value
Gender	Male	71(64.5)	70(63.6)	0.88*
	Female	39(35.5)	40(36.4)	
Social class	Low	64(58.2)	63(57.3)	0.94*
	Middle	42(38.2)	42(38.2)	
	Upper	4(3.6)	5(4.5)	

Note: Test statistics = *Chi-Square test.
Abbreviation: SCD, sickle cell disease.

Table 2 Pattern of Past Treatments and Care-Seeking Practices of Participants

Characteristics	SCD Patients N=110 n (%)	Controls N=110 n (%)	P value
General pattern of how treatment was received			
Our Hospital: Yes	110.0 (100.0)	110.0 (100.0)	
Other places alongside our hospital: Yes	66 (60.0)	35 (31.8)	**<0.0001***
No	44 (40.0)	75 (68.2)	
Sites where treatment was received outside our hospital			
ªPrivate hospital: Yes	32 (29.1)	15 (13.7)	**0.0052***
No	78 (70.9)	95 (86.3)	
ªPatent Medicine shop: Yes	55 (50.0)	35 (31.8)	**0.006***
No	55 (50.0)	75 (68.2)	
ªTraditional care: Yes	10 (9.1)	6 (5.5)	0.437†
No	100 (90.9)	104 (94.5)	
ªFaith-based centers: Yes	27 (24.5)	11 (9.0)	**0.007†**
No	83 (75.5)	99 (90.0)	
Type of treatment/care received aside drugs (orthodox and non-orthodox) within past 1year			
Blood transfusions: Yes	51 (46.4)	25 (22.7)	**0.0002***
No	59 (53.6)	85 (77.3)	
Surgical operations: Yes	1 (0.9)	2 (1.8)	1.000†
No	109 (99.1)	108 (98.2)	
Prayer at faith based (religious) centres: Yes	27 (24.5)	25 (22.7)	0.751*
No	83 (75.5)	85 (77.3)	
Use of biologic products (herbal medicines at traditional healing center): Yes	10 (9.1)	6 (5.5)	0.437†
No	100(90.9)	104(94.5)	
Scarifications (at traditional healing center): Yes	8(7.3)	5(4.5)	0.284†
No	102(92.7)	105(95.5)	

Notes: Test statistics = †Fisher's exact test, *Chi-Square test. ªMany individual patients received treatments at multiple places, P values in bold fonts indicate statistical significance.
Abbreviation: SCD, sickle cell disease.

and 4 with a median of 2 (mean 1.8±0.8). The commonest reason for admission was for VOC (severe bone pains) and this was among 51 (70.8%) of the admitted group. Other comorbidities/complications for which the SCD cohort members were admitted are as shown in Table 3.

Association Between SCD Cohort Characteristics and Their Health-Seeking Behavior

As shown in Table 4, lack of health insurance (p<0.0001), belonging to low social class (p=0.012) and presence of VOC (p<0.0001) in the preceding year were all associated with poor health-seeking behavior on Chi-Square analysis.

Determinants/Predictors of Poor Health-Seeking Behavior Among SCD Patients

As shown in Table 5, SCD patients who were not enrolled in a health insurance scheme were 18 times more likely to have poor health-seeking behavior (OR=18.38, 95% CI (4.41–76.57), p value= <0.0001). Also, the absence of VOC within the preceding year reduces the risk of poor health-seeking behavior among SCD patients by 91.5% (OR=0.085, 95% CI (0.028–0.258), p value= <0.0001). No other determinants/predictors of poor HSB were identified (Table 5).

Table 3 Associated Admissions' Comorbidities/Complications Among the SCD Patients

Comorbidities	Number of Patients Admitted (N=72) n(%)
VOC	51(70.8)
Malaria	33 (45.8)
Severe anemia	33 (45.8)
Stroke	10 (13.9)
Sepsis	5 (6.9)
Osteomyelitis	5 (6.9)
Priapism	3 (4.2)
Infected Leg ulcer	2 (2.8)
Pneumonia	2 (2.8)

Note: Some patients had multiple comorbidities/complications.
Abbreviation: VOC, vaso-occlusive crisis.

Table 4 Relationship Between Sociodemographic, Clinical Attributes and Types of Health-Seeking Behavior Among SCD Patients

Variables	Good Health-Seeking Behavior N=49 n(%)	Poor Health-Seeking Behavior N=61 n(%)	P value
Sex Male (N=71)	30 (61.2)	41 (67.2)	0.376*
Female (N=39)	19 (38.8)	20 (32.8)	
Social class			**0.012***
Upper (N=4)	3 (6.2)	1 (1.6)	
Middle (N=42)	23 (46.9)	19 (31.2)	
Lower (N=64)	23 (46.9)	41 (67.2)	
NHIS insured			**<0.0001†**
Yes (N=21)	16 (32.6)	5 (8.2)	
No (N=89)	33 (67.4)	56 (91.8)	
Admitted within past 1 year			0.53*
Yes (N=72)	31 (63.3)	41 (67.2)	
No (N=38)	18 (36.7)	20 (32.8)	
VOC within past 1 year			**<0.0001***
Yes (N=51)	16 (32.6)	35 (57.4)	
No (N=59)	33 (67.4)	26 (42.6)	

Notes: Test statistics †Fisher's exact test, *Chi-Square test. P values in bold fonts indicate statistical significance.
Abbreviations: VOC, vaso-occlusive crisis; NHIS, National Health Insurance Scheme; SCD, sickle cell disease.

Discussion

The cause and nature of a disease condition as well as local culture, accessibility of affordable orthodox health care and experiences/pieces of advice from locals who have suffered similar disease are factors that tend to influence the health-seeking behavior of the affected individual. In this study, both the SCD and non-SCD patients received treatment outside orthodox care. However, SCD patients were more affected when compared to their non-SCD counterparts. This may be due to the nature of SCD being more frequently associated with recurrent complications. Also, the fact that SCD standard-of-care management is relatively expensive and borne out-of-pocket in our environment may be a factor.[29,30]

Table 5 Binary Logistic Regression for Predictors/Determinants of Poor Health-Seeking Behavior Among SCD Patients

Predictor Variables	Odds Ratio	95% CI	P value
Enrolled on NHIS			
NO	18.384	4.414 −76.571	**<0.0001**
YES			
Social Class			
Lower	1.488	0.601–3.683	0.39
Middle and upper			
VOC within past 1 year			
NO	0.085	0.028–0.258	**<0.0001**
YES			
Sex			
Male	0.720	0.289–1.793	0.480
Female			

Note: P values in bold fonts indicate statistical significance.
Abbreviations: CI, confidence interval; NHIS, National Health Insurance Scheme; SCD, sickle cell disease; VOC, vaso-occlusive crisis.

Patent medicine shops ranked highest among the places where our study participants sought treatment, accounting for 50% amongst the SCD patients and 32% amongst the non-SCD patients. The participation of patent medicine vendors (PMV) in healthcare delivery especially in low middle-income countries such as Nigeria is well documented.[31–34] The implications of this for the care of SCD patients may include delay before presentation at the hospital as the time taken to consult the PMVs and waiting time to see the effect of care provided by the PMVs add up to time taken before presentation at the hospital. In addition, some might have received interventions that could worsen their clinical condition as many of the operators of these PMVs have limited knowledge on the care for SCD patients. It might also add up to the economic burden on the family as some of them who had visited the PMVs might need to still visit the hospitals for the same condition, thus increasing the cost of healthcare with the possibility of tilting them to catastrophic health expenditure.[29,30]

Looking at it from another perspective, the high patronage of PMVs suggests that there is a need to educate and train the operators of the PMVs appropriately on knowing their limitations. Also, the government needs to enforce regulations guiding their operation.

Our finding of more SCD patients patronizing faith-based centers is supported by finding by previous authors who reported that prayers, herbal medicine and spiritual sources and other mind-body therapies were commonly used healthcare services employed by caregivers of SCD patients and other chronic diseases across Nigeria[10,18,24,28,35] and other parts of Africa.[17,20,36,37] These findings may be because of the chronic nature of SCD and the need for lifelong treatments/repeated visits to the hospital for follow-up hence, the desire for the cure of the disease condition. They may, therefore, seek miracles, at some faith-based centers and other non-orthodox facilities. Furthermore, it may also reflect that many people in Nigeria and some African countries have strong beliefs in religious institutions for healing and healthcare services as previously reported.[17,24,28,35] This, therefore, suggests the need for community awareness on appropriate care for common clinical manifestations of SCD as some of the people in the community often term children suffering from SCD as afflicted by spirits/devils because of the nature and clinical presentation of SCD and as such, believe that these children need spiritual and other non-orthodox care. Religious leaders may therefore have a role to play in counseling SCD patients with regards to effective care as earlier suggested given their possible prime roles in SCD control in Nigeria.[38]

The observation that more SCD patients received blood transfusion is not surprising given the chronic hemolytic nature of SCD and its commonly associated crises, particularly pain and anemic crises, which often involve the use of several therapeutic approaches including blood transfusion in their management.[1–5,39,40]

Although both groups tended to have an equal rate of therapeutic scarifications at non-orthodox traditional places (80%) vs (83.3%). Nevertheless, the majority of the 13 participants 8/13 (62%) who had scarifications, were SCD patients. The higher preponderance of body scarifications among the SCD patients is not surprising, given that the practice is used by traditional healers to introduce herbs and other substances into different parts of the patient's body through incisions, thus producing scars after healing. These scars are found usually over the limbs and abdomen, which are the common sites of SCD-related pain crisis. Despite the wide acceptability of complementary therapies in the SSA,[14] some of the practices at these alternative care centres are inimical to health and pose an increased risk of contracting other serious diseases such as human immunodeficiency virus, hepatitis

and tetanus that could be transmitted by the use of contaminated instruments.[41,42] This further suggests the need for more collaboration between the formal sector (orthodox care) and the informal healthcare providers so as to educate the latter group and discourage them from harmful practices to SCD patients and their any other clients. The recent recognition being accorded traditional and complementary medicine in Nigeria is a welcome development and steps taken in the right direction[43] and should therefore be given all the needed supports.

That the lack of a health insurance predicted poor HSB among the SCD patients is not surprising given the observation by previous authors that the high rate of affordability, ease of access, perceived good healthcare and good response to treatment are key reasons while Nigerians patronize non-orthodox health care.[17,28,44] These are benefits that clients enrolled in the health insurance scheme are expected to enjoy. Also, most healthcare cost for SCD in Nigeria is usually borne out-of-pocket with the tendency towards financial catastrophe which previous studies have hinted that could push caregivers of SCD patients who are not protected by health insurance to poverty.[29,30] Therefore, the SCD patients in this study, might have taken advantage of these factors to patronize non-orthodox health care and practiced poor HSB. These observations as found may be a reflection of the roles of poverty in the overall outlook of SCD in Nigeria.[45]

Our findings raise the need for universal health coverage for SCD patients as previously canvased.[29,30] Doing so will further alleviate the burden of care for the disease and help fulfill the call for more efforts and actions to scale up SCD care in the study location and other parts of the world where the condition is quite prevalent.[46]

Nevertheless, our observation of the association of VOC with poor HSB in this study contrasts with findings by Busari et al[28] who observed that VOC did not discriminate regarding whether or not their study participants patronized non-orthodox care in Lagos, Nigeria. Possible explanations for this disparity could stem from the fact that, while their study involved a mixed population of both adults and pediatric age group, ours was purely among the latter group with the likelihood that caregivers of children with SCD in this study could not probably able to bear the repeated sights of seeing their young wards suffering repeated bouts of severe VOC without taking actions to ameliorate their sufferings and such actions could involve seeking help from diverse places. Hence, the possibility of seeking helps from complementary care centres in order to

find succor for their wards. More so, VOC was the most common reason for which our study participants were hospitalized.

Limitations

This study is limited by its being both hospital and questionnaire based, with the possibility of recall bias, not totally reflecting the entire community practice. Also, some respondents might not have volunteered the whole information more so, because of the face-to-face contact with the research assistants which could put subtle pressure on the caregivers (respondents). Despite these limitations, the study is the first from the study location and it established that the health-seeking behavior for children with SCD was suboptimal and proposed several possibilities for the findings as well as suggesting some approaches towards addressing the situation.

Conclusion

This study revealed that the majority of SCD patients in our study locality exhibited poor HSB by seeking care from non-hospital and unorthodox sources including patent medicine shops, faith-based centers and traditional healers. Some practices at these places included the use of scarifications and other non-orthodox care methods which have huge implications for grave health consequences. Guardians of SCD patients need to be made aware of the risks associated with poor HSB so as to discourage such a practice. More enrollment into health insurance scheme and the prevention of VOC will lessen the burden of poor HSB among SCD patients. Finally, there is a need to recognize other non-orthodox care facilities in the study locality and possibly, most parts of the SSA in order to allow for policy makers and stakeholders to train and monitor their activities for effective healthcare delivery.

Abbreviations

SCD, sickle cell disease; HSB, health-seeking behavior; VOC, vaso-occlusive crisis; PMV, patent medicine vendor.

Ethical Approval

This study was performed according to the Declaration of Helsinki on research involving human subjects. Ethical approval was obtained from the Ethics and Research Committee of the EKSUTH and written informed consent was obtained from the guardians/parents of the children (patients and controls) as well as patients' assents where applicable and they were assured of the confidentiality of their responses. Data were stored on a computer after personal identifiers had been removed and were only accessible to the research team with a password.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest with respect to this study.

References

1. Charkravorty S, Williams TN. Sickle cell disease: a neglected chronic disease of increasing global health importance. *Arch Dis Child*. 2015;100:48–53. doi:10.1136/archdischild-2013-303773
2. Galadanci N, Wudil BJ, Balogun TM, et al. Current sickle cell disease management practices in Nigeria. *Int Health*. 2014;6:23–28. doi:10.1093/inthealth/iht022
3. Piel FB, Patil AP, Howes RE, et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. *Lancet*. 2013;381:142–151. doi:10.1016/S0140-6736(12)61229-X
4. Adekile AD. What's new in the pathophysiology of sickle cell disease. *Med Princ Pract*. 2013;22:311–312.
5. Piel FB, Steinberg MH, Rees DC. Sickle cell disease. *N Engl J Med*. 2017;376:1561–1573. doi:10.1056/NEJMra1510865
6. Piccin A, Fleming P, Eakins E, McGovern E, Smith OP, McMahon C. Sickle cell disease and dental treatment. *J Ir Dent Assoc*. 2008;54:75–79.
7. Piccin A, Murphy C, Eakins E, et al. Insight into the complex pathophysiology of sickle cell anaemia and possible treatment. *Eur J Haematol*. 2019;102:319–330. doi:10.1111/ejh.13212
8. Diaku-Akinwumi IN, Abubakar SB, Adegoke SA, et al. Blood transfusion services for patients with sickle cell disease in Nigeria. *Int Health*. 2016;8(5):330–335. doi:10.1093/inthealth/ihw014
9. Wade DT, Halligan PW. Do biomedical models of illness make for good health care system. *BMJ*. 2004;329:1398–1401. doi:10.1136/bmj.329.7479.1398
10. Afolabi MO, Daropale VO, Irinoye AI, Adegoke AA. Health seeking behavior and student perception of health care services in a university community in Nigeria. *Health*. 2013;5:817–824. doi:10.4236/health.2013.55108

11. Siddiqui MS, Siddiqui MK, Sohage AA. Health seeking behavior of the people; knowledge, attitudes and practices(KAP) study of the people of urban slum Areas of Karachi. *Prof Med J*. 2011;18:626–631.

12. Oberoi S, Chaudhary N, Patnack S, Singh A. Understanding health seeking behavior. *J Family Med Prim Care*. 2016;5:463–464. doi:10.4103/2249-4863.192376

13. Shaikh BT, Hatcher J. Health seeking behavior & health service utilization in Pakistan: challenging the policy makers. *J Public Health (Bangkok)*. 2004;27:49–54. doi:10.1093/pubmed/fdh207

14. Zhang T, Liu X, Bromley H, Tang S. Perceptions of tuberculosis and health seeking behavior in rural Inner Mongolia, China. *Health Policy*. 2007;81:155–165. doi:10.1016/j.healthpol.2005.12.009

15. Munthali A, Braathen SH, Grut L, Kamaleri Y, Ingstad B. Seeking care for epilepsy and its impact on households in a rural district in southern Malawi. *Af J Disabil*. 2013;2:54. doi:10.4102/ajod.v2i1.54

16. EhsanulHuq KATM, Moriyama M, Zaman K, et al. Health seeking bahaviour and delayed management of tuberculosis patients in rural Bangladesh. *BMC Infect Dis*. 2018;18:515. doi:10.1186/s12879-018-3430-0

17. James PB, Wardle J, Steel A, Adam J. Traditional, complementary and alternate medicine use in sub-Saharan Africa: a systematic review. *BMJ Global Health*. 2018;3:e000895. doi:10.1136/bmjgh-2018-000895

18. Ojinnaka CN, Aronu AE, Ojinnaka GC, Uwaezuoke NA, Bisi-Onyemaechi AJ. Health seeking behavior of care-givers of children with epilepsy in a resource poor country. *Acta Sci Neurol*. 2019;2.

19. Famuyiwa OO, Aina OF. Mother's knowledge of sickle cell anaemia in Nigeria. *Int Q Community Health Educ*. 2010;30:69–80. doi:10.2190/IQ.30.1.f

20. Sinkala D, Fleming LC, Silwimba F, Jacobsen KH. Health services access for young children with sickle cell anaemia in the chilubi District of Zambia. *Med J Zambia*. 2018;45:133–137.

21. Olatunya OS, Albuquerque DM, Adekile AD, Costa FF. Evaluation of sociodemographic, clinical, and laboratory markers of sickle leg ulcers among young nigerians at a Tertiary Health Institution. *Niger J Clin Pract*. 2018;21:882–887. doi:10.4103/njcp.njcp_4_18

22. Hajian-Tilaki K. Sample size estimation in epidemiologic studies. *Caspian J Intern Med*. 2011;2(4):289–298.

23. Lagunju IA. Complementary and alternative medicines use in children with epilepsy in Ibadan, Nigeria. *Afr J Med Med Sci*. 2013;42:15–23.

24. Oshikoya Oshikoya KA, Senbanjo IO, Njokanma OF, Soipe A. Use of complementary and alternative medicines for children with chronic health conditions in Lagos, Nigeria. *BMC Complement Altern Med*. 2008;8:66. doi:10.1186/1472-6882-8-66

25. Kirkwood BR, Sterne JAC. *Calculating the Required Sample Size. Essential Medical Statistics*. 2nd ed. Massachusetts: Blackwell science; 2003:413–428.

26. Olatunya OS, Albuquerque DM, Santos MNN, Kayode TS, Adekile A, Costa FF. Haptoglobin gene polymorphism in patients with sickle cell anemia: findings from a Nigerian Cohort Study. *Appl Clin Genet*. 2020;13:107–114. doi:10.2147/TACG.S246607

27. Oyedeji GA. Socio-economic and cultural background of hospitalised children in Ilesha. *Niger J Paediatr*. 1985;12(4):111–117.

28. Busari AA, Mufutau MA. High prevalence of complementary and alternative medicine use among patients with sickle cell disease in a tertiary hospital in Lagos, South West, Nigeria. *BMC Complement Altern Med*. 2017;17:299. doi:10.1186/s12906-017-1812-2

29. Brown BJ, Okereke JO, Lagunju IA, Orimadegun AE, Ohaeri JU, Akinyinka OO. Burden of health-care on carers of children with sickle cell disease in Nigeria. *Health Soc Care Community*. 2010;18 (3):289–295. doi:10.1111/j.1365-2524.2009.00903.x

30. Olatunya OS, Ogundare EO, Fadare JO, et al. The financial burden of sickle cell disease on households in Ekiti, Southwest Nigeria. *Clinicoecon Outcomes Res*. 2015;7:545–553. doi:10.2147/CEOR.S86599

31. Prach LM, Treleaven E, Isiguzo C, Liu J. Care seeking at patent and proprietary medicine vendors in Nigeria. *BMC Health Serv Res*. 2015;15:231. doi:10.1186/s12913-015-0895-z

32. Liu J, Prach LM, Treleaven E, et al. The role of drug vendors in improving basic health care services in Nigeria. *Bull World Health Organ*. 2016;94:267–275. doi:10.2471/BLT.15.154666

33. Onwujekwe O, Chukwuogo O, Ezeoke U, Uzochukwu B, Eze S. Asking people directly about preferred health seeking behavior yields invalid response: an experiment in south east Nigeria. *J Public Health (Bangkok)*. 2011;33:93–100. doi:10.1093/pubmed/fdq065

34. Chirdan OO, Zoakah AI, Ejembi CL. Impact of health education on home treatment and prevention of malaria in Jengre, north central Nigeria. *Ann Afr Med*. 2008;7:112–119. doi:10.4103/1596-3519.55667

35. Gideon OO, Chioma AP. Indigenous complimentary health seeking behavior among caregivers of sickle cell disorder in Nigeria. *J Genet Disor Genet Rep*. 2015;4:2. doi:10.4172/2327-5790.1000126

36. Nnko S, Bukenya D, Kavishe BB, et al. Chronic diseases in North-west Tanzania and Southern Uganda: public perceptions of terminologies, aetiologies, symptoms and preferred management. *PLoS One*. 2015;10(11):e0142194. doi:10.1371/journal.pone.0142194

37. Tusuubira SK, Nakayinga R, Mwambi B, Odda J, Kiconco S, Komuhangi A. Knowledge, perception and practices towards sickle cell disease: a community survey among adults in Lubaga division, Kampala, Uganda. *BMC Public Health*. 2018;18:561. doi:10.1186/s12889-018-5496-4

38. Olatunya OS, Babatola AO, Ogundare EO, et al. Perceptions and practice of early diagnosis of sickle cell disease by parents and physicians in a southwestern state of Nigeria. *World Sci J*. 2020;2020:4801087. doi:10.1155/2020/4801087

39. Diwe KC, Iwu AC, Uwakwe K, et al. prevalence and pattern of sickle cell disease among children attending tertiary and non tertiary health care institutions in a south Eastern state, Nigeria: a 10 year survey. *J Res Med Dent Sci*. 2016;4(3):75–81. doi:10.5455/jrmds.2016432

40. Saganuwan AS. The pattern of sickle cell disease patients from Northwestern Nigeria. *Clin Med Insights Ther*. 2016;8:53–57. doi:10.4137/CMT.S38164

41. Garve R, Garve M, Turp JC, Fobil JN, Meyer CG. Scarification in sub-Saharan Africa: social skin, remedy and medical import. *Trop Med Int Health*. 2017;22(6):708–715. doi:10.1111/tmi.12878

42. Hrdy DB. Cultural practices contributing to the transmission of human immunodeficiency virus in Africa. *Rev Infect Dis*. 1987;9 (6):1109–1119. doi:10.1093/clinids/9.6.1109

43. Lawal S Nigeria government takes steps to promote alternative medicine. 2019. Available from: https://allafrica.com/stories/201912060178.html. Accessed November 26, 2020.

44. Aina O, Gautam L, Simkhada P, Hall S. Prevalence, determinants and knowledge about herbal medicine and non-hospital utilisation in southwest Nigeria: a cross-sectional study. *BMJ Open*. 2020;10: e040769. doi:10.1136/bmjopen-2020-040769

45. Olatunya OS. Poverty and severity of childhood sickle cell disease. *Br J Haematol*. 2020;190(6):819–821. doi:10.1111/bjh.16816

46. Ware RE. Is sickle cell anemia a neglected tropical disease? *PLoS Negl Trop Dis*. 2013;7(5):e2120. doi:10.1371/journal.pntd.0002120

The Hemarthrosis-Simulating Knee Model: A Useful Tool for Individualized Education in Patients with Hemophilia (GEFACET Study)

Sophie le Doré, Nathalie Grinda, Emmanuelle Ferré, Valerie Roussel-Robert, Birgit Frotscher, Pierre Chamouni, Sandrine Meunier, Sophie Bayart, Edita Dolimier, Francoise Truong-Berthoz & Emmanuelle de Raucourt

ⓐ Open Access Full Text Article

ORIGINAL RESEARCH

The Hemarthrosis-Simulating Knee Model: A Useful Tool for Individualized Education in Patients with Hemophilia (GEFACET Study)

Sophie le Doré[1]
Nathalie Grinda[2]
Emmanuelle Ferré[1]
Valerie Roussel-Robert[3]
Birgit Frotscher[4]
Pierre Chamouni[5]
Sandrine Meunier [ID][6]
Sophie Bayart[7]
Edita Dolimier[8]
Francoise Truong-Berthoz[9]
Emmanuelle de Raucourt[1]

[1]Haemophilia Treatment Centre, Hôpital Mignot, Versailles, France; [2]Regional Hemophilia Centre, Hôpital de Bicêtre (AP-HP), Kremlin-Bicêtre, France; [3]Regional Hemophilia Treatment, Hôpital Cochin (AP-HP) Centre, Paris, France; [4]Haemophilia Treatment Centre, Hôpital de Brabois, Vandoeuvre-Les-Nancy, France; [5]Regional Hemophilia Treatment Centre, Centre Hospitalier Universitaire de Rouen, Rouen, France; [6]Hospices Civils de Lyon – Unité d'Hémostase Clinique, Hôpital Cardiologique Louis Pradel, Bron, France; [7]Haemophilia Treatment Centre, Hôpital Pontchaillou, Centre Hospitalier Universitaire de Rennes, Rennes, France; [8]Baxalta France SAS, a Takeda Company, Paris, France; [9]Baxalta GmbH, a Takeda Company, Zurich, Switzerland

Correspondence: Emmanuelle de Raucourt
Service d'Hématologie Biologique, Clinique des Anticoagulants (CAC), Unité Inserm U 1148, Hôpital Beaujon, Clichy, France
Tel +33 01 40 87 55 41
Fax +33 01 40 87 56 83
Email emmanuelle.de-raucourt@aphp.fr

Background: Hemophilic arthropathy is a major complication in patients with severe hemophilia. A plastic knee model has been developed for the therapeutic education of patients to promote improved care management and self-treatment skills. The objective of this study was to evaluate the impact of this hemarthrosis-simulating artificial knee (HSAK) on patients' knowledge of their disease and its treatment.

Methods: In this observational study, the impact of HSAK was assessed during individualized education in patients with severe/moderately severe hemophilia A or B at seven hemophilia treatment centers in France. Participants provided written informed consent and completed questionnaires to assess knowledge of their disease (score range: 0–7) and knowledge of their treatment (score range: 0–4). Questionnaires were completed before, immediately after and 6 months after HSAK use. The scores obtained before and after the use of the HSAK were compared.

Results: The participants comprised 32 children, 29 teenagers, and 31 adults. The mean (SD) disease knowledge score increased significantly in all age groups of patients from 4.5 (2.0) to 5.9 (1.5; $p<0.001$) immediately after the training and remained unchanged at 6 months. Mean (SD) treatment knowledge scores were unchanged, but Wilcoxon signed rank testing showed a significant increase after the training course that was maintained at 6 months in children and teenagers.

Conclusion: These findings suggest that an individualized training course can enhance the understanding of hemophilia in patients of all ages, especially in children and teenagers, and that the HSAK may assist in improving patients' management of their disease.

Keywords: hemarthrosis, hemophilia, knee joint, patient education as topic, therapeutic education

Introduction

One of the major complications of hemophilia, especially in patients with severe or moderate disease, is recurrent joint bleeding leading to musculoskeletal complications.[1,2] If intra-articular bleeding is poorly controlled it can lead to the development of chronic synovitis and multi-articular arthropathy.[3,4] Diagnosis and treatment of these bleeding episodes must be delivered as early as possible and intensive treatment should be performed until the resolution of symptoms.[5–10] Early prophylactic treatment could avoid the development of hemophilic arthropathy.[11–14] Low compliance with prophylaxis may limit the effectiveness of treatment.[15] Education of patients with hemophilia and their families helps reduce mortality
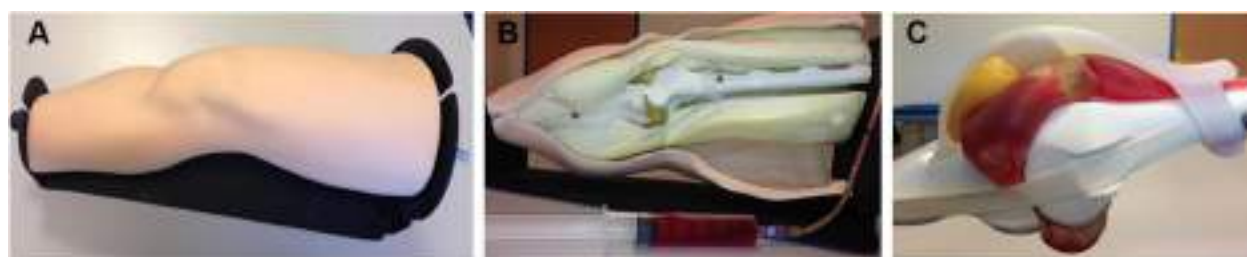
Figure 1 HSAK overview: the outer view (**A**), inner view (**B**), and fillable artificial joint capsule (**C**) of the hemarthrosis-simulating artificial knee.

and morbidity and may boost their quality of life.[16,17] This education should include information about the nature of the disease and its complications as well as the therapeutic products available and home treatment strategies, including self-administered intravenous infusions and self-management. Lifelong supervision of patients is essential to reinforce self-treatment, self-management, adherence and coping strategies.[18,19]

The hemophilia treatment center (HTC) at the Hôpital de Versailles, France, has developed a novel hemarthrosis-simulating artificial knee model (HSAK) (Figure 1) for use as a therapeutic education (TE) tool. The knee model was initially tested on a small group of patients at the Versailles HTC. After reports of improvements in the management of care and patients' self-treatment skills, an observational study was designed to evaluate the impact of this tool on patients' knowledge. This study was called GEFACET, the French acronym for "Artificial Knee and Therapeutic Education."

Materials and Methods

The study was conducted between May 2015 and February 2017 at seven hemophilia treatment centers in France: (1) Hôpital de Versailles, Versailles, (2) Regional Hemophilia Centre, Hôpital de Bicêtre (AP-HP), Kremlin-Bicêtre, (3) Regional Hemophilia Treatment Centre, Hôpital Cochin (AP-HP), Paris, (4) Haemophilia Treatment Centre, Hôpital de Brabois, Vandoeuvre-Les-Nancy, (5) Regional Hemophilia Treatment Centre, Centre Hospitalier Universitaire de Rouen, Rouen, (6) Hospices Civils de Lyon – Unité d'Hémostase Clinique, Hôpital Cardiologique Louis Pradel, Bron, and (7) Haemophilia Treatment Centre, Hôpital Pontchaillou, Centre Hospitalier Universitaire de Rennes, Rennes. Investigators (physicians, physiotherapists, psychologists, nurses) invited all patients with severe or moderately severe hemophilia A or B who were receiving therapeutic education with the HSAK to participate in the study. All

participants (patients or their legal representatives) provided written informed consent to take part in the study, which was undertaken in accordance with the Declaration of Helsinki. This project on educational intervention with the HSAK was strictly observational. Only questionnaires were used during the study and no clinical or biological data were recorded. The CCTIRS (French Advisory Board on Data Processing and Methodology) granted a favorable opinion. Authorization from CNIL (French Data Privacy Regulatory Agency) was obtained on 16th February 2015: authorization number 914,637.

The knee model was initially developed for healthcare staff training to visualize synovial fluid aspiration. It was then adapted to provide a visual effect of hemarthrosis on the anatomy of the knee joint and the impact of treatment (Figure 1). The model includes a pouch that can be filled with red liquid representing blood, thereby simulating hemarthrosis. When the red liquid is injected, the external aspect of the knee swells and the accumulation of blood in the joint is visible. A second pouch filled with a clear liquid is then used, showing the effect of treatment with coagulation factor concentrate on hemarthrosis. When this clear liquid is injected, the red liquid is progressively removed and the knee recovers a normal aspect. The presentation was standardized for all sites and is described in the Supplementary Section.

Participants were asked to complete three questionnaires specifically developed for the study. Two questionnaires were completed on the day of enrollment (D0). The first collected biometric data (age and sex). The second assessed knowledge of hemophilia and management of hemarthrosis; it was completed before the use of the HSAK. Immediately after completing the training course with the HSAK, patients or relatives were asked to complete the second questionnaire with an additional question on the HSAK. Six (±1) months later (M6 visit), participants were invited to complete a third questionnaire, which was identical to that used just after the education

session with HSAK at enrollment but included a new question relating to possible treatment stoppage since inclusion. The M6 questionnaire was completed whenever possible at the HTC or at home.

It was left to the discretion of the investigator or patient's legal representative to determine whether minors were able to complete the questionnaires alone. However, all three questionnaires had to be completed by the same person during the study. Patient responses were coded by investigators taking into account age differences.

The primary endpoint of the survey was the score that assessed patient knowledge of the disease. Unanswered questions or incorrect answers scored 0; correct answers scored 1. Disease knowledge was calculated on the basis of four questions, with a possible total mark of 7 points. This score was calculated at D0 pre-TE, then at D0 post-TE, and finally at M6.

Another score (graded from 0 to 4) was calculated for the secondary endpoint, which assessed patients' knowledge of their treatment. This covered patient appreciation of HSAK use as well as patient understanding of the disease and treatment of bleeding episodes. Information on treatment stoppage was provided at M6. Questionnaires and score assessment are detailed in the Supplementary Section.

To ensure the quality of results, data were double entered and data management consistency tests were conducted on the clinical database. Before analysis, a data entry quality control was performed on a representative number of case report forms. Statistical analyses were performed using SASR software (Version 9.4, SAS Institute, North Carolina State University, United States). Quantitative data were described by their numbers, mean, standard deviation (SD), median and extreme values. Qualitative data were described by their numbers and percentage. 95% bilateral confidence intervals (CI) were provided when deemed relevant. The significance threshold (Type I error) was set to $\alpha = 5\%$. The pre-education and post-education scores and sub-scores as well as scores and sub-scores found at D0 and M6 were compared using appropriate statistical tests: Wilcoxon signed rank test (for questions with at least 3 answers) or MacNemar test (for binary questions).

Results

Thirty-two children (<12 years of age), 29 teenagers (12–18 years), and 31 adults (>18 years) met the enrollment criteria and were eligible for the study. All 92 patients were male; median age was 14 years, ranging from 6 to 68 years. Most patients (81.5%) had severe hemophilia A. Eight dropouts were observed, either at the patient's request (n=6) or lost to follow-up (n=2). The M6 visit was therefore completed by 91.3% of enrolled patients.

At baseline, the median (mean ± SD) score for disease knowledge in all patients was 5 (4.5±2.0). This significantly increased after the TE session to 7 (5.9±1.5) (p<0.001), and remained unchanged at M6: 7 (5.9±1.4) (Table 1). The change in disease knowledge score was

Table 1 Scores of Knowledge of Disease and Its Treatment Before and After HSAK Use

Age Group	Score of Knowledge of Disease			Score of Knowledge of Treatment		
	D0 Before TE	D0 After TE	M6	D0 Before TE	D0 After TE	M6
Children N Mean ± SD	32 3.4 ± 2.1	32 5.4 ± 1.8 [a]*	31 5.7 ± 1.4 [b]*	32 2.2 ± 0.8	32 3.0 ± 0.7 [a]*	31 3.0 ± 0.8 [b]*
Teenagers N Mean ± SD	29 4.6 ± 1.7	29 6.3 ± 1.3 [a]*	26 5.9 ± 1.5 [b]*	29 2.7 ± 0.6	29 3.2 ± 0.6 [a]*	26 3 ± 0.6
Adults N Mean ± SD	31 5.5 ± 1.7	31 6.1±1.2 [a]*	27 6 ± 1.3	31 2.8 ± 0.5	31 2.9 ± 0.5	27 3 ± 0.5
All N Mean ± SD	92 4.5 ± 2	92 5.9 ± 1.5 [a]*	84 5.9 ± 1.4 [b]*	92 2.6 ± 0.7	92 3 ± 0.6 [a]*	84 3 ± 0.7 [b]*

Notes: Changes in mean disease- and treatment-related knowledge scores [a]between D0 pre-TE and D0 post-TE and [b]between D0 pre-TE and the M6 visit. *p<0.001 as determined by Wilcoxon signed rank test (nonparametric intraindividual data comparison).
Abbreviations: D0, day of enrollment; M6, 6 (±1) months after enrollment; TE, therapeutic education.

significant (p<0.001) in all groups of patients: children, teenagers and adults at D0 after TE (Table 1). For all patients, the disease awareness score had significantly improved at the M6 visit compared with the D0 pre-TE score (p<0.001), with a median increase (mean ± SD) of 1 (1.3±2.0). Sub-group analysis revealed that the change in disease knowledge score at the M6 visit was significant only in children and teenagers (Table 1).

At baseline, the median (mean ± SD) score for treatment knowledge was 3 (2.6±0.7). This remained at 3 (3.0 ±0.6) after the TE session and at M6 (3.0±0.7) (Table 1). A Wilcoxon signed rank test revealed that the score had significantly increased after TE (p<0.001), and that this increase was maintained at M6 (p<0.001). Sub-group analysis revealed that this increase was significant only in children and teenagers at D0 after TE and in children at M6 (Table 1).

In addition, 61 (66.3%) and 48 (52.2%) patients commented on D0 and at the M6 visit, respectively, that the HSAK helped them "a lot" to understand hemarthrosis after receiving TE. Similarly, most patients (64.1%) considered that the HSAK helped them "a lot" to understand explanations provided by healthcare providers on D0 after TE; this figure was 47% at the M6 visit. However, patients expressed mixed opinions about whether their disease knowledge or understanding of how hemarthrosis is treated had been improved through training.

Of the 84 patients who completed the M6 visit, only one patient (1.2%) stopped prophylaxis in the 6 months prior to the M6 visit.

Discussion

This study aimed to assess the impact of using the HSAK on disease- and treatment-related knowledge in male patients, most of whom had severe hemophilia A. The HSAK used in this TE program for children, adolescents, and adults improved the knowledge of the disease among these patients and their caregivers. Overall, this improvement was maintained 6 months after TE compared with the baseline (ie, before using the HSAK), although this improvement was significant only in children and teenagers at M6 compared with D0 before the educational intervention. Interestingly, age group analysis also showed that improvement of the knowledge of treatment at D0 after TE was statistically significant for children and teenagers but not for adults. These findings suggest that an individualized program provided at any age can enhance the understanding of hemophilia, especially in children

and teenagers. In addition, our results suggest that the HSAK, as a novel educational device, could enable patients to be more effective in looking after themselves.[20]

This study also showed that most patients had a high level of knowledge regarding their treatment and were aware of the value of prophylaxis, even when joint pain and swelling had subsided. Educating patients and their caregivers about the disease and treatments to prevent bleeding damage is essential for ensuring treatment adherence: caregiver acceptance, infrequent bleeds, venous access, and treatment compliance have been reported to be possible obstacles to adherence in pediatric patients with hemophilia.[21–24] Alternatively, educational efforts could specifically focus on patients entering the transitional period from childhood to adolescence, because adolescents appear to be more reluctant to use treatment than younger patients.[25] Interestingly in the present study, improvements of disease and treatment knowledge were generally greater in young patients than in adults.

This study is limited owing to several biases inherent in the way the data were collected: patient questionnaires can be interpreted differently and are sometimes completed indifferently by patients (because they have to reply three times to the same questions). However, few studies have reported evaluation of educational materials and educational intervention is usually evaluated on patient questionnaires.[20,26,27] The interval between TE and the M6 visit (±1) also differed between patients, and might have affected how their knowledge levels were assessed 6 months after training. In addition, there was no standard method for dealing with dropouts in this study. Some patients who did not attend their M6 visit when the clinical research associate (CRA) monitored the study sites were excluded from the study, whereas data were collected from some patients who returned their questionnaire more than 7 months after the baseline visit but prior to the CRA monitoring visit. Furthermore, no comparison was made between TE without the HSAK and TE with the HSAK, although this could be done in a further study. Finally, the small sample size included in each sub-group was a limitation although this size may be relevant given that hemophilia is a rare disease.

Despite these limitations it is noteworthy that about two-thirds of patients said they appreciated the use of this device; the HSAK was especially appreciated by younger patients because its visual nature allowed them to understand more easily. These findings should be

confirmed by further studies, and it may be possible to develop similar models for other joints.

Conclusions

The results of the GEFACET study, based on the completion of questionnaires after training performed by healthcare providers using the HSAK, showed a significant improvement in patient knowledge concerning hemophilia and its treatment, especially in children and teenagers. They suggest that the HSAK could be used as a support tool for TE, and might be valuable for helping patients with hemophilia to improve their disease management in an everyday setting. These findings should be confirmed by further studies.

Abbreviations

CRA, clinical research associate; D0, day of enrollment visit; GEFACET, artificial knee and therapeutic education [in French]; HSAK, hemarthrosis-simulating artificial knee; HTC, hemophilia treatment center; M6, 6-month visit; TE, therapeutic education.

Acknowledgments

Funding

Disclosure

References

1. Lobet S, Hermans C, Lambert C. Optimal management of haemophilic arthropathy and hematomas. *J Blood Med*. 2014;5:207–218. doi:10.2147/jbm.s50644
2. Rodriguez-Merchan EC. Articular bleeding in hemophilia. *Cardiovasc Hematol Disord Drug Targets*. 2016;16(1):21–24. doi:10.2174/1871529x16666160613114506
3. Rodriguez-Merchan EC. Musculoskeletal complications of hemophilia. *HSS J*. 2010;6(1):37–42. doi:10.1007/s11420-009-9140-9
4. Rodriguez-Merchan EC. Musculo-skeletal manifestations of haemophilia. *Blood Rev*. 2016;30(5):401–409. doi:10.1016/j.blre.2016.04.008
5. Rodriguez-Merchan EC, De la Corte-rodriguez H, Jimenez-Yuste V. Joint aspiration of acute tense knee haemarthroses in adult haemophilia A patients. *Thromb Res*. 2013;132(6):778–779. doi:10.1016/j.thromres.2013.09.025
6. De la Corte-rodriguez H, Rodriguez-Merchan EC, Alvarez-Roman MT, Martin-Salces M, Romero-Garrido JA, Jimenez-Yuste V. Accelerating recovery from acute hemarthrosis in patients with hemophilia: the role of joint aspiration. *Blood Coagul Fibrinolysis*. 2019;30(3):111–119. doi:10.1097/MBC.0000000000000803

7. Rodriguez-Merchan EC. Management of musculoskeletal complications of hemophilia. *Semin Thromb Hemost*. 2003;29(1):87–96. doi:10.1055/s-2003-37942

8. Liddle AD, Rodriguez-Merchan EC. Evidence-based management of the knee in hemophilia. *JBJS Rev*. 2017;5(8):e12. doi:10.2106/JBJS.RVW.16.00100

9. De la Corte-rodriguez H, Rodriguez-Merchan EC. The role of physical medicine and rehabilitation in haemophiliac patients. *Blood Coagul Fibrinolysis*. 2013;24(1):1–9. doi:10.1097/MBC.0b013e32835a72f3

10. De la Corte-rodriguez H, Rodriguez-Merchan EC, Alvarez-Roman T, Martin-Salces M, Jimenez-Yuste V. 'Do not do' recommendations in hemophilia. *Cardiovasc Hematol Disord Drug Targets*. 2020;20(3):168–174. doi:10.2174/1871529X20666200305111323

11. Rodriguez-Merchan EC, Hedner U, Heijnen L, et al. Prevention of haemophilic arthropathy during childhood. May common orthopaedic management be extrapolated from patients without inhibitors to patients with inhibitors? *Haemophilia*. 2008;14(Suppl 6):68–81. doi:10.1111/j.1365-2516.2008.01892.x

12. Rodriguez-Merchan EC, Jimenez-Yuste V, Aznar JA, et al. Joint protection in haemophilia. *Haemophilia*. 2011;17(Suppl 2):1–23. doi:10.1111/j.1365-2516.2011.02615.x

13. Rodriguez-Merchan EC. Prevention of the musculoskeletal complications of hemophilia. *Adv Prev Med*. 2012;2012:201271. doi:10.1155/2012/201271

14. Berntorp E, Astermark J, Björkman S, et al. Consensus perspectives on prophylactic therapy for haemophilia: summary statement. *Haemophilia*. 2003;9(Suppl 1):1–4. doi:10.1046/j.1365-2516.9.s1.17.x

15. Pérez-Robles T, Romero-Garrido JA, Rodriguez-Merchan EC, Herrero-Ambrosio A. Objective quantification of adherence to prophylaxis in haemophilia patients aged 12 to 25 years and its potential association with bleeding episodes. *Thromb Res*. 2016;143:22–27. doi:10.1016/j.thromres.2016.04.015

16. El Dakhakhny AM, Hesham MA, Hassan TH, El Awady S, Hanfy MM. Impact of health instructions on improving knowledge and practices of haemophilia A adolescents: a single centre experience. *Haemophilia*. 2014;20(4):e260–e266. doi:10.1111/hae.12431

17. D'Ivernois JF, Gagnayre R. *Learning to Educate the Patient: Pedagogical Approach*. 5th ed. [French]. Paris, France: Maloine; 2016.

18. Schrijvers LH, Beijlevelt-van der ZANDE M, Peters M, Schuurmans MJ, Fischer K. Learning intravenous infusion in haemophilia: experience from the Netherlands. *Haemophilia*. 2012;18(4):516–520. doi:10.1111/j.1365-2516.2012.02752.x

19. Schrijvers LH, Schuurmans MJ, Fischer K. Promoting self-management and adherence during prophylaxis: evidence-based recommendations for haemophilia professionals. *Haemophilia*. 2016;22(4):499–506. doi:10.1111/hae.12904

20. Phadnis S, Kar A. The impact of a haemophilia education intervention on the knowledge and health related quality of life of parents of Indian children with haemophilia. *Haemophilia*. 2017;23(1):82–88. doi:10.1111/hae.13070

21. Castaman G, Rocino A, Mazzucconi MG, Zanon E, Gagliano F, Molinari AC. Prophylaxis therapy in paediatric patients with haemophilia: a survey of clinical management trends in Italy. *Blood Transfus*. 2015;13(4):631–638. doi:10.2450/2015.0333-14

22. Berntorp E. Joint outcomes in patients with haemophilia: the importance of adherence to preventive regimens. *Haemophilia*. 2009;15(6):1219–1227. doi:10.1111/j.1365-2516.2009.02077.x

23. Petrini P. Identifying and overcoming barriers to prophylaxis in the management of haemophilia. *Haemophilia*. 2007;13(Suppl 2):16–22. doi:10.1111/j.1365-2516.2007.01501.x

24. Geraghty S, Dunkley T, Harrington C, Lindvall K, Maahs J, Sek J. Practice patterns in haemophilia A therapy – global progress towards optimal care. *Haemophilia*. 2006;12(1):75–81. doi:10.1111/j.1365-2516.2006.01189.x

25. Petrini P, Seuser A. Haemophilia care in adolescents – compliance and lifestyle issues. *Haemophilia*. 2009;15(Suppl 1):15–19. doi:10.1111/j.1365-2516.2008.01948.x

26. De la Corte-rodriguez H, Rodriguez-Merchan EC, Alvarez-Roman T, Martin-Salces M, Garcia-Barcenilla S, Jimenez-Yuste V. Health education and empowerment in adult patients with haemophilia. *Expert Rev Hematol*. 2019;12(11):989–995. doi:10.1080/17474086.2019.1650640

27. Lambert C, Meité N, Sanogo I, Lobet S, Hermans C. Development and evaluation of appropriate, culturally adapted educational tools for Ivoirian patients with haemophilia, haemophilia carriers and their families. *Haemophilia*. 2019;25(5):838–844. doi:10.1111/hae.13818

Perceptions About Blood Transfusion Therapy Among the General Public and Healthcare Professionals in the Qassim Region of Saudi Arabia

Ashwag S Alsharidah, Haifa A Alsuhaibani, Basma S Almansour & Mansour S Alsharidah

Published online: 11 Mar 2021.

Submit your article to this journal 🖉

Article views: 232

View related articles 🗗

View Crossmark data 🗗

Citing articles: 1 View citing articles 🗗

ORIGINAL RESEARCH

Perceptions About Blood Transfusion Therapy Among the General Public and Healthcare Professionals in the Qassim Region of Saudi Arabia

Ashwag S Alsharidah[1]
Haifa A Alsuhaibani[2]
Basma S Almansour[3]
Mansour S Alsharidah[1]

[1]Department of Physiology, College of Medicine, Qassim University, Buraidah, Qassim, Saudi Arabia; [2]Department of Pharmacy Practice, College of Pharmacy, Qassim University, Buraidah, Qassim, Saudi Arabia; [3]Department of Medicinal Chemistry, College of Pharmacy, Qassim University, Buraidah, Qassim, Saudi Arabia

Purpose: Blood transfusion is a conventional therapeutic procedure; however, the perceptions of general public and healthcare professionals (HCPs), especially physicians and nurses, remain unclear, although the insights of HSPs may affect the treatment decision. This study aimed to assess the awareness of HCPs and the public about blood transfusion risks and consent in Qassim region of Saudi Arabia, to uncover the factors that may influence such perceptions.

Patients and Methods: This study used two different closed questionnaires that were distributed electronically between February and March 2018 among the population and HCPs in Qassim region.

Results: A total of 400 general public participants and 135 HCPs completed the survey. Among the surveyed participants, 70% believed that blood transfusion therapy was safe. The perceived risk of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) was the highest among all complications (74%). Furthermore, 88.2% of respondents were willing to accept a blood transfusion as a therapeutic measure, primarily from a first-degree relative, although the remaining 11.8% rejected the idea of a transfusion due to fear of medical error. From the HCP survey, 80% were previously involved in a blood transfusion therapy consent process. HCPs typically reported explaining the benefits, risks, and alternatives described in the consent form (74.1%, 67.4%, and 53.3%, respectively).

Conclusion: Our results indicated that despite the current high level of acceptance and knowledge regarding blood transfusions, additional educational efforts remain necessary to increase public awareness of blood transfusion therapy.

Keywords: consent, blood therapy, alternative therapy, blood bank, adverse reaction

Introduction

Blood transfusion is a therapeutic intervention often used in the hospital for a variety of purposes and typically saves lives.[1] Blood transfusion therapy is a complex treatment that should be performed under the supervision of healthcare professionals (HCPs), particularly physicians and nurses. Blood transfusion therapy may include health risks; therefore, the blood transfusion treatment must be monitored, starting from the beginning of the process, with the selection of a blood donor, and includes immunohematology, serology testing of blood units, and post-process evaluations.

Although blood transfusion is a commonly used therapeutic procedure, it is not a risk-free intervention.[2] Many risks may be associated with a blood transfusion, such as transfusion-transmitted infections, transfusing an incorrect blood product, hyperkalemia, hemolytic reactions, transfusion-related circulatory overload (TACO),

Correspondence: Ashwag S Alsharidah
Department of Physiology, College of Medicine, Qassim University, Buraidah, Qassim, 51491, Saudi Arabia
Tel +966 541719777
Email ashriedt@qu.edu.sa

transfusion-related acute lung injury (TRALI), volume and iron overload, and acute allergic reactions.[3,4] The transfusion of incorrect blood components represents the largest cause of adverse incidents associated with blood transfusion therapy. The United States Food and Drug Administration considers TACO to be the second-most common cause of death associated with blood transfusion therapy.[4] Bolton-Maggs and Cohen (2013) estimated the risk of infection from transfusion and found that 1 in 1.3 million transfusions was associated with the reciprocal expression of hepatitis B virus (HBV).[4] Similarly, 1 in 28 million transfusions are associated with hepatitis C virus (HCV) transmission, and 1 in 6.7 million transfusions have been associated with the transmission of human immunodeficiency virus/acquired immune deficiency syndrome (AIDS/HIV).[5]

The purposes of blood transfusions, the anticipated benefits, and the expected outcomes should be explained and clarified to the patient before transfusion. Patient consent must be obtained by qualified HCPs who must clarify and be aware of all possible serious hazards of transfusion (SHOTs), in addition to the therapeutic benefits. Subsequently, the consent form should be signed by the patient before the transfusion procedure is initiated.[6] All HCPs should be aware of the detailed procedures, the associated risks, and the requirements for performing blood transfusions and be able to explain these concepts to patients prior to obtaining consent form signatures. HCPs should be able to address any doubts expressed by the patient, which will increase patient safety and awareness.

A study performed in Pakistan found that 56 (16%), out of the 350 surveyed patients, were aware of the possibility of HIV, HBV, and HCV infection associated with transfusion. However, only 2% of the patients in the study reported being asked for their consent, whereas the remaining 98% percent were neither asked nor provided with information regarding consent. In contrast, 236 (67%) of the patients believed that receiving a transfusion from a close friend was associated with lower infection risk than receiving blood from a blood bank.[7]

Locally, Al-Drees conducted a similar study in Riyadh, Saudi Arabia, and found that infection is the primary reason given by the 20% of 609 participants who stated that they would reject a blood transfusion even if they need one. Furthermore, 84.5% of participants stated a preference for receiving blood from a relative or a friend. Moreover, 11.5% believed that blood transfusion was a serious medical procedure, could be harmful, and carried risks of infection, whereas only 55.1% agreed that blood banks provide contamination-free blood.[8]

The current study aimed to assess the perception and awareness among the general public and among HCPs regarding the risks, associated diseases, and necessity of blood transfusions in Qassim region of Saudi Arabia and to identify the factors that influence these perceptions. This knowledge could enable HCPs to better understand their patients and allow them to better guide their patients through the decision-making process when faced with the decision to accept blood transfusion therapy, in addition to preventing the risks associated with the procedure.

Patients and Methods
Study Design, Sample Size, and Inclusion Data

A descriptive, exploratory, cross-sectional study with a quantitative, comparative design was conducted in Qassim region of Saudi Arabia. The study was conducted over four weeks, from 25 February to 25 March 2018. The minimum sample size for the study was calculated as 384, based on the population of Qassim region in 2016.[9] We collected 400 responses to account for the possibility of missing data from some of the survey responses. We recruited both males and females with ages ranging from 20 to 60 years. Informed consent was obtained from all participants. We limited study participation to residents of Qassim region of Saudi Arabia. We received 135 HCPs (physicians and nurses) responses after informed consent was obtained. This study was conducted in accordance with the Declaration of Helsinki. Ethical approval for the study was obtained from the subcommittee of Health Research Ethics in Qassim University, Buraidah, Saudi Arabia.

Data Collection Tool

Two structured surveys were designed based on an extensive literature review. The surveys were distributed online to the public and to HCPs (physicians and nurses).[2,3,10] The questionnaires were originally written in English, but we translated them into Arabic prior to distribution to ensure better understanding by the respondents. The questionnaires were distributed electronically and containing close-ended questions. Questionnaires have many advantages over other data collection tools, such as being inexpensive, easy to perform, and able to collect many findings and outcomes for analysis in a single format.

The general public survey included 24 questions on demographic data, educational level, and their willingness to accept a blood transfusion from a blood bank, expected risks, information regarding transfusion alternatives, and awareness of consent.

Another survey assessed the HCPs' impressions with 15 questions about the importance of the transfusion consent process, blood transfusion risks, the importance of the patient's acceptance of blood transfusion therapy, and the benefits and alternatives to blood transfusion.

Statistical Analysis

The questionnaire responses were analyzed using the Statistical Package for the Social Science (SPSS Inc. Chicago, IL, USA), version 23. Categorical variables are described as frequencies and percentages. Descriptive analyses were performed using the Chi-square test to test the significance of associations between categorical variables. The level of significance was set to $P < 0.05$.

Results

Participants Characteristics

This study aimed to identify community perceptions, awareness, and knowledge regarding blood transfusion therapy. Our community-aimed survey obtained a total of 400 responses, and the population characteristics are described in Table 1.

Participants' Knowledge of Blood Transfusion Therapy Safety

The majority of participants thought that blood transfusion was safe [283 (70.8%)], although 117 (29.2%) participants expressed the perception that transfusions were not safe. More than half of the participants [237 (59.2%)] thought that the blood groups of the donor and recipient were required to be matched for blood transfusion, whereas the remaining participants either thought that type-matching was not mandatory [135 (33.8%)] or indicated a lack of knowledge [28 (7%)]. Nearly half of the participants did not know whether alternatives to blood transfusion existed [202 (50.5%)], and nearly a third of them thought that no alternatives to blood transfusion were available [129 (32.3%)]; only a few participants stated that they believed alternatives to blood transfusion existed [69 (17.2%)].

Table 1 Demographic Characteristics of Participants

General Public Characteristics (n = 400)		n (%)
Sex	Female	268 (67%)
	Male	132 (33%)
Age	20 to 29 years	215 (53.8%)
	30 to 39 years	58 (14.5%)
	40 to 49 years	50 (12.5%)
	50 to 60 years	77 (19.2%)
Education	Basic education (Primary/intermediate)	8 (2%)
	Secondary	66 (16.5%)
	Bachelor's	294 (73.5%)
	Post-graduate education	32 (8%)
HCP characteristics (n = 135)		n (%)
Sex	Female	77 (57%)
	Male	58 (43%)
Profession	Physician	73 (54.1%)
	Nurse	62 (45.9%)
Specialty	Hematology	19 (14.1%)
	Internal medicine	18 (13.3%)
	Surgery	18 (13.3%)
	Obstetrics and Gynecology	7 (5.2%)
	Pediatrics	5 (3.7%)
	Anesthesiology	6 (4.4%)
	Others	62 (45.9%)
Seniority	Consultants	25 (24%)
	Specialists	35 (33.7%)
	Residents	44 (42.3%)

Abbreviation: HCP, healthcare provider.

Awareness Regarding Blood Transfusion Complications

Participants were more aware of the risks of chronic post-transfusion complications than the risks of acute complications. Nearly three-fourths of participants [296 (74%)] were aware of the possibility of transmission of or infection by HIV/AIDS, and nearly two-thirds [249 (62.3%)] were aware of the risks of transmission of or infection by HBV and HCV. However, knowledge of acute post-transfusion complications was poor, and only one-third of participants knew that allergic reactions, fever, and hemolysis were potential acute post-transfusion complications and only one-fifth of them [85 (21.3%)] knew that electrolyte imbalance was a potential acute post-transfusion complication.

Acceptance and Awareness of Participants Towards Blood Transfusion Therapy Consent Forms

The majority of participants believed that the provision of consent prior to blood transfusion therapy was mandatory [335 (83.8%)], whereas only a few indicated that consent was not mandatory [65 (16.2%)]. The aspects that the participants emphasized that HCPs should discuss during the written consent process, represented in terms of the frequency and percentage of responses and multiple answer was allowed, were as follows: possible side effects [262 (65.5%)], medical benefits [257 (64.2%)], possible risks [244 (61%)], and alternative treatments [185 (46.2%)].

Acceptance of Participants Toward Blood Transfusion Therapy

The majority of participants stated that they would accept a blood transfusion if their doctor recommended a blood transfusion or informed them that they required a transfusion [353 (88.2%)], whereas the remaining respondents [47 (11.8%)] stated that they would refuse a transfusion, even if told a transfusion was necessary. Among those who were willing to accept a transfusion (n = 353), when asked who they would prefer to be the donors, their preferred donors, represented by frequencies and percentages, were as follows: first-degree relatives [206 (58.4%)]; any person, it does not matter [130 (36.8%)]; and husband/wife [92 (26.1%)]. Among those who stated that they would refuse a transfusion (n = 47), the following

barriers were noted: fear of medical errors during the transfusion process [24 (51.1%)]; fear of transmission of or infection by HBV, HCV, and HIV [14 (29.8%)]; and insufficient alternatives [9 (19.1%)] (Table 2).

Participants' Education Levels and Awareness of Post-Transfusion Complications

We asked the participants about their awareness of various complications that may occur after blood transfusion therapy (allergic reactions, such as itching, erythema, skin rash, shortness of breath, and anaphylaxis; fever and hemolysis (red blood cell destruction); disturbance and imbalance of electrolytes in the body). Most participants had a low level of awareness regarding the potential complications following blood transfusion therapy, which did not differ significantly across different education levels (Table 3).

Consent Perception and Experiences of HCPs

Among the surveyed HCPs, 108 (80%) were involved in or attended a blood transfusion consent process, whereas 27 (20%) had not. Among the 135 HCPs, 129 (95.6%) agreed that the consent process for blood transfusion was necessary, whereas the remaining 6 (4.4%) thought that consent was not necessary. A total of 120 (88.9%) HCPs thought that obtaining the patient's consent before performing a blood transfusion should be mandatory, whereas 15 (11.1%) thought that consent should not be mandatory. In addition, 119 (88.1%) HCPs thought that all hospitals should adopt and apply

Table 2 Acceptance Among Participants of Blood Transfusion Therapy (n = 400)

Attitude	n (%)
If your doctor suggested blood transfusion therapy, and he informed you that you required this blood transfusion, would you accept it?	
Accepted	353 (88.2%)
Refused	47 (11.8%)
Those who accepted blood transfusions preferred the following donors (n = 353)	
First-degree relatives	206 (58.4%)
Any person, it does not matter	130 (36.8%)
Husband/Wife	92 (26.1%)
Second-degree relatives	57 (16.1%)
Friends	11 (3.1%)
Far relatives (such as tribe members)	2 (0.5%)
Those who refused blood transfusions reported the following barriers (n = 47)	
Fear of medical errors during a blood transfusion	24 (51.1%)
Fear of HBV/HCV/HIV and other blood-borne infections	14 (29.8%)
Insufficient alternatives	9 (19.1%)

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

Table 3 Relationship Between Education Level and the Potential for Acute Allergic Reaction to Blood Transfusion Therapy (n = 400)

Character		Education n (%)				p-value
		Basic n = 8	**Secondary** n = 66	**Bachelor's** n = 294	**Post-Graduate** n = 32	
Awareness of allergic reaction as a complication of blood transfusion	Yes	0 (0%)	18 (27.3%)	108 (36.7%)	10 (31.2%)	0.021
	No	4 (50%)	13 (19.7%)	34 (11.6%)	4 (12.5%)	
	Did not know	4 (50%)	35 (53%)	152 (51.7%)	18 (56.3%)	

a transfusion consent policy, whereas 7 (5.2%) thought that such policies should not be adopted, and 9 (6.7%) did not know. More than three-fourths of HCPs [102 (78.5%)] were satisfied with the transfusion consent process used for blood transfusion therapy at the hospital, whereas the remaining respondents [28 (21.5%)] were unsatisfied.

HCPs who believed that the transfusion consent procedure form adds knowledge to the patients were among the majority [119 (88.1%)], and only a few expressed the opinion that the consent process did not provide knowledge to the patients [16 (11.9%)]. A total of 100 (74.1%) of the HCPs who reported being involved in or attending a transfusion consent process stated that they explained the benefits of blood transfusion when they obtained the transfusion consent, whereas the remaining [8 (5.9%)] HCPs involved in or attending a transfusion consent process and did not

fully explain the benefits of blood transfusions when they obtained transfusion consent, and 27 (20%) were not involved in or never attended a transfusion consent process. Among HCPs who were involved in or attended a transfusion consent process, the majority stated that they explained the risks of blood transfusion when they obtained transfusion consent [90 (66.6%)], whereas the 18 (13.3%) did not explain the risks of blood transfusion, and 27 (20%) were not involved in the transfusion consent process. In addition, 72 (53.3%) of HCPs who were involved in or attended a transfusion consent process reported explaining the alternatives to blood transfusions, whereas 36 (26.7%) of those involved in the transfusion consent process did not explain alternatives to blood transfusion, and 27 (20%) were not involved in the transfusion consent process (Table 4).

Table 4 HCPs Practice of Blood Transfusion Therapy Consent (n = 135)

Character	n (%)
Transfusion consent procedure form provides knowledge to patients who will undergo the consent process	
Yes	119 (88.1%)
No	16 (11.9%)
Do you explain the benefits of blood transfusion when obtaining transfusion consent?	
Yes	100 (74.1%)
No	8 (5.9%)
Not involved in the consent process	27 (20%)
Do you explain the risks of blood transfusion when obtaining transfusion consent?	
Yes	90 (66.6%)
No	18 (13.3%)
Not involved in the consent process	27 (20%)
Do you explain alternatives to blood transfusion when obtaining transfusion consent?	
Yes	72 (53.3%)
No	36 (26.7%)
Not involved in the consent process	27 (20%)

Abbreviation: HCP, healthcare provider.

Discussion

Over recent years, identifying the variables that affect the awareness and perceptions of Saudis regarding blood transfusion therapy has become crucial, as World Health organization, stated an increase in voluntary blood donations from 2013 to 2018.[11] The present study strengthens our understanding and knowledge of the perceptions and knowledge of both the general public and HCPs in terms of blood transfusion therapy.

The demand for blood transfusion therapy is increasing due to the growing population of Saudi Arabia and the increasing number of healthcare facilities; therefore, maintaining the safety of blood transfusion therapy has become essential. HIV/AIDS and HBV/HCV were the highest perceived complications in our study, with 74% and 62.3% of respondents indicating that they perceived the transmission of these viruses to be risks of blood transfusion, respectively. Locally, a study was conducted to measure the prevalence of HBV markers among blood donors between 2013 and 2015 in Qassim regional hospitals, which found identified 0.42% positive units.[12]

Blood transfusion therapy has many known risks and side effects, including allergic reactions.[3] Post-transfusion allergies are the result of several plasma proteins, which can cause an anaphylactic reaction.[13]

Hemolytic transfusion reactions can cause the lysis of transfused red blood cells due to immunological incompatibility.[14] Approximately one-third of our participants believed that blood transfusion therapy might cause fever and hemolysis.

Hyperkalemia related to transfusion depends not only on the potassium concentration in the red blood cell unit but also on the volume and rate of red blood cell administration.[15] However, only 21.3% of our participants were aware of the possibility of electrolyte disturbance caused by blood transfusion therapy. Raza et al, in their study, reported that 4% of 125 patients suffered from hyperkalemia after receiving blood transfusion therapy.[16]

After the donation process, donated blood goes through an extensive screening procedure to identify a variety of viruses and parasites at the blood banks in Saudi Arabia. In 2008, Aldrees's results stated that 55.1% of patients believed that blood transfusion therapy was a safe procedure.[8] Furthermore, Vetter et al, in 2014, found that only 15% of surgical patients rated blood transfusion therapy as a very risky procedure.[3] In our study, only 17.2% of our participants were aware of blood transfusion therapy

alternatives. Similarly, 18.4% of 294 surgical patients in the study reported by Vetter et al were aware of blood transfusion therapy alternatives.[3]

HCPs' opinions and attitudes towards blood transfusion are vital for assessing the credibility of the obtained consent. Our study highlighted their perceptions of transfusion consent and their consent-associated practices. The majority of the HCPs were previously involved in a blood transfusion therapy consent process (80%). In Oman, 77% of the physicians declared their previous involvement in blood transfusion consent.[6] Locally, at Buraidah Central Hospital, emergency blood transfusion therapy consent is mandatory prior to blood component release from the blood bank.

A study by Cheung et al found that all surveyed patients reported having a conversation regarding the reason for receiving blood transfusion therapy. Although 85% of participants agreed that the benefits of the procedure were discussed, only one-third of the patients stated that the risks of infection transmission were discussed. Only 24% stated that HCPs discussed alternatives prior to blood transfusion therapy procedures.[17]

The consent form contains information regarding blood transfusion therapy benefits, risks, and alternatives. Similar results to ours were found in a study by Al-Riyami et al, in which 80% of the surveyed physicians thought that the process of obtaining blood transfusion consent provides the patient with knowledge prior to the blood transfusion therapy.[6] However, in Davis et al, the majority of HCPs (83 out of 123) felt that the information provided during the consent process regarding blood transfusion therapy procedures was insufficient.[18]

A study by Freidman et al showed that all surveyed residents primarily discuss the improvement of anemia during the consent process.[2] In contrast, in Ohio, United States, patients interviewed after blood transfusion therapy reported that pre-transfusion written consent was not sufficient to explain the benefits and risks of the procedure, and they reported concerns about the safety of the blood supply.[19]

Conclusion

Most of the surveyed general public thought that blood transfusion was safe, and we found that the general awareness of allergic reactions as potential blood transfusion complications increased with education level. The major reason stated for refusing blood transfusion therapy was fear of blood-borne infections. The survey responses indicated that the participants were aware of the possible risks

associated with the blood transfusion process. HCPs' responses suggested the need to modify the current consent process by including more information about the benefits and risks to ensure that patients are provided with a full understanding of the procedure and to minimize the chances that HCPs will omit any information.

The major limitation of this study was the paucity of time. As we distributed the survey to approximately 600 HCPs but only received 135 HCP, which is less than the one-third that was expected. The response rate of the study survey was 22.5%. This low response rate is considered a result of the short data collection time and is a significant limitation and may contribute to non-response bias.

The present study reflects a positive attitude among general public towards the safety of blood transfusion. Our results also indicated the need for additional educational efforts to increase public awareness of blood transfusion therapy.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Allard S. Blood transfusion. *Medicine*. 2013;41(4):242–247. doi:10.1016/j.mpmed.2013.01.010
2. Friedman M, Arja W, Batra R, et al. Informed consent for blood transfusion. *Am J Clin Pathol*. 2012;138(4):559–565. doi:10.1309/AJCP2TN5ODJLYGQR
3. Vetter T, Adhami L, Porterfield J, Marques M. Perceptions about blood transfusion. *Anesth Analg*. 2014;118(6):1301–1308. doi:10.1213/ANE.0000000000000131
4. Bolton-Maggs P, Cohen H. Serious Hazards of Transfusion (SHOT) haemovigilance and progress is improving transfusion safety. *Br J Haematol*. 2013;163(3):303–314. doi:10.1111/bjh.12547
5. Loor G, Koch C, Sabik J, Li L, Blackstone E. Implications and management of anemia in cardiac surgery: current state of knowledge. *J Thorac Cardiovasc Surg*. 2012;144(3):538–546. doi:10.1016/j.jtcvs.2012.04.014
6. Al-Riyami A, Al-Marshoodi I, Zia F, et al. Transfusion consent in Oman: physicians' perception at a tertiary care university hospital. *Oman Med J*. 2016;31(4):253–257. doi:10.5001/omj.2016.50
7. Habibullah S, Dahar S, Ashraf J, Shakeel Aamir M. To determine patients' perceptions and attitudes for blood transfusion. *Pak J Med Res*. 2016;55(2):48–50.
8. Al-Drees A. Attitude, belief and knowledge about blood donation and transfusion in Saudi population. *Pak J Med Sci*. 2008;24(1):74–79.
9. Demographic survey [Internet]. 2016 [cited March 23, 2018]. Available from: https://www.stats.gov.sa/sites/default/files/ar-demographic-research-2016_5.pdf. Accessed February 26, 2021.
10. Abdul-Aziz B, Lorencatto F, Stanworth S, Francis J. Patients' and health care professionals' perceptions of blood transfusion: a systematic review. *Transfusion*. 2017;58(2):446–455. doi:10.1111/trf.14404
11. World Health Organization. Blood safety and availability[Internet]. 2020 [Cited February 3, 2021] Available from: https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability. Accessed February 26, 2021.
12. Alharbi A, Alouffi S, Alcantara J, et al. Prevalence of hepatitis B virus markers among blood donors in Qassim Region, Saudi Arabia. *Int J Pharm Res Allied Sci*. 2017;6(1):99–106.
13. Matsuyama N, Yasui K, Amakishi E, et al. The IgE-dependent pathway in allergic transfusion reactions: involvement of donor blood allergens other than plasma proteins. *Int J Hematol*. 2015;102 (1):93–100. doi:10.1007/s12185-015-1794-0
14. Davenport R, Bluth M. Hemolytic transfusion reactions. In: Simon T, editor. *Rossi's Principles of Transfusion Medicine*. 5th ed. New York: John Wiley & Sons; 2016:642–651.
15. Smith H, Farrow S, Ackerman J, Stubbs J, Sprung J. Cardiac arrests associated with hyperkalemia during red blood cell transfusion: a case series. *Anesth Analg*. 2008;106(4):1062–1069. doi:10.1213/ane.0b013e318164f03d
16. Raza S, Ali Baig M, Chang C, et al. A prospective study on red blood cell transfusion related hyperkalemia in critically Ill patients. *J Clin Med Res*. 2015;7(6):417–421. doi:10.14740/jocmr2123w
17. Cheung D, Lieberman L, Lin Y, Callum J. Consent for blood transfusion: do patients understand the risks and benefits? *Transfus Med*. 2014;24(5):269–273. doi:10.1111/tme.12141
18. Davis R, Vincent C, Sud A, et al. Consent to transfusion: patients' and healthcare professionals' attitudes towards the provision of blood transfusion information. *Transfus Med*. 2012;22(3):167–172. doi:10.1111/j.1365-3148.2012.01148.x
19. Adams KW, Tolish D. Blood transfusion: the patient's experience. *Am J Nurs*. 2011;111(9):24–30. doi:10.1097/01.NAJ.0000405057.23324.a8

Impact of CD105 Flow-Cytometric Expression on Childhood B-Acute Lymphoblastic Leukemia

Rasha A Elkholy, Mohamed H Fouda, Eslam E Elhawary, Reem A Elkholy & Ola A Elshora

Published online: 16 Mar 2021.

Submit your article to this journal

Article views: 157

View related articles

View Crossmark data

Citing articles: 1 View citing articles

ORIGINAL RESEARCH

Impact of CD105 Flow-Cytometric Expression on Childhood B-Acute Lymphoblastic Leukemia

Rasha A Elkholy [1]
Mohamed H Fouda[1]
Eslam E Elhawary[2]
Reem A Elkholy[3]
Ola A Elshora[1]

[1]Clinical Pathology Department, Faculty of Medicine, Tanta University, Tanta, Egypt; [2]Pediatrics Department, Faculty of Medicine, Tanta University, Tanta, Egypt; [3]Pharmacology Department, Faculty of Medicine, Tanta University, Tanta, Egypt

Background: CD105 (Endoglin) is a receptor of the transforming growth factor-Beta (TGF-β) superfamily. It is expressed in angiogenic endothelial cells and is considered a powerful marker of angiogenesis and a potential main player in the pathogenesis of vascular diseases as well as tumor progression. CD105 expression was correlated with poor prognosis in many types of solid malignancies, however, its influence on hematological neoplasms is still an area of interest.

Purpose: To assess the flow-cytometric expression of CD105 in childhood B-acute lymphoblastic leukemia (B-ALL) and its relation to disease response after the induction chemotherapy.

Subjects and Methods: Eighty children newly diagnosed with B-ALL were screened for flow-cytometric expression of CD105 at time of diagnosis, then they were followed up to detect their response to induction therapy.

Results: CD105 was expressed in 41.2% of B-ALL patients. Higher expression of CD105 was observed in high and very high-risk groups. The multivariate analysis considered CD105 positivity as an independent prognostic marker for response to induction therapy. Values higher than 2.5 Specific fluorescence indices (SFIs) and 35% expression were sensitive predictors to induction failure.

Conclusion: CD105 can be considered as a potential prognostic marker for the detection of response to induction therapy in childhood B-ALL, and it can serve to optimize treatment decisions.

Keywords: B-acute lymphoblastic leukemia, endoglin, CD105

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder representing clonal expansion and arrest of maturation of lymphoid progenitor cells in the bone marrow, blood, and extramedullary sites.[1] The incidence of ALL generally follows a bimodal distribution, with its first peak occurring in childhood and the second around the age of 50.[2]

With proper risk stratification, ALL responds well to chemotherapy, yet early mortality during the induction phase of chemotherapy is not uncommon. Besides, the high relapse rate remains a major problem.[3] Therefore, identification of new prognostic markers will not only aid in increasing the accuracy of patients' risk stratification but also will minimize the chances of relapse by optimizing therapy at the early stages of treatment.[4]

Flow-cytometry immunophenotyping is considered a potent technology used to identify cell membrane antigens.[5] The identification of surface antigens on

Correspondence: Rasha A Elkholy
Department of Clinical Pathology, Faculty of Medicine, Tanta University, Aljaysh Street, Tanta, 31951, Egypt
Tel +20 1224489930
Fax +2 040/3407734
Email rashaelkholy26@gmail.com

leukemic cells is essential for the assignment of the proper treatment plan and is also valuable for assessing prognosis and searching for applicable markers to detect minimal residual disease.[6]

CD105 (Endoglin) is a homodimeric transmembrane co-receptor that interacts with transforming growth factor-Beta (TGF-β) receptors type I and III, consequently adjusting angiogenesis by regulating proliferation, differentiation, and endothelial cell migration.[7] It is expressed on the surface of endothelial cells, stromal cells, melanocytes, and different hematopoietic cells.[8–10] It is highly expressed as well on the surface of activated vascular endothelial cells but not or weakly expressed on the normal quiescent vessels. Therefore, it has been proposed as a marker of angiogenesis.[9] In contrast, lack of CD105 expression is diagnostic for hereditary hemorrhagic telangiectasia type −1, a disease characterized by multiple vascular malformations.[11]

Buhring et al[12] reported that CD105 was not detectable on normal bone marrow CD34 positive hemopoietic precursor cells, mature T, B, natural killer, and myeloid cells, but was instead present on a subset of glycophorin A-positive mononuclear cells. In a subsequent study, Rokhlin et al[13] demonstrated the existence of two CD105 positive populations in bone marrow: B-lineage precursor cells and pro-erythroblasts. The expression of CD105 on hemopoietic precursor cells serves as a mediator affecting quiescence and enables long-term repopulation.[14]

Regarding hematological malignancies, CD105 expression was reported in myelodysplastic syndrome,[15] ALL,[16,17] and acute myeloid leukemia (AML).[16–19] CD105 positive blast cells exhibit higher leukemogenic activity when compared to their negative counterpart.[20]

Cosimato, and his colleagues,[16] extensively studied the expression of CD105 on blast cells of acute leukemia patients and reported that CD105 was expressed in the majority of B-ALL cases, and only the most immature types expressed this antigen, as it was consistently absent in "Burkitt-like" mature B-ALL without referring to its effect on patient's prognosis. Also, Poręba et al[17] reported that CD105 expression in patients with ALL was limited to a small group of patients and needs to be confirmed on a larger group. So, adequate information about its prognostic impact in B-ALL is still worth further evaluation.

The current work aimed to assess the CD105 flow-cytometric expression in B-ALL pediatric patients and its relation to disease response after the induction phase of chemotherapy.

Subjects and Methods

The current study was carried out on 80 children newly diagnosed with B-ALL referred to Hematology/Oncology Unit, Pediatric Department, Tanta University Hospitals, and Pediatric Oncology Unit, Tanta Cancer Center from June 2017 to August 2020.

Cases were diagnosed based on clinical presentation, complete blood count (CBC), bone marrow (BM) examination, morphological and cytochemical smears as well as immunophenotyping.

Risk stratification was done according to Children's Oncology Group (COG) protocols.[21] Cases were classified into three groups; standard-risk group (54 cases, 67.5%), high-risk group (17 cases, 21.25%), and very high-risk group (9 cases, 11.25%).

Initial investigations included; automated CBC on ERMA PCE-210N cell counter (Tokyo, Japan) with an examination of Giemsa-stained smears, Liver and renal function tests, and lactate dehydrogenase enzyme (LDH) on a fully automated chemistry analyzer (Konelab Prime 60i, Thermo-scientific, Vantaa, Finland), erythrocyte sedimentation rate (ESR), and cerebrospinal fluid (CSF) cytological examination. BM aspiration samples were evaluated through Giemsa-stained smears, and acute leukemia was diagnosed by the presence of ≥20% blast cells. Philadelphia (Ph) chromosome was detected by fluorescence in-situ hybridization (FISH).

Immunophenotyping analysis was done on BM samples collected into EDTA-containing tubes using the four-color flow cytometry Becton Dickinson (BD) FACS Calibur instrument (Becton Dickinson, San Diego, California, USA), using the Cell Quest software (Becton Dickinson, version 3, verify software House Topsham, ME, USA). Before each run, calibrated beads provided by the manufacture were used to adjust the compensation of different fluorochromes. Mouse isotopic controls were used as negative controls to exclude autofluorescence. At least 10.000 events/tubes were acquired for each analysis. Blast cells were identified based on dim/intermediate CD45 Peridinin Chlorophyll Protein Complex (Per-CP) expression versus log side scatter characteristics (CD45/SSC gating strategy). The gated fluorescence dot plot was evaluated for positive cells using cursor position from the dot plot of isotypic controls. The internal negative control was checked using normal cells in the sample that lacked the antigen, while the internal positive control was checked using the normal cells in the sample that expressed the antigen.

B-ALL was diagnosed using the acute leukemia panel that included the following combinations of surface markers: CD45/CD14/CD117/CD34, CD45/HLA-DR/CD10/CD38, CD45/CD64/CD19/CD20, CD45/CD7/CD33, CD45/CD2/CD13, and the cytoplasmic markers anti TDT/anti MPO, anti CD79a and anti cyt μ, markers were supplied by Becton Dickinson (BD biosciences, Mountain View, California). B-ALL blast cells were identified by positive expression of CD19 in addition to CD10 and/or CD79a.

Immunophenotyping analysis of CD105 using CD105 fluorescein isothiocyanate (FITC) labeled monoclonal antibody, supplied by BD biosciences, Catalog number 561443 Clone 266 (RUO). The total leucocytic count (TLC) was adjusted to 10^6 cells/tube. Cells were incubated with 5μ of CD45 Per-CP, and 10 μ of CD105 FITC in the dark, at room temperature, for 25 min. Red blood cells were then lysed with 1 mL of BD FACS lysing solution for 20 min before centrifugation, the cells were then washed twice with 0.5 mL of phosphate buffer saline (PBS), and suspended in 300 μL of PBS to be ready for the flow cytometer acquisition. Blast cells were selectively gated using CD45/SSC strategy, and the percent of blast cells expressing CD105 was determined within this population using a cutoff value for positivity >5%.[17] Specific fluorescence indices (SFIs) were calculated by dividing median fluorescence intensity (MFI) of CD105 by MFI of negative isotype control, positive expression was defined as SFIs >1.5[19] (Figures 1 and 2).

After being fully investigated at diagnosis, all the patients received induction chemotherapy, according to the protocol adopted by the treating centers; the Modified St Jude Children's Research Hospital (SJCRH) Total Therapy XV Protocol.[22] At the end of induction therapy on day 28, all patients were reevaluated by CBC and BM samples. Remission was identified by the absence of peripheral blood blasts and BM blast cells less than 5%. Refractoriness to therapy was defined by the presence of greater than 5% BM blasts and/or CNS infiltration by leukemic cells. Those who did not achieve complete remission were reassigned to more intensified treatment protocols.[22]

Statistical Analysis

Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov–Smirnov test was used to verify the normality of the distribution of variables. Comparisons between groups for categorical variables were assessed using the Chi-square test [Fisher or Monte Carlo (MC)]. Student's t-test was used to compare two groups of normally distributed quantitative variables while the Mann Whitney test was used to compare two groups of non-normally distributed quantitative variables. For non-normally distributed quantitative variables more than two groups Kruskal Wallis test was used and followed by the Post Hoc test (Dunn's for multiple comparisons test). Pairwise comparison Spearman coefficient was used to correlate between quantitative variables. Univariate and multivariate logistic regression analyses were used to determine the related co-variables associated with poor response to induction therapy. The Receiver operating characteristic curve (ROC) was used to determine the diagnostic characteristics of the marker. The optimal cutoff value was assessed via the Youden index. The significance of obtained results was judged at the ≤0.05 level.[23]

Results

This prospective study was conducted on eighty children newly diagnosed with B-ALL. They were 50 boys (62.5%) and 30 girls (37.5%) with a male to female ratio of 1.6:1, their ages ranged from 1 to 15 years with a median value 7.3 years.

At diagnosis, the mean and standard deviation (SD) of the hemoglobin (Hb) level was 7.9±1.8 (gm/dl), blast percentage in peripheral blood (P.B) 38±23.3 (%), and ESR 82±15.3 (m/h). The median and range for the TLC was 20 (1–110) x10^3/cmm, platelets count 65 (10–260) x10^3/cmm, blast percentage in bone marrow 82.5 (50–98) %, LDH level 822 (340–3200) IU/L.

As regard immunophenotyping results, CD19 was expressed in all cases 80/80 (100%) of B-ALL, CD10 in 66/80 (82.5%) cases, CD34 in 71/80 (88.7%) cases, cytoplasmic μ in 12/80 (15%) cases, CD20 in 9/80 (11.2%) cases, with aberrant expression of CD33 and CD13 in 6/80 (7.5%) and 15/80 (18.7%) cases respectively. Based on these data, patients were classified according to EGIL classification as follows: pro-B-ALL (5 cases), common B-ALL (63 cases), pre-B-ALL (3 cases), and mature B-ALL (9 cases).

Positive expression of CD105 on BM samples was detected in 33/80 (41.2%) patients, and it ranged from 8–90%, 2–8 SFIs with a median value of 36% and 3 SFIs respectively, while negative expression was detected in 47/80 (58.8%) patients, and ranged from 0.5–5%,
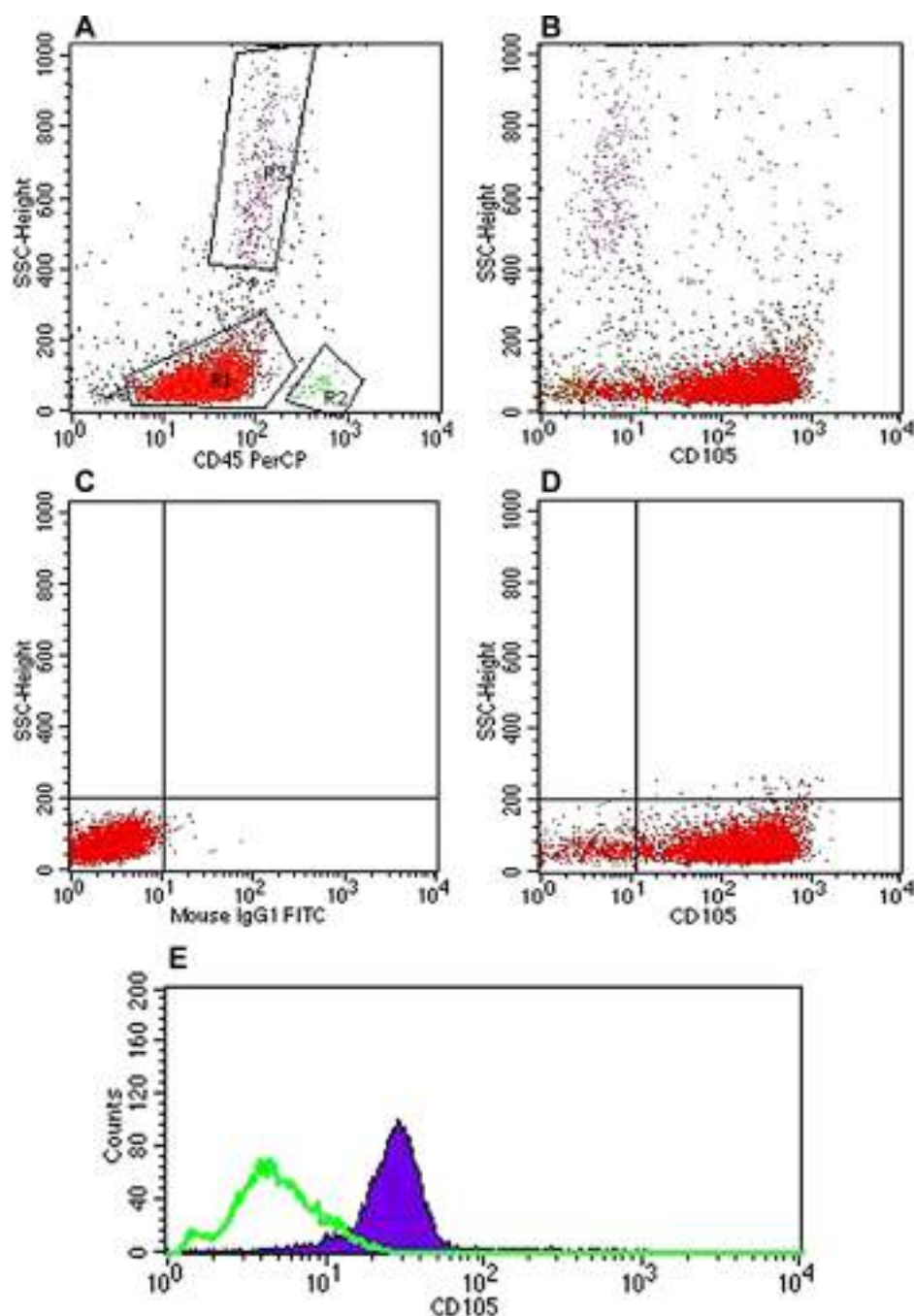
Figure 1 Flow cytometry analysis of CD105 positive case. (**A**) Dot plot showed gating using CD45/SSC strategy, blast cells (R1), normal lymphocytes (R2) and granulocytes (R3). (**B**) Scatter dot plot showing blast cells positive for CD105 expression in combination with normal lymphocytes and granulocytes negative for CD105 expression. (**C**) Dot plot showing the mouse IgG1 isotypic negative control. (**D**) Dot plot showing positive CD105 expression on gated blast cells. (**E**) Histogram showing positive CD105 (solid violet curve) versus negative control (green colored curve).

0.5–1.4 SFIs with a median value of 3% and 1 SFIs respectively.

CD105 expression in B-ALL subtypes was positive in (3/5) Pro B-ALL, (29/63) common B-ALL, (1/3) Pre B-ALL, while it was absent (0/9) in all cases of "Burkitt-like" mature B-ALL.

We found variations between CD105 percentage, SFIs and the patients' risk stratification, with statistically significant difference between the standard-risk and the very high-risk groups (P-value=0.045, and 0.035 respectively) as shown in (Table 1). No statistically significant difference was observed between the CD105 positive and the
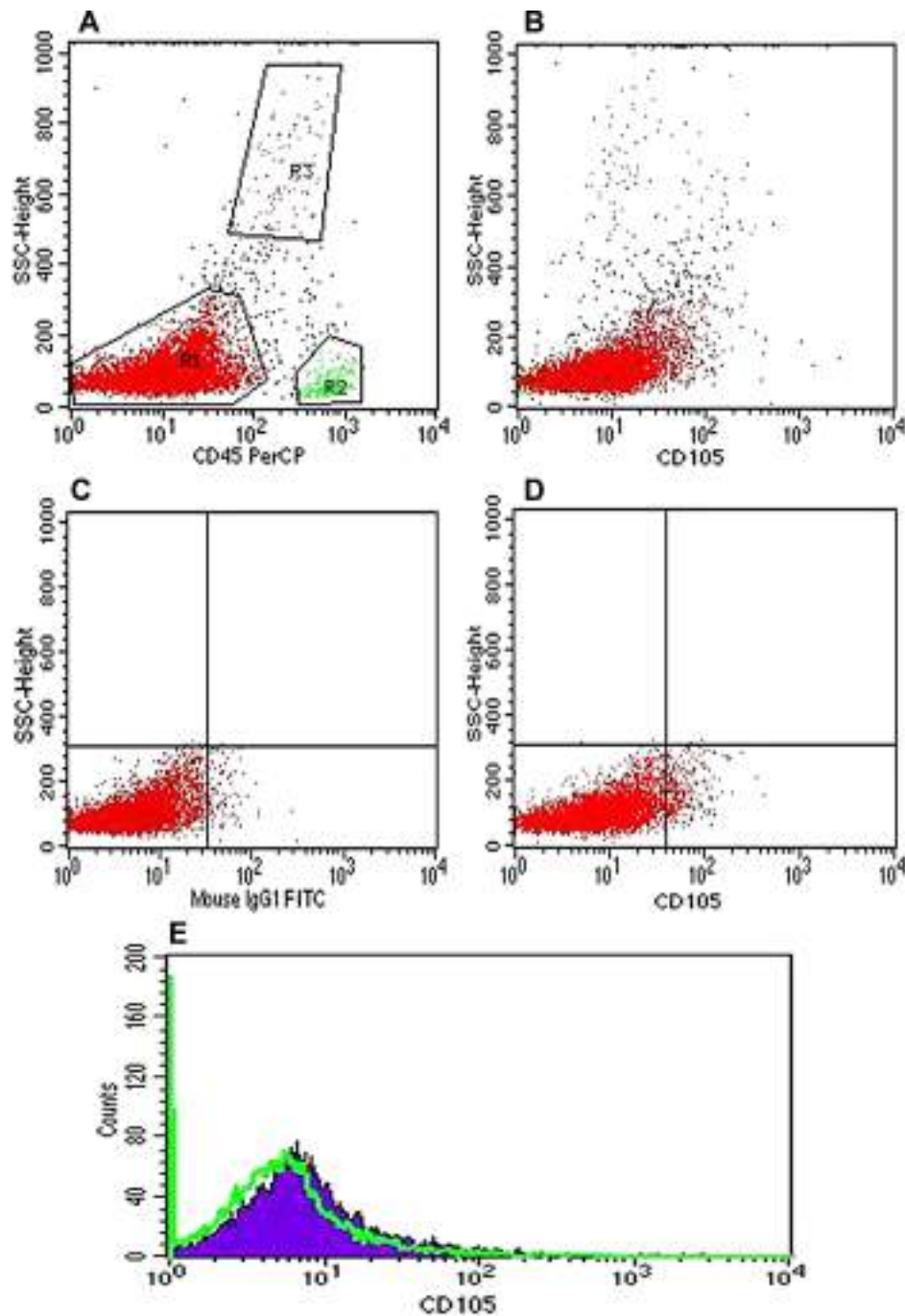
Figure 2 Flow cytometry of analysis of CD105 negative case. (**A**) Dot plot showed gating using CD45/SSC strategy, blast cells (R1), normal lymphocytes (R2) and granulocytes (R3). (**B**) Scatter dot plot showing blast cells, normal lymphocytes and granulocytes all negative for CD105 expression. (**C**) Dot plot showing the mouse IgG1 isotypic negative control. (**D**) Dot plot showing negative CD105 expression on gated blast cells. (**E**) Histogram showing negative CD105 (solid violet curve) versus negative control (green colored curve).

negative groups as regard clinical and laboratory parameters (P-value > 0.05) (Table 2).

CD105 expression had a weak positive correlation (<0.3) with age and TLC, LDH, and a weak negative correlation (<0.3) with CD20 as presented in (Table 3).

At follow up, 15 out of 80 patients were excluded from the statistical analysis (9 patients chose to complete induction at other centers, 3 of them were among the mature B-ALL group, the other 6 mature B-ALL patients were intentionally excluded as they received a different treatment protocol).

Table 1 The Pattern and Range of CD105 Expression in B-ALL Cases as Regards Risk Stratification

	Standard Risk (54 Patients)	High Risk (17 Patients)	Very-High (9 Patients)	P-value	Post Hoc Test
CD105 SFIs Median (Min. – Max.)	1.2 (0.5–6)	1.3 (0.5–6)	6 (1–8)	0.035*	P_1:0.239, P_2:0.013*, P_3:0.168
CD105% Median (Min. – Max.)	4 (0.5–60)	4 (2–60)	50 (1–90)	0.045*	P_1:0.169, P_2:0.022*, P_3:0.282

Notes: *Significant at P ≤ 0.05. P_1: p-value for comparing between standard and high risk. P_2: p-value for the association between standard and very high. P_3: p-value for the association between high risk and very high.
Abbreviation: SFIs, specific fluorescence indices.

The remaining 65 cases were followed till the end of induction. Remission was achieved by 51/65 (78.4%) cases; 36 of them had a negative expression of CD105 and 15 had CD105 positive expression. 10/65 cases (15.4%) were refractory to the first induction cycle, one of them was CD105 negative while and 9 were CD105

Table 2 The Impact of CD105 Expression Pattern on B-ALL Patient's Characteristics

Parameters	CD105 Negative Group (47 Patients)	CD105 Positive Group (33 Patients)	P-value
Age (years) Median (Min. – Max.)	6.5 (1.5–14)	8.5 (1–15)	0.109
Hemoglobin (gm/dl) Mean ± SD.	8.1 ± 1.6	7.7 ± 2	0.394
Total leucocytic count (×10³/cmm) Median (Min. – Max.)	18 (1–110)	30 (1.7–96)	0.052
Platelets count (×10³/cmm) Median (Min. – Max.)	57 (10–260)	70 (15–185)	0.384
Peripheral blood blasts (%) Mean ± SD.	36.2 ± 20.8	40.6 ± 26.7	0.324
Bone marrow blasts (%) Median (Min. – Max.)	80 (50–98)	90 (59–98)	0.634
Lactate dehydrogenase (IU/L) Median (Min. – Max.)	787 (340–2300)	900 (444–3200)	0.095
Erythrocyte sedimentation rate (m/h) Mean ± SD.	82.6 ± 15.4	81.1 ± 15.3	0.667
Sex Male Female	32 (68.1%) 15 (31.9%)	18 (54.5%) 15 (45.5%)	^{FE}p= 0.218
Central nervous system infiltration Absent Present	45 (95.7%) 2 (4.3%)	30 (90.9%) 3 (9.1%)	^{FE}p= 0.644
Philadelphia chromosome Negative Positive	46 (97.9%) 1 (2.1%)	28 (84.8%) 5 (15.2%)	^{FE}p= 0.077
Testicular infiltration Present Absent	2 (4.3%) 45 (95.7%)	4 (12.1%) 29 (87.9%)	^{FE}p= 0. 224

Abbreviation: FE, Fisher exact.

Table 3 Correlation Between CD105 SFIs and CD105% with Different Parameters

	CD105 SFIs		CD105%	
	r_s	P-value	r_s	P-value
Age (years)	0.298	0.007*	0.293	0.008*
Hemoglobin (gm/dl)	−0.151	0.181	−0.105	0.354
Total leucocytic count (×10³/cmm)	0.261	0.019*	0.230	0.040*
Platelets count (×10³/cmm)	−0.039	0.731	−0.025	0.824
Peripheral blood blasts (%)	0.059	0.606	0.081	0.473
Bone marrow blasts (%)	0.113	0.320	0.189	0.092
Lactate dehydrogenase (IU/L)	0.236	0.035*	0.199	0.077
Erythrocyte sedimentation rate (m/h)	0.018	0.871	−0.036	0.754
CD19 (%)	0.041	0.721	0.018	0.874
CD20 (%)	−0.232	0.038*	−0.179	0.113

Notes: *Significant at P ≤ 0.05. r_s: Spearman coefficient.
Abbreviation: SFIs, specific fluorescence indices.

positive expression. Four children died during the induction stage, one of them was CD105 negative and the other three were CD105 positive. Deaths were due to be sepsis with severe neutropenia in three children and intracranial hemorrhage with severe thrombocytopenia in one child.

The difference between the CD105 positive and the negative group regarding the response to therapy was highly significant (X^2 14.383*[MC]P-value <0.001).

To establish the effect of CD105 expression on response to induction therapy, logistic regression analyses were performed to detect the related covariables that can be associated with poor response to induction therapy. In univariate analysis, Ph chromosome positivity, and CD105 positivity were significantly associated with poor response

to therapy (P-value ≤0.05). In multivariate analysis, CD105 positivity remained the only significant independent factor associated with poor response to induction therapy (P-value ≤0.05). (Table 4).

As CD105 positivity was significantly correlated to poor response to induction therapy, we used the Receiver-operating characteristic (ROC) curve analysis to establish the most suitable cutoff value after exclusion of mature B-ALL cases, missed cases, and deaths. ROC curve analysis showed that CD105 expression >35% and SFIs >2.5% is highly significant to differentiate between good and poor responders to induction therapy with high diagnostic efficacy (AUROC 0.948 and 0.928 respectively) (Table 5) and (Figure 3).

Table 4 Univariate and Multivariate Analysis for the Parameters Affecting Response to Induction Therapy

	Univariate		Multivariate	
	P-value	OR (95% C.I)	P-value	OR (95% C.I)
Age (>8 versus. ≤8 years)	0.232	2.325 (0.582–9.283)		
Sex (female versus. male)	0.213	2.400 (0.605–9.522)		
Hemoglobin (>7.8 gm/dl versus. ≤7.8 gm/dl)	0.195	0.381 (0.088–1.640)		
Total leucocytic count (>20×10³/cmm versus. ≤20×10³/cmm)	0.280	2.143 (0.538–8.540)		
Platelets count (>65×10³/cmm versus ≤65×10³/cmm)	0.865	1.125 (0.290–4.366)		
Peripheral blood blasts (>40% versus. ≤40%)	0.385	1.833 (0.468–7.187)		
Bone marrow blasts (>89% versus. ≤89%)	0.161	2.841 (0.659–12.239)		
Lactate dehydrogenase (>856 IU/L versus. ≤856 IU/L)	0.998	–		
Erythrocyte sedimentation rate (>85 m/h versus. ≤85 m/h)	0.683	0.750 (0.189–2.980)		
Central nervous system infiltration (present versus. absent)	0.635	1.778 (0.166–19.065)		
Philadelphia chromosome (positive versus. negative)	0.049*	12.500 (1.012–154.397)	0.110	14.820 (0.54–405.82)
CD105 (positive versus negative)	0.005*	21.600 (2.511–185.80)	0.007*	22.807 (2.34–221.84)

Notes: *Significant at P ≤ 0.05. The number between brackets in columns 1 represent cutoff used was the median of the cases included.
Abbreviations: OR, odds ratio; C.I, confidence interval.

Table 5 Performance (AUC, Sensitivity, Specificity) for CD105 SFIs and CD 105% to Discriminate Between Poor Responder (n=10) and Good Responder (n=51)

	AUC	P-value	95% C.I	Cutoff	Sensitivity	Specificity	PPV	NPV
CD105 SFIs	0.928	<0.001*	0.800–1.057	>2.5	90.0	92.16	69.2	97.9
CD105%	0.948	<0.001*	0.867–1.029	>35	90.0	94.12	75.0	98.0

Note: *Significant at P ≤0.05.
Abbreviations: AUC, area under the curve; C.I, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

Discussion

The TGF-β co-receptor CD105 plays a major role in fetal, adult, and malignant angiogenesis. Previous studies reported the CD105 expression on tumor vessels of a variety of neoplasms.[8] Although, CD105 is now well known to be expressed on malignant cells in various hematopoietic malignancies, data on its prognostic relevance in especially leukemias are still not adequate.[19]

CD105 positivity in the current study was present in 41.2% of cases diagnosed with B-ALL, this is lower than the values presented by Cosimato et al[16] who reported positive CD105 expression in 68.4% of B-ALL cases, this difference can be attributed to the relatively small number of cases involved in our study. They also found that CD105 was absent in all cases of mature B-ALL, and expression levels were higher among the high and the very high-risk groups compared to the standard group, which is similar to our findings.
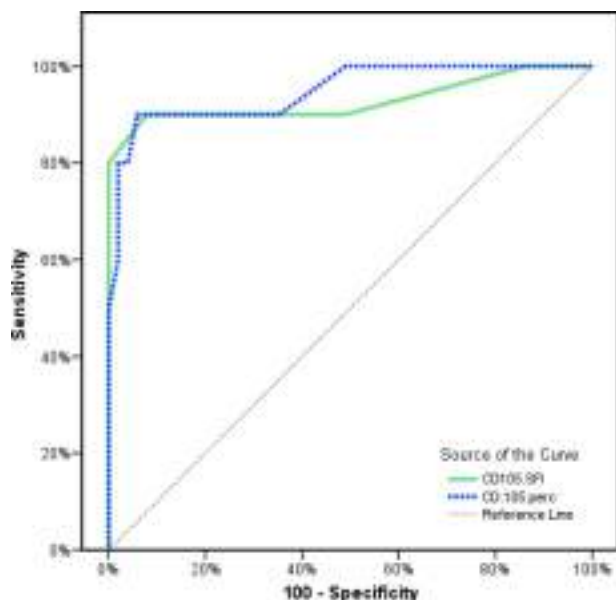
Figure 3 ROC curve for CD105 SFIs and CD105%to discriminate between poor and good responder.

Although the study of Poręba et al[17] reported that no statistically significant correlation was present between CD105 expression and age, gender, LDH level, blast percentage in P.B or BM, we were able to detect a weak positive correlation with age, TLC, and LDH level and a weak negative correlation between CD105 SFIs and CD20 expression.

Patients with positive CD105 expression showed a higher rate of induction failure. Moreover, the multivariate analysis showed that CD105 positivity is an independent factor that associated with poor outcome to induction therapy. This highlights the importance of CD105 as a prognostic marker in B-ALL. This is comparable to what Kauer et al[19] found in their study of CD105 in AML, where they correlated it to poor outcome and failure of response to chemotherapy, and they recommended the use of CD105 expression as a prognostic marker in AML, which can help optimize follow up and treatment decisions for AML patients. They attempted to explain the inferior outcome in CD105 positive AML patients by suggesting that CD105 contributes to dysregulation of TGF- β dependent and TGF- β independent signaling pathways and enhances angiogenesis which gives a better chance for survival of malignant cells with increasing the risk of minimal residual disease (MDR). Also, Xu et al[24] suggested that resistance to intensive therapy might be explained by the presence of hypoxia inducible factor 1 alpha induced multi-drug resistance transporters in CD 105 high AML blast cells.

To our knowledge, this is the first study that reported the impact of CD105 flow-cytometric expression on the response to induction therapy in B-ALL. Therefore, we established a cutoff value of > 35% and >2.5 SFIs as calculated by the ROC curve to identify patients who are at risk for induction failure that may require intensive therapy from the start to improve outcome.

Novel therapeutic targets for acute leukemia are urgently needed and successful treatment of acute leukemia remains a clinical challenge.[25] For ALL, although significant progress has been made in the last decade, in

cases of refractory or relapsed ALL, second-line chemotherapy has shown a poor effect, rarely resulting in long-term survival.[26] Thus, there is a critical need for new therapeutic options. Monoclonal antibodies are promising agents because they deliver their therapeutic effects with minimal toxicity.[27]

CD105 is a promising target that can be used for tumor imaging and prognosis and it has therapeutic potential in patients with solid tumors and other neoplastic diseases with increased angiogenesis.[18] Dourado and his colleagues,[21] studied the use of monoclonal antibody TRC105 to prevent the engraftment of primary AML blasts and inhibit leukemia progression following disease establishment, but in B-ALL, TRC105 alone was ineffective due to the shedding of soluble CD105. However, in both B-ALL and AML, TRC105 synergized with reduced intensity myeloablation to inhibit leukemogenesis, indicating that TRC105 may represent a novel therapeutic option for B-ALL and AML. So, measurement of CD105 may be of great value not only to assess the prognosis but also in targeted therapy for B-ALL.

The results of our study support the important role of CD105 in leukemia progression and response to induction therapy which is one of the major contributors of B-ALL risk stratification.

The limitations of the present study included the relatively small number of patients included in this study, and the association between CD105 flow-cytometric expression and its soluble level was not investigated. Therefore, it is recommended to extend this research on a large patients cohort, investigate the coupled measurement of CD105 on blast cells and its soluble form in serum. Also, extend the follow-up duration to assess the impact of CD105 expression on the overall survival and disease-free survival, as well as studying the stability of the marker after therapy.

Conclusion

CD105 can be considered a potential marker for the prognosis of pediatric patients with B-ALL, as patients who showed expression higher than 35% and 2.5 SFIs were at higher risk for induction failure. So, CD105 may serve to optimize treatment decisions for B-ALL patients.

Data Sharing Statement

Data can be provided on request.

Ethics Approval and Consent to Participate

Acknowledgments

Author Contributions

All authors made essential contributions to study design, analysis and interpretation of data; took part in drafting the article and revising it critically for important intellectual content; agreed to submit to the current journal, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors declare that they have no conflicts of interest.

References

1. Terwilliger T, Abdul-Hay MJ. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. *Blood Cancer J.* 2017;7 (6):e577–e577. doi:10.1038/bcj.2017.53

2. Paul S, Kantarjian H, Jabbour EJ. Adult acute lymphoblastic leukemia. *Mayo Clin Proc.* 2016;91(11):1645–1666. doi:10.1016/j. mayocp.2016.09.010

3. Tzortzatou-Stathopoulou F, Papadopoulou AL, Moschovi M, Botsonis A, Tsangaris GT. The low relapse rate in children with acute lymphoblastic leukemia after risk –directed therapy. *J Pediatr Hematol Oncol.* 2001;23(9):591–597. doi:10.1097/00043426-200112000-00008

4. Rashed WM, Hamza MM, Matboli M, Salem SI. MicroRNA as a prognostic biomarker for survival in childhood acute lymphoblastic leukemia: a systematic review. *Cancer Metastasis.* 2019;38 (4):771–782. doi:10.1007/s10555-019-09826-0

5. Xu B, Hu C, Miao XD, et al. Immunophenotyping of 106 adult patients with acute leukemia by flow cytometry. *Di Yi Jun Yi Da Xue Xue Bao.* 2003;23(10):1043.

6. Khoury H, Dalal BI, Nevill TJ. Acute myelogenous leukemia with t (8; 21)–identification of a specific immunophenotype. *Leuk Lymphoma*. 2003;44(10):1713–1718. doi:10.1080/1042819031000116698

7. Minhajat R, Mori D, Yamasaki F, Sugita Y, Satoh T, Tokunaga O. Organ-specific endoglin (CD105) expression in the angiogenesis of human cancers. *Pathol Int*. 2006;56(12):717–723. doi:10.1111/j.1440-1827.2006.02037.x

8. Fonsatti E, Vecchio LD, Altomonte M, et al. Endoglin: an accessory component of the TGF-β-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. *J Cell Physiol*. 2001;188(1):1–7. doi:10.1002/jcp.1095

9. Dallas NA, Samuel S, Xia L, et al. Endoglin (CD105): a marker of tumor vasculature and potential target for therapy. *Clin Cancer Res*. 2008;14(7):1931–1937. doi:10.1158/1078-0432.CCR-07-4478

10. Brum ST, Demasi AP, Stelini RF, Cintra ML, de Araujo VC, Soares AB. Endoglin is highly expressed in human mast cells. *Appl Immunohistochem Mol Morphol*. 2019;27(8):613–617. doi:10.1097/PAI.0000000000000668

11. McAllister KA, Grogg KM, Johnson DW, et al. Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet*. 1994;8(4):345–351. doi:10.1038/ng1294-345

12. Bühring HJ, Müller CA, Letarte M, et al. Endoglin is expressed on a subpopulation of immature erythroid cells of normal human bone marrow. *Leukemia*. 1991;5(10):841.

13. Rokhlin OW, Cohen MB, Kubagawa H, et al. Differential expression of endoglin on fetal and adult hematopoietic cells in human bone marrow. *J Immunol*. 1995;154(9):4456–4465.

14. Borges L, Oliveira VK, Baik J, Bendall SC, Perlingeiro RC. Serial transplantation reveals a critical role for endoglin in hematopoietic stem cell quiescence. *Blood*. 2019;133(7):688–696. doi:10.1182/blood-2018-09-874677

15. Della Porta MG, Malcovati L, Invernizzi R, et al. Flow cytometry evaluation of erythroid dysplasia in patients with myelodysplastic syndrome. *Leukemia*. 2006;20(4):549–555. doi:10.1038/sj.leu.2404142

16. Cosimato V, Scalia G, Raia M, et al. Surface endoglin (CD105) expression on acute leukemia blast cells: an extensive flow cytometry study of 1002 patients. *Leuk Lymphoma*. 2018;59(9):2242–2245. doi:10.1080/10428194.2017.1416366

17. Poręba M, Jaźwiec B, Poręba R, Kuliczkowski K. Expression of CD105 antigen in patients with acute leukemia, malignant lymphoma, and multiple myeloma in active phase of the disease. *Adv Clin Exp Med*. 2006;15(6):1023–1028.

18. Chakhachiro ZI, Zuo Z, Aladily TN, et al. CD105 (endoglin) is highly overexpressed in a subset of cases of acute myeloid leukemias. *Am J Clin Pathol*. 2013;140(3):370–378. doi:10.1309/AJCPG8XH7ZONAKXK

19. Kauer J, Schwartz K, Tandler C, et al. CD105 (Endoglin) as negative prognostic factor in AML. *Sci Rep*. 2019;9(1):1. doi:10.1038/s41598-019-54767-x

20. Dourado K, Baik J, Oliveira VK, et al. Endoglin: a novel target for therapeutic intervention in acute leukemias revealed in xenograft mouse models. *Blood*. 2017;129(18):2526–2536. doi:10.1182/blood-2017-01-763581

21. Borowitz MJ, Devidas M, Hunger SP, et al.; Children's Oncology Group. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. *Blood*. 2008;111(12):5477–5485. PMID: 18388178; PMCID: PMC2424148. doi:10.1182/blood-2008-01-132837.

22. Pui CH, Relling MV, Sandlund JT, Downing JR, Campana D, Evans WE. Rationale and design of Total Therapy Study XV for newly diagnosed childhood acute lymphoblastic leukemia. *Ann Hematol*. 2004;83:S124–6. doi:10.1007/s00277-004-0850-2

23. Dawson BD, Trapp RG. *Basic & Clinical Biostatistics*. 3rd ed. New York, NY: Medical Publication Division, Lange Medical Book/McGraw-Hill; 2001:161–218.

24. Xu P, Wang M, Jiang Y, Ouyang J, Chen B. The association between hypoxia inducible factor 1α and multidrug resistance of acute myeloid leukemia. *Transl Cancer Res*. 2017;6(1):198–205. doi:10.21037/tcr.2017.01.10

25. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukemia. *Lancet*. 2013;381(9881):1943–1955. doi:10.1016/S0140-6736(12)62187-4

26. Dias A, Kenderian SJ, Westin GF, Litzow MR. Novel therapeutic strategies in acute lymphoblastic leukemia. *Curr Hematol Malig Rep*. 2016;11(4):253–264. doi:10.1007/s11899-016-0326-1

27. Farhadfar N, Litzow MR. New monoclonal antibodies for the treatment of acute lymphoblastic leukemia. *Leuk Res*. 2016;49:13–21. doi:10.1016/j.leukres.2016.07.009

Correlation of Automated Chemiluminescent Method with Enzyme-Linked Immunosorbent Assay (ELISA) Antibody Titers in Convalescent COVID-19 Plasma Samples: Development of Rapid, Cost-Effective Semi-Quantitative Diagnostic Methods

Rachelle Mendoza, Michael Silver, Alejandro R Zuretti, Manan Christian, Ballabh Das, Allen J Norin, Patrick Borgen, Jenny Libien & Martin H Bluth

METHODOLOGY

Correlation of Automated Chemiluminescent Method with Enzyme-Linked Immunosorbent Assay (ELISA) Antibody Titers in Convalescent COVID-19 Plasma Samples: Development of Rapid, Cost-Effective Semi-Quantitative Diagnostic Methods

Rachelle Mendoza [ⓘD][1]
Michael Silver[2,3]
Alejandro R Zuretti[1,4]
Manan Christian[4]
Ballabh Das[5]
Allen J Norin[5]
Patrick Borgen[6]
Jenny Libien[1,4]
Martin H Bluth[4]

[1]Department of Pathology, SUNY Downstate Health Sciences University, Brooklyn, NY, USA; [2]School of Public Health, SUNY Downstate Health Sciences University, Brooklyn, NY, USA; [3]Department of Medicine, Maimonides Medical Center, Brooklyn, NY, USA; [4]Department of Pathology, Maimonides Medical Center, Brooklyn, NY, USA; [5]Department of Medicine, SUNY Downstate Health Sciences University, Brooklyn, NY, USA; [6]Department of Surgery, Maimonides Medical Center, Brooklyn, NY, USA

Correspondence: Martin H Bluth
Blood Transfusion and Donor Services, Clinical Laboratories, Translational Research (Pathology), Maimonides Medical Center, 4802 10th Avenue, Brooklyn, NY, 11219, USA
Email MBluth@maimonidesmed.org

Background: We investigated the utility of an automated chemiluminescent SARS-CoV-2 IgG antibody assay platform in quantifying the amount of binding antibodies present in donated convalescent plasma.

Methods: A total of 179 convalescent plasma units were analyzed for the presence of SARS-CoV-2 IgG antibodies using the Beckman-Coulter chemiluminescent immunoassay (CLIA) platform. The equipment-derived numerical values (S/Co ratio) were recorded. Aliquots from the same units were subjected to enzyme-linked immunosorbent assay (ELISA) that detects IgG antibodies against the receptor-binding domain (RBD) of the SARS-CoV-2 S1 protein. The relationship between ELISA titers and CLIA S/Co values was analyzed using linear regression and receiver operating characteristics (ROC) curve.

Results: Twenty-one samples (11.7%) had S/Co values of less than 1.0 and were deemed negative for antibodies and convalescent plasma had S/Co values between >1.0 and 5.0 (70/ 179, 39.1%). Fifteen units (8.4%) had negative ELISA titer. The majority of the units (95/ 179. 53.1%) had titers ≥1:1024. The sensitivities of ELISA to CLIA were comparable (90.5% vs 88.3%, respectively; p=0.18). There was positive linear correlation between CLIA S/Co values and ELISA IgG titer (Rho = 0.75; Spearman's rank = 0.82, p-value = <0.0001). The agreement between the two methods was fair, with a κ index of 0.2741. Using the ROC analysis, we identified a CLIA S/Co cutoff value of 8.2, which gives a sensitivity of 90% and a specificity of 82% in predicting a titer dilution of ≥1:1024.

Conclusion: The utility of automated antibody detection systems can be extended from simply a screening method to a semi-quantitative and quantitative functional antibody analysis. CLIA S/Co values can be used to reliably estimate the ELISA antibody titer. Incorporation of chemiluminescent-based methods can provide rapid, cost-effective means of identifying anti-SARS-CoV-2 antibody titers in donated plasma for use in the treatment of COVID-19 infection.

Keywords: COVID-19, ELISA, chemiluminescence, antibody, titers

Introduction

Coronavirus disease 2019 (COVID-19), caused by a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the cause of a pandemic that has infected over 49.7 million people worldwide with a mortality

of 2.4% to date.[1] Due to the record-breaking economic and social impact of COVID-19, there is a need to optimize all resources to improve outcome and minimize the spread of the virus. A key goal is to increase the availability of treatment options for the severely ill COVID-19 patients, one of which is convalescent plasma.

Initial case reports from China have shown immediate beneficial clinical results in COVID-19 patients transfused with convalescent plasma.[2–4] The viral loads were decreased after transfusion, coinciding with increased levels of neutralizing COVID-19 antibody. In a match-controlled study done in the United States,[5] patients who received COVID-19 convalescent plasma (CCP) were more likely to have improved oxygen requirement by day 14 post-transfusion as compared to those who did not receive CCP. The recipients also had significantly longer survival, especially non-intubated patients.[5] Another study involving approximately 5000 patients observed better survival among recipients who were given CCP units that contain high antibody levels based on chemiluminescent intensity value.[6]

Recent investigations on recovered COVID-19 patients revealed low antibody levels among those with mild symptoms, especially among the healthy and younger individuals.[7,8] Healthy and younger individuals are most likely to be eligible for blood product donation. Another study observed rapid decline in IgG antibodies among recovered COVID-19 patients with mild symptoms from the time they were screened for donation up to plasmapheresis.[9] Although the FDA has issued emergency use authorization (EUA) for the use of convalescent plasma, it does not strictly implement antibody testing prior to transfusion of the blood product.[10] This has great implication on the quality of convalescent plasma units that are available in blood centers.

Neutralizing antibody assays, considered as the gold standard for detection of anti-COVID function, are assessed using native or pseudotype virus in cellular assay.[11] The disadvantages of this analysis include the limited number of facilities that have the capacity and expertise to perform this assay, lengthy turn-around time and high cost.[12,13] However, antibody levels can also be estimated using an antibody binding assay such as an enzyme-linked immunoassay (ELISA). A study on COVID-19 patients showed good correlation between ELISA antibody titer and neutralizing antibody levels.[14] In particular, the linear correlation with neutralizing antibody levels was seen in ELISA assays that detected

immunoglobulins (IgG) antibodies against the receptor-binding domain (RBD) of the viral spike protein S1.[14] Although easier to perform than neutralization assays, ELISA assays are limited by long turn-around time and higher costs.

Chemiluminescent immunoassay (CLIA) is considered as a qualitative antibody assay that detects binding antibodies to viral antigens, similar to the principle of an ELISA. The test relies on mixing patient samples with a known viral protein, buffer reagents, and specific enzyme-labeled antibodies that allow a light-based, luminescent read-out.[15–17] The amount of light (radiance) emitted from each sample is relative to the number of antibodies present in a patient sample.[17] This type of assay can assess the presence of multiple types of antibodies, including IgG, IgM, and IgA, is automated, has a short turn-around time (~45 minutes) and can be implemented with minimal cost in already existing clinical laboratory platforms.[18] However, this method has not been optimized to quantify the amount of antibodies. Currently, FDA has not authorized the use of automated chemiluminescent assays for screening of antibodies in potential donors of COVID-19 convalescent plasma.

At Maimonides Medical Center, antibody testing is performed using a Beckman Coulter platform that utilizes CLIA that detects IgG antibodies against the SARS-CoV-2 RBD. The intensity value retrieved from the platform, based on the sample-to-cut-off (S/Co) ratio, reflects the amount of anti-SARS-CoV-2 antibodies present in the sample. Since this platform utilizes the RBD of the virus, there is a good chance that the results of this assay may correlate with the number of neutralizing antibodies. However, to date, no study has been published correlating the S/Co value with an ELISA or neutralization titer in the setting of COVID-19. Such a correlation would be crucial to provide a more cost-efficient and rapid antibody testing process that is readily available and can be utilized for convalescent plasma donor screening.

Methods
Plasma Collection

Convalescent plasma was obtained from the Blood Donation Service at Maimonides Medical Center. Convalescent plasma was donated by recovered COVID-19 patients with confirmed diagnosis via clinical laboratory test and were symptom free for at least 28 days. Some CCP donors were initially screened for the presence of antibodies via Dynex Agility™ instrument through a reference laboratory (Table

Table 1 Characteristics of Volunteer Donors and Their Donated Plasma

Characteristics	Median (IQR)/N (%)
Age, years	39.9 (30.3–49.7)
Antibody test prior to donation	
Performed	91 (50.8)
Not performed	88 (49.2)
Antibody test result prior to donation (S/Co)	2.3 (1.7–3.1)
CCP chemiluminescent results	
0.02 to 1	21 (11.7)
>1-5	71 (39.1)
>5-10	32 (17.9)
>10-20	27 (15.1)
>20	29 (16.2)
Donated plasma ELISA titers	
Negative	15 (8.4)
1:32	2 (1.1)
1:64	7 (3.9)
1:128	10 (5.6)
1:256	19 (10.6)
1:512	31 (17.3)
1:1024	35 (19.6)
1:2048	22 (12.3)
1:4096	22 (12.3)
1:8192	5 (2.8)
1:16,384	8 (4.5)
1:32,768	3 (1.9)

1). An aliquot of plasma or serum was collected and stored at −20°C for subsequent antibody testing at the time of plasma donation. This study was approved by the IRB/Research Committee at Maimonides Medical Center. The requirement to obtain informed consent from the subjects has been waived by the IRB in accordance with 45 C.F.R. § 46.116(d) and the guidelines outlined in the Declaration of Helsinki were followed.

The plasma or serum samples were analyzed for anti-SARS-CoV-2 RBD IgG antibodies using the Beckman Coulter chemiluminescent immunoassay (CLIA) in accordance with manufacturer instructions (https://www.beckmancoulter.com/en/products/immunoassay/access-sars-cov-2-igg-antibody-test). This CLIA test utilizes a recombinant SARS-CoV-2 protein specific for the receptor-binding domain (RBD) of the S1 protein. Results of this assay are based on the sample signal-to-cut-off (S/Co) ratio, with values <1.0 and >/= 1.0 corresponding to negative and positive results, respectively. The S/Co values reflect relative levels of anti-SARS-CoV-2 IgG antibodies.

ELISA IgG

Assay controls and serum samples were diluted to 1:64 followed by 2-fold dilution up to 1:32,768 and added to a 96-well microtiter plate (Thermo Scientific Immulon, Waltham, MA, USA) that was coated with SARS-CoV-2 recombinant RBD (Mount Sinai Medical Center, New York, NY, USA). A secondary anti-human IgG (Fab specific) antibody labeled with horse radish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to form a specific complex of antigen-antibody bound to the plate surface. The binding reaction was then enhanced visually with SIGMAFAST™ OPD (Sigma-Aldrich, St. Louis, MO, USA) substrate generating a yellow color for positive specimens. After application of the stop solution (3M Hydrochloric acid), the color changed from yellow to orange and optical density was measured at 490 nm. When the absorbance value was greater than the cut-off value (OD490 = 0.15) at a minimum dilution of 1:64, the specimen was reported as a positive result and the corresponding titer reported.

Statistical Analysis

The analysis population included COVID-19 convalescent plasma samples collected at the Blood Donation Service, therefore, no sample size calculations were performed. Correlation between CLIA and ELISA titer was analyzed using Spearman's rank, R square and κ index. The sensitivity and specificity of the two methods were analyzed using a 2x2 table. Linear relationship between the two assays was also assessed using a linear regression model, though due to violation of model assumptions, both CLIA and ELISA values were transformed to maintain the normality of residuals. The square-root of the CLIA assay was used to predict the log-transformed ELISA. The outputs of this model were then transformed back into raw units to show the relationship between the assays. A logistic regression model was created, as well, to predict the probability of a positive ELISA result (titer ≥ 1:1024). A CLIA S/Co cut-off value of 8.2 was determined using the ROC curve to meet 90% sensitivity and 82% specificity.

A two-sided P value of less than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using SAS version 9.4 (Cary, NC) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA).

Results

A total of 179 donated convalescent plasma units were included in this study. The inclusion criteria were mild

COVID-19 symptoms and positive RT-qPCR tests on nasopharyngeal swab samples. Females and those younger than 18 years of age were excluded. The characteristics of the donors are summarized in Table 1.

Aliquots of all units were analyzed using the Beckman Coulter CLIA platform and the relative amount of antibodies as measured by the S/Co value was recorded. Twenty-one samples (21/179; 11.7%) had S/Co values of less than 1.0 and were deemed negative for antibodies. Fifteen units that had S/Co values >1.0 (15/179; 8.4%) had negative ELISA titers. The majority of the units had a titer of ≥1:1024 (95/179, 53.1%). The sensitivity of ELISA was comparable to the sensitivity of CLIA (90.5% vs 88.3%, respectively; p-value = 0.18; Table 2). Table 3 shows the distribution of CLIA S/Co values and ELISA IgG antibody titers of all donated convalescent plasma. Samples with S/Co values of less than 10 had ELISA IgG titers ranging from 0 (negative) to 1:4096. Those with S/Co values of 10–20 had titers varying from 1:128 to 1:4096. Samples with CLIA S/Co value higher than 20 had titers of 1:2048 to 1:32,768.

Using linear regression, there is positive linear correlation between CLIA S/Co values and ELISA IgG titer (Rho = 0.75; Spearman's rank = 0.82, p-value = <0.0001; Figure 1). The agreement between the two methods was fair, with a κ index of 0.2741. A linear regression model was developed using the square root of the S/Co value and logarithmic transformation of the equivalent ELISA IgG titer (Table 4). Using this linear regression model, an estimate of the equivalent ELISA IgG titer when given a CLIA S/Co value can be computed using the following formula:

$$Raw\ Titer\ Dilution = e^{4.8949+0.70612\sqrt{CLIA\frac{S}{Co}value}}$$

Table 5 provides correlation of representative CLIA intensity to its expected titer concentration. Further, a predictive statistical file was created using this formula for more exact conversions from CLIA values to expected titer determinations (Supplementary Table). This file enables the user to enter any CLIA S/Co value and will

Table 2 Sensitivity of the Antibody Tests Compared to SARS-CoV-2 Viral RNA RT PCR

	Chemiluminescent Assay	**ELISA**
Sensitivity	88.3%	90.5%
No.	158/179	162/179
95% CI	82.5–91.8%	83.6–92.6%

Table 3 CLIA S/Co Values and ELISA Titers of Donated Convalescent Plasma

CLIA S/Co Values	**N (%)**	**ELISA IgG Titer Equivalent**
0.02 to 1	21 (11.7)	Neg to 1:512
>1 to 5	70 (39.1)	Neg to 1:4096
>5 to 10	32 (17.9)	Neg to 1:4096
>10 to 20	27 (15.1)	1:128 to 1:4096
>20	29 (16.2)	1:2048 to 1:32,768

ELISA IgG Titer	**N (%)**	**CLIA S/Co Value Equivalent**
Neg	15 (8.4)	0.02 to 6.25
1:32	2 (1.1)	0.37 to 0.5
1:64	7 (3.9)	0.09 to 9.71
1:128	10 (5.6)	0.25 to 11.51
1:256	19 (10.6)	0.05 to 7.66
1:512	31 (17.3)	0.81 to 19.65
1:1024	35 (19.6)	1.2 to 15.52
1:2048	22 (12.3)	2.32 to 26.62
1:4096	22 (12.3)	1.92 to 44.12
1:8192	5 (2.8)	23.48 to 34.26
1:16,384	8 (4.5)	28.08 to 63.4
1:32,768	3 (1.9)	28.82 to 52.64

Abbreviations: CLIA, chemiluminescent immunoassay, S/Co, sample to control intensity ratio, ELISA, enzyme-linked immunosorbent assay, Neg, negative, "0.

automatically generate the equivalent ELISA IgG titer with 95% confidence intervals.

A receiver operating characteristic (ROC) curve analysis was utilized to determine the optimal CLIA S/Co value that will reliably correspond to a high ELISA IgG titer (>1:1024). This analysis identified that a CLIA S/Co cut-off value of 8.2 gives a sensitivity of 90% and a specificity of 82% in predicting a titer dilution of more than 1:1024 (Figure 2).

Discussion

Our results showed significant linear correlation between the CLIA S/Co values and ELISA IgG titers. This indicates that comparable antibody quantification can be reliably derived from the results of an automated antibody detection system. This observation agrees with the conclusion of a similar study that analyzed the quantification capability of 8 commercially available immunoassays, two of which were CLIA-based.[19] They found that the automated systems provided the highest sensitivities (up to 98%), and one of the CLIA platforms had the best overall quantitative correlation to the neutralization titer (Rho=0.729).

Neutralization assays are the gold standard for assessing specific immunity and a benchmark for
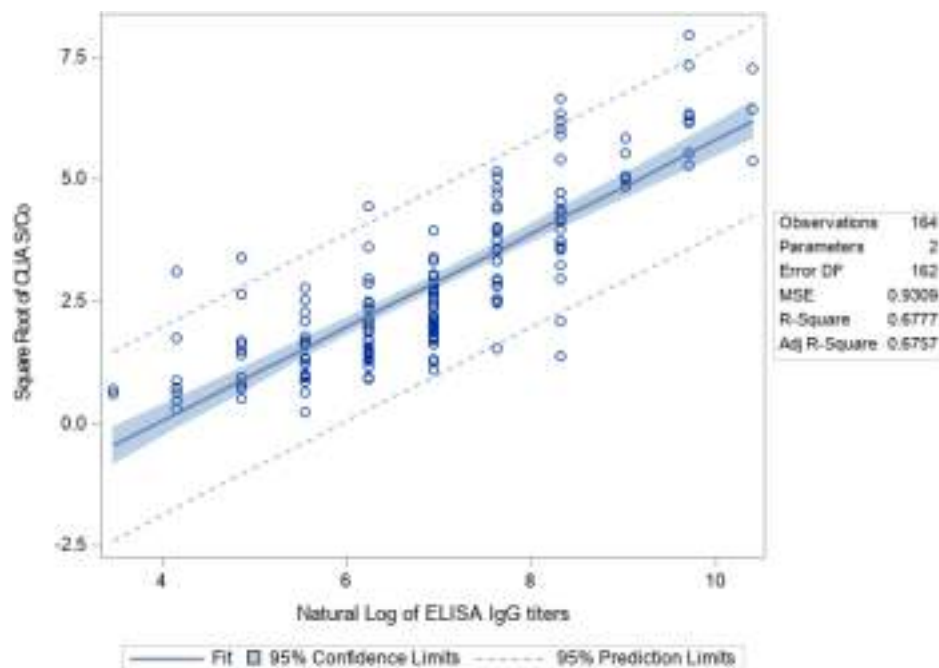
Figure 1 Linear regression analysis on the relationship of CLIA S/Co values and ELISA IgG titers.

other antibody assays. These tests are very complex, require incubation times of 5–7 days and a biosafety level 3 laboratory, limiting the routine use of these assays on a large scale.[20,21] Previous studies have shown positive correlation between neutralizing titer and ELISA that detects IgG antibodies against the SARS-CoV-2 S1 protein[19] or IgG antibodies against the receptor-binding domain of the S1 protein.[14] Similar studies correlating CLIA S/CO with ELISA assessment have been reported in other infectious disease spaces such as hepatitis,[22] measles[23] mycoplasma[24] and HIV[25] for diagnosis as well as donor infectious disease screening, where applicable. In a similar manner, the results of this study demonstrated correlation between ELISA antibody titer and CLIA S/Co values. Since ELISA titers correlate with the amount of neutralizing antibody, our results extend

this association to support the use of automated CLIA-based systems as a fast and convenient method of estimating neutralizing antibody quantities in convalescent plasma.

Only about a third of convalescent plasma units collected at our center had S/Co values of at least 8.2 and antibody titers of more than 1:1024. Preliminary evidence with COVID-19 suggests that patients with mild symptoms may develop very low titer antibodies.[14,26,27] One study observed that 18% of convalescent COVID-19 donors had undetectable neutralizing titers in their plasma samples collected an average of 30 days after the onset of symptoms.[28] A larger study performed by NYBC showed that more than half of plasma donors had low neutralizing titers and that there was a large variation in antibody titers among donors.[29] There is an existing challenge to screen convalescent plasma donors for antibody titers, as

Table 4 Linear Regression Model of the Root of Intensity as a Predictor of the Logarithmic Value of ELISA IgG Titer

Parameter Estimates							
Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|	95% Confidence Limits	
Intercept	1	4.89496	0.12611	38.82	<0.0001	4.64593	5.14399
Root of CLIA S/Co	1	0.70612	0.03826	18.45	<0.0001	0.63056	0.78168

Table 5 Representative CLIA Intensity to Expected Titer Concentrations

CLIA S/Co Values	Estimated ELISA IgG Antibody Titer		
	Estimated ELISA IgG Antibody Titer, Mean	Lower 95%	Upper 95%
0	1:133.6	1:104.2	1:171.4
1	1:270.7	1:195.7	1:374.5
2	1:362.7	1:254.1	1:517.7
3	1:454.0	1:310.5	1:663.7
4	1:548.5	1:367.6	1:818.4
5	1:648.0	1:426.6	1:984.2
6	1:753.4	1:488.1	1:1162.9
7	1:865.4	1:552.4	1:1355.8
8	1:984.5	1:619.8	1:1563.9
9	1:1111.3	1:690.6	1:1788.3
10	1:1246.3	1:765.0	1:2030.2
15	1:2058.5	1:1197.6	1:3538.4
20	1:3142.7	1:1747.4	1:5652.0
25	1:4562.2	1:2437.5	1:8538.9
30	1:6390.3	1:3293.3	1:12,399.7
35	1:8711.7	1:4343.2	1:17,474.0
50	1:19,692.3	1:8997.1	1:43,101.0
60	1:31,714.9	1:13,769.7	1:73,046.9
70	1:49,157.5	1:20,365.0	1:118,657.2

Abbreviations: CLIA, chemiluminescent immunoassay; S/Co, sample to control intensity ratio; ELISA, enzyme-linked immunosorbent assay.

Figure 2 Receiver operating characteristic (ROC) curve showing an area under the curve (AUC) of 0.9143. The orange line denotes a CLIA S/Co cutoff value of 8.2.

evidence shows that the majority of mild COVID-19 patients may not develop an adequate level of anti-SARS - CoV-2 antibodies to provide a therapeutic benefit. Screening of donated convalescent plasma units for antibody is, therefore, an essential step to ensure the efficacy of convalescent plasma as a therapeutic product with viral-neutralizing capacity.

Testing for antibodies should be performed to first confirm the presence of antibodies. Subsequent identification of CCP units that provide higher concentrations of anti-COVID (neutralizing) antibodies may provide a more appropriate logic of selecting optimal CCP units for patient administration. The utility of automated antibody detection systems can be extended

from simply a screening method to a semi-quantitative and quantitative functional antibody analysis. CLIA S/Co values can be used to estimate the ELISA antibody titer, and this can immediately provide crucial information on dosing of convalescent plasma. As a semi-quantitative method, a CLIA S/Co cut-off value of 8.2 can be used to reliably detect convalescent plasma donors or units with more than 1:1024 IgG antibody titer (90% sensitivity; 82% specificity); this value is well above the recently published FDA guidance of S/Co) ≥3.3 indicative of high titer CCP further relating the values to functional anti-viral titer-based activity using alternative instrumentation and/or methodologies.[30]

Application of additional approaches to assess the anti-COVID antibody concentration in CCP is paramount. In addition, methodological differences can introduce differences in results. For example, chemiluminescence methodology is able to determine total Ig (IgM, IgG, IgA) whereas ELISA determines only IgG. This may very well contribute towards the observed differences when a sample may have high percentage of IgM (giving high CLIA number), and low IgG (low titer) and vice versa. Although certain instruments have been promoted and approved to serve this need, many institutions and blood centers lack access to such instruments thereby causing a bottleneck in reference lab identification of

appropriate products for transfusion. Expansion to additional CLIA instrumentation can reduce the strain of CCP assessment and offer the ideal plasma product for patients who require such.

Furthermore, the methodology employed by CLIA instrumentation could be used to examine response to COVID-19 vaccination and, depending on target, provide distinction between immune response to COVID-19 infection from the immune response to vaccination.

The rapid turnover and cost benefit of such a CLIA based screening approach provides additional value. The testing can be easily implemented into existing chemiluminescent systems and provides opportunity costs for run time and personal skill set allocation. For example, in certain cases, time to result can be reduced from 180 minutes (ELISA) to 30 minutes (CLIA). In addition, adaptation can be automated in a CLIA setting and may require a lower degree of operator expertise (particularly if automated) than with ELISA.[22] This can beneficially impact the operational logic of the clinical laboratory to optimize efficiency of motion, human capital as well as resource allocation.

Acknowledgments

Funding

Disclosure

No conflicting relationship exists for any author.

References

1. World Health Organization. Coronavirus disease (COVID-19) Weekly Epidemiological Update and Weekly Operational Update. Available from: https://www.who.int/emergencies/diseases/novel-coronavirus -2019/situation-reports. Accessed 10 November 2020.
2. Duan K, Liu B, Li C, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *PNAS*. 2020;117(17):94 90–9496. doi:10.1073/pnas.2004168117
3. Shen C, Wang Z, Zhao F, et al. Treatment of 5 critically Ill patients with COVID-19 with convalescent plasma. *JAMA*. 2020;323 (16):1582–1589. doi:10.1001/jama.2020.4783
4. Chen L, Xiong J, Bao L, Shi Y. Convalescent plasma as a potential therapy for COVID-19. *Lancet Infect Dis*. 2020;20(4):398–400. doi:10.1016/S1473-3099(20)30141-9
5. Liu STH, Lin HM, Baine I, et al. Convalescent plasma treatment of severe COVID-19: a propensity score–matched control study. *Nat Med*. 2020;26:1708–1713. doi:10.1038/s41591-020-1088-9
6. Joyner MJ, Senefeld JW, Klasses SA, et al. Effect of convalescent plasma on mortality among hospitalized patients with COVID-19: initial three-month experience. *MedRxiv*. doi:10.1101/2020.08.12. 20169359
7. Wu F, Liu M, Wang A, et al. Evaluating the association of clinical characteristics with neutralizing antibody levels in patients who have recovered from mild COVID-19 in Shanghai, China. *JAMA Intern Med*. 2020;180(10):1356. doi:10.1001/jamainternmed.2020.4616
8. Choe P, Kang C, Suh H, et al. Antibody responses to SARS-CoV-2 at 8 weeks postinfection in asymptomatic patients. *Emerg Infect Dis*. 2020;26(10):2484–2487. doi:10.3201/eid2610.202211
9. Ibarrondo FJ, Fulcher JA, Goodman-Meza D, et al. Rapid decay of anti–SARS-CoV-2 antibodies in persons with mild COVID-19. *N Engl J Med*. 2020;383(11):1085–1087. doi:10.1056/NEJMc2025179
10. FDA. Recommendations for Investigational COVID-19 Convalescent Plasma. Available from: https://www.fda.gov/vac cines-blood-biologics/investigational-new-drug-ind-or-device- exemption-ide-process-cber/recommendations-investigational- COVID-19-convalescent-plasma. Accessed 16 November 2020.
11. Nie J, Li Q, Wu J, et al. Establishment and validation of a pseudovirus neutralization assay for SARS-CoV-2. *Emerg. Microbes Infect*. 2020;9:680–686. doi:10.1080/22221751.2020.174 3767
12. Tang YW, Schmitz JE, Persing DH, Stratton CW. The laboratory diagnosis of COVID-19 infection: current issues and challenges. *J Clin Microbiol*. 2020;58:e00512–20. doi:10.1128/JCM.00512- 20
13. Tan CW, Chia WN, Qin X, et al. A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2– spike protein–protein interaction. *Nat Biotechnol*. 2020;38(9):10 73–1078. doi:10.1038/s41587-020-0631-z
14. Salazar E, Kuchipudi SV, Christensen PA, et al.Relationship between Anti-Spike Protein Antibody Titers and SARS-CoV-2 In Vitro Virus Neutralization in Convalescent Plasma. Preprint. bioRxiv. 2020;. doi:10.1101/2020.06.08.138990.
15. Campbell AK, Patel A. A homogeneous immunoassay for cyclic nucleotides based on chemiluminescence energy transfer. *Biochem J*. 1983;216(1):185–194. doi:10.1042/bj2160185
16. Vo-Dinh T. Chemiluminescence. *Encyclopedia of Applied Physics*. 2003. doi:10.1002/3527600434.eap063
17. Wang YFW, Kobayashi M. *Antibody Detection: Principles and Applications/Advanced Techniques in Diagnostic Microbiology*. Springer US; 2013:53–73.
18. Cinquanta L, Fontana DE, Bizzaro N. Chemiluminescent immunoassay technology: what does it change in autoantibody detection? *Auto Immun Highlights*. 2017;8:9. doi:10.1007/s13317-017-0097-2
19. Weidner L, Gansdorfer S, Unterweger S, et al. Quantification of SARS-CoV-2 antibodies with eight commercially available immunoassays. *J of Clin Vir*. 2020;129:104540. doi:10.1016/j.jcv.20 20.104540
20. Kohmer N, Westhaus S, Rühl C, Ciesek S, Rabenau HF. Clinical performance of different SARS-CoV-2 IgG antibody tests. *J. Med. Virol*. 2020;1–5. doi:10.1002/jmv.26145
21. Perera RAPM, Mok CKP, Tsang OTY, et al. Serological assays for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), March 2020. *Eurosurveillance*. 2020;25(16). doi:10.2807/1560- 7917.ES.2020.25.16.2000421

22. Madiyal M, Sagar S, Vishwanath S, Banerjee B, Eshwara VK, Chawla K. Comparing Assay Performance of ELISA and Chemiluminescence Immunoassay in Detecting Antibodies to Hepatitis B Surface Antigen. *J Clin Diagn Res*. 2016;10:DC22–DC25. doi:10.7860/JCDR/2016/24108.8921

23. de Ory F, Minguito T, Balfagón P, Sanz JC. Comparison of chemiluminescent immunoassay and ELISA for measles IgG and IgM. *APMIS*. 2015;123(8):648–651. doi:10.1111/apm.12413

24. Chen D, Zhang Y, Xu Y, et al. Comparison of chemiluminescence immunoassay, enzyme-linked immunosorbent assay and passive agglutination for diagnosis of *Mycoplasma pneumoniae* infection. *Ther Clin Risk Manag*. 2018;14:1091–1097. doi:10.2147/TCRM.S159227

25. Chang L, Zhao J, Guo F, et al. Comparative evaluation and measure of accuracy of ELISAs, CLIAs, and ECLIAs for the detection of HIV infection among blood donors in China. *Can J Infect Dis Med Microbiol*. 2020;2020:2164685. doi:10.1155/2020/2164685

26. Klein S, Pekosz A, Park H-S, et al. Sex, age, and hospitalization drive antibody responses in a COVID-19 convalescent plasma donor population. *J Clin Invest*. 2020;130(11):6141–6150. doi:10.1172/JCI142004

27. Madariaga MLL, Guthmiller JJ, Schrantz S, et al. Clinical predictors of donor antibody titre and correlation with recipient antibody response in a COVID-19 convalescent plasma clinical trial. *J Intern Med*. 2020. doi:10.1111/joim.13185

28. Robbiani DF, Gaebler C, Muecksch F, et al. Convergent antibody responses to SARS-CoV-2 in convalescent individuals. *Nature*. 2020;584(7821):437–442. doi:10.1038/s41586-020-2456-9

29. Luchsinger LL, Ransegnola BP, Jin DK, et al. Serological assays estimate highly variable SARS-CoV_2 neutralizing antibody activity in recovered COVID-19 patients. *J. Clin. Microbiol*. 2020. doi:10.1128/JCM.02005-20

30. https://www.fda.gov/media/141477/download. Accessed March 2, 2021.

Obstacles to Early Diagnosis and Treatment of Inherited von Willebrand Disease: Current Perspectives

Giancarlo Castaman & Silvia Linari

REVIEW

Obstacles to Early Diagnosis and Treatment of Inherited von Willebrand Disease: Current Perspectives

Giancarlo Castaman
Silvia Linari

Center for Bleeding Disorders and Coagulation, Department of Oncology, Careggi University Hospital, Florence, Italy

Abstract: Von Willebrand disease (VWD), the most common inherited bleeding disorder, is highly heterogeneous, and its early diagnosis may be difficult, especially for mild cases and in qualitative von Willebrand factor (VWF) defects. Appropriate VWD diagnosis requires the combination of personal and/or family history of bleeding and abnormal VWF laboratory testing. The use of bleeding assessment tools has been helpful in standardizing bleeding history collection and quantification of bleeding symptoms to select patients who may benefit of further hemostatic testing. Type 1 and 3 VWD which represent quantitative VWD variants are relatively easy to diagnose. The diagnosis of type 2 VWD requires multiple assessments to evaluate the effects induced by the responsible abnormality on the heterogeneous functions of VWF. Sensitive and reproducible tests are needed to evaluate different VWF activities, starting from measuring VWF-platelet interaction. In the recent years, several increasingly sensitive, rapid and automated assays have been developed, but they are not widely available so far. Genetic testing for VWD diagnosis is not a common practice because VWF gene is very large and highly polymorphic and therefore it is used only in specific cases. It is evident that the early and correct VWD diagnosis allows optimal management of bleeding and situations at risk. Tranexamic acid, desmopressin, replacement therapy with plasma-derived concentrates with a variable content of VWF and FVIII, or the new recombinant VWF are the different therapeutic options available. Careful VWD classification guides treatment because desmopressin is widely used in type 1 while replacement therapy is the cornerstone of treatment for type 2 and 3 variants.
Keywords: von Willebrand disease, von Willebrand factor, bleeding history, laboratory assays, desmopressin, replacement therapy

Correspondence: Giancarlo Castaman
Center for Bleeding Disorders and Coagulation, Department of Oncology, Careggi University Hospital, Largo G. Brambilla 3, Florence, 50134, Italy
Tel +39 055 7947587
Fax +39 055 7947794
Email giancarlo.castaman@unifi.it

Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder, characterized by an extreme variability of clinical manifestations and laboratory phenotypes. The clinical spectrum varies from subjects with dubious, mild symptoms to patients with spontaneous, severe, sometimes life-threatening bleeds. Due to the complexities of clinical and laboratory assessment, the diagnosis can be difficult, especially in mild forms, so that the disease may be under-diagnosed also among symptomatic patients.

In this paper, we analyze the actual obstacles to early diagnosis and subsequent treatment of VWD.

VWD

VWD is a bleeding disorder characterized by a deficiency and/or an abnormality of von Willebrand factor (VWF), an adhesive glycoprotein that plays an essential function in both primary and secondary hemostasis. VWF is required for platelet adhesion to the subendothelium exposed by a vascular injury and also in mediating platelet–platelet interactions together with fibrinogen, thus enhancing the hemostatic process. Furthermore, VWF is the carrier of coagulation factor VIII (FVIII), driving it to the site of vascular damage and modulating its proteolytic degradation.[1,2] VWD is extremely heterogeneous from a genetic and clinical point of view. The inheritance can be autosomal dominant or recessive, although VWD usually presents with an autosomal dominant inheritance. Thus, patients often report other relatives with a history of bleeding, although severity may differ among affected members belonging to same family. In epidemiological studies, VWF deficiency is identified in about 1% of the general population, but only about 0.01% has clinically significant bleeding symptoms.[3,4] The actual prevalence is probably between these two extreme values and some people could have undiagnosed VWD-related bleeding.

Biology of VWF

VWF is a large multimeric adhesive plasma glycoprotein synthesized in megakaryocytes and endothelial cells.[5–7] The biosynthesis and organization of VWF involves a complex intracellular pathway and defects at any step of which may cause a decreased plasma VWF level or dysfunction. The gene coding for VWF is located on the short arm of chromosome 12 (12p13.2) and it includes 52 exons.[8] VWF has a partial pseudogene located on chromosome 22 with 97% homology with the authentic *VWF* gene.[9] The presence of this pseudogene can complicate genetic analysis and contribute to a pathophysiological mechanism of gene conversion.

The primary translation protein contains 2813 amino acids, composed by a prepeptide of 22 amino acid, a propeptide 741 amino acid long and the mature VWF subunit of 2050 amino acids. The different binding abilities of VWF are scattered over four repeated domains (D1, D2, D', D3, A1, A2, A3, D4, C1-C6, CK).[10,11] The D'-D3 domains are essential for binding to FVIII. The VWF A1 domain is important in binding VWF to platelets through the platelet receptor glycoprotein, GPIb, and contains binding sites for heparin and collagen. The A2 domain contains the cleavage site which regulates post-secretion processing of VWF size by the VWF-cleaving protease, ADAMTS13. The A3 domain contains a binding site for collagen. The C1 domain contains a unique RGDS (Arg-Gly-Asp-Ser) sequence responsible for binding to the glycoprotein GPIIb/IIIa complex on platelet surface.

The cleavage of the signal peptide prompts the VWF subunits to dimerize at the C-terminal region through intermolecular disulfide bridges in the endoplasmic reticulum. Then, the acidic pH and high calcium concentration in the Golgi induce the building of VWF multimers through bridging through disulfide residues between D3 domains. The propeptide remains non-covalently bound to the VWF multimer inside the cell until fully cleavage allows for the separate release into plasma. VWF is stored in α-granules of platelets or endothelial Weibel-Palade bodies[11] and secreted in plasma or abluminally to subendothelium through a constitutive and a regulated pathway. The subsequent proteolysis by ADAMTS13 produces variably sized multimers,[12] whose clearance occurs later by macrophages in the liver and spleen.[13]

VWD Clinical Manifestations and Classification

The quantitative deficiency and/or qualitative abnormality of VWF lead to a variable risk of bleeding, depending on severity of the deficiency of VWF and FVIII. VWD clinical manifestations include predominantly mucosal bleeding (epistaxis, menorrhagia, gastrointestinal bleeds), surgery or trauma-related bleeding and joint bleeding, especially for patients with more severe FVIII deficiency.

VWD is classified into three main types categorizing quantitative (type 1 and 3) or qualitative (type 2) VWF abnormalities (Table 1).[14,15]

Unlike type 3, type 1 VWD (60–70% of cases) presents milder VWF deficiency (10–30 U/dL), with normal or slightly reduced FVIII levels. The inheritance transmission is autosomal dominant and missense amino acid changes represent the majority of causative mutations. Bleeding manifestations are mild. VWF is virtually absent in patients with type 3 (1–2% of cases), and FVIII levels are usually very low. Most of these patients are homozygous or compound heterozygous for null alleles in the *VWF* gene.[16] The remaining 20–25% of patients have Type 2VWD, further divided into four subtypes which take into account distinct pathophysiological mechanisms and laboratory features.[14,17]

Table I Classification of Von Willebrand Disease

	Quantitative Deficiency of VWF
Type 1	Partial quantitative deficiency of VWF- autosomal dominant transmission (~60–70% of all cases)
Type 3	Virtually complete deficiency of VWF - autosomal recessive transmission (~ 1–2% of all cases)
	Qualitative Deficiency of VWF
Type 2	Qualitative deficiency of VWF (~ 25–30% of all cases)
Type 2A	Qualitative variants with decreased platelet-dependent function associated with the absence of high and intermediate-molecular–weight VWF multimers
Type 2B	Qualitative variants with increased affinity for platelet GpIb
Type 2M	Qualitative variants with decreased platelet-dependent function not caused by the absence of high-molecular–weight VWF multimers
Type 2N	Qualitative variants with markedly decreased affinity for FVIII

Note: Adapted with permission from Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the subcommittee on von Willebrand factor. J Thromb Haemost. 2006;4:2103–2114.15.

Typically, type 2A is caused by mutations resulting in a decreased binding to platelets caused by the absence of significant reduction of high and intermediate-molecular-weight VWF multimers and. In type 2B, the mutant VWF shows an increased affinity for GpIb on platelet surface, which induces a rapid clearance of VWF. Type 2M is characterized by a decreased in or absent binding to GpIb, with an apparently intact VWF multimer structure. Type 2N (Normandy) VWF presents variable binding ability for FVIII with a shortened FVIII half-life. Most type 2 are usually inherited as a dominant trait with high penetrance and expressivity apart from type 2N which is transmitted in a recessive manner.

VWD Treatment

The correction of the dual hemostatic defect represents the aim of treatment in VWD. Bleeding history together with FVIII and VWF and levels in plasma allow to define the bleeding risk in each patient. When FVIII level is greater than 40–50 U/dL, the risk of serious bleeding is limited and tranexamic acid alone may allow to control most minor bleeds. In patients with a more severe defect or VWF as in qualitative variants, VWF and FVIII plasma levels must be corrected to allow for effective hemostasis. The available therapeutic strategies are based on increasing VWF and FVIII by releasing VWF from stores in endothelial cells triggered with desmopressin (DDAVP)

or replacing missing VWF with plasma-derived (pd) coagulation factor concentrates containing FVIII and VWF.

DDAVP (1-deamino-8-D-arginine-vasopressin) is a synthetic analog of the antidiuretic hormone vasopressin[18,19] which can be given by intravenous, subcutaneous, or intra-nasal route. In responsive patients, a peak of the released FVIII and VWF > 50 U/dL is usually obtained 60 minutes after its administration. Usually, 0.3 µg/kg is used for intravenous or subcutaneous administration, and 300 µg in adults and 150 µg in children with intranasal spray. DDAVP is cheap and carries no risk of viral transmission; however, progressive reduction in magnitude of response (tachyphylaxis) is observed after repeated closely spaced doses due to the cellular depletion of VWF/FVIII. DDAVP is mostly efficacious in patients with type 1 VWD with normal VWF content in cells and baseline VWF and FVIII levels >10 U/dL.[20] In type 2B, DDAVP causes a transient occurrence or aggravation of thrombocytopenia and is considered contraindicated[21] while heterogeneous patterns are observed in other type 2 varieties.[14,22] Type 3 patients do not show any significant increase post-administration.

Plasma-derived VWF-FVIII (pd-VWF/FVIII) concentrates are the treatment of choice when DDAVP is not useful.[14,22] Several intermediate and high-purity pd-VWF/FVIII concentrates are available (Table 2). All these concentrates proved effective and safe in VWD although they have different VWF and FVIII content and show variable lack of high molecular weight (HMW) multimers.[23] There are several national or international guidelines on the dosages to be used according to different clinical situations.[14,24,25] A significant accumulation of FVIII may occur after repeated infusions of pd-VWF/FVIII since FVIII endogenously synthesized is stabilized by the infused VWF thus adding to that exogenously provided.[26,27] This phenomenon may lead to the risk of deep vein thrombosis or cardiovascular complications, especially if other pro-thrombotic risk factors are present.[28] Thus, daily monitoring of FVIII:C to maintain plasma levels below 150 U/dL is recommended to prevent thrombotic risk especially during major surgery. Also, a pd-VWF high purity concentrate containing little FVIII (Wilfactin ®; LBF, Les Ulis, France) could be chosen in patients with mild FVIII reduction. In this case, the co-administration of a priming dose of FVIII is recommended in patients with basal FVIII:C levels <30 U/dL, when immediate hemostasis is required, since 6–8 hours are necessary for endogenous FVIII to reach levels higher

Table 2 Pd-VWF/FVIII Concentrates Licensed in Europe

Product	Brand	Purification	Viral Inactivation	VWF:RCo/Ag (Ratio)	VWF:RCo/FVIII (Ratio)
Alphanate	Grifols	Heparin ligand chromatography	S/D + dry heat (80°, 72h)	0.47 ± 0.1	0.91 ± 0.2
Fanhdi	Grifols	Heparin ligand chromatography	S/D + dry heat (80°, 72h)	0.47 ± 0.1	1.04 ± 0.1
Haemate P	CSL Behring	Multiple precipitation	Pasteurization (60°, 10h)	0.59 ± 0.1	2.45 ± 0.3
Immunate	Baxter	Ion exchange chromatography	S/D + vapor heat (60°, 10h)	0.47	1.1
Wilate	Octapharma	Ion exchange + size exclusion chromatography	S/D + dry heat (100°, 2h)	–	0.9
Wilfactin	LFB	Ion exchange + affinity	S/D, 35 nm filtration, dry heat (80°, 72h)	0.95	50
Veyvondi/ VonVendi	Shire/ Takeda	Chinese Hamster Ovary (CHO) cell line co-expressing the VWF and FVIII genes, in absence of any animal or other human plasma proteins; purified by immune-affinity chromatography	None	1.16 ± 0.25	>100

Abbreviations: VWF, von Willebrand factor; RCo, ristocetin co-factor; Ag, antigen; FVIII, factor VIII; S/D, solvent/detergent.

than 50–60 U/dL after infusion.[29] Recently, a human recombinant VWF (r-VWF) Vonicog alpha has been produced by using a genetically engineered Chinese Hamster Ovary (CHO) cell line co-expressing VWF and FVIII genes and purified by immune-affinity chromatography and approved in some countries.[30] Vonicog alpha is a >99% pure r-VWF molecule with the full range of VWF multimers because ADAMTS13 is not present during the manufacturing process. Therefore, also the ultra-large multimers (ULMs) are present, which are the most active forms,[31] similarly to those observed after release in plasma from Weibel-Palade bodies of endothelial cells. These ULMs are however rapidly cleaved by ADAMTS-13 when infused for treatment. Several studies in VWD patients have demonstrated the efficacy and safety for treating spontaneous bleeding and during surgery.[32–34] Two Phase III studies are assessing its safety and efficacy in children and for prophylaxis.

VWD Diagnosis

The diagnosis of VWD may be complex, especially in mild form, due to the extreme clinical variability, the difficult standardization of diagnostic tests, the presence of physiological and pathological variables influencing the plasma level of VWF. VWF plasma levels vary with age, increase during pregnancy, physical activity, inflammation, infections and cancer. Furthermore, plasma VWF levels are influenced by blood group with VWF levels 25–30% lower in type-0

individuals than in those with other blood groups.[35] Subjects with blood group 0 show a VWF accelerated clearance[36] and an enhanced ADAMTS13-mediated proteolysis.[37] Thus, low VWF level alone does not suggest the presence of VWD and the cut-off for the diagnosis especially in type 1 is still discussed.[38] However, it has been proposed as a diagnostic threshold the presence of VWF level <30 U/dL[14,24] because an increased risk of bleeding is evident[39] and the chance of detecting a causative mutation is very high.[40] Subjects with levels between 30 and 50 U/dL may represent a difficult challenge.[41,42] These subjects, and especially the females, may have increased bleeding scores, but inconsistent linkage to VWF locus and mutations identified roughly in 50% only and they have mostly blood group 0.[41,42] The category "Low VWF" has been designed for these very frequent individuals.[41] From the therapeutic point of view, these patients can be safely and effectively managed by using antifibrinolytics and/or DDAVP.[43]

Therefore, the definite diagnosis of VWD requires the combination of personal history of bleeding, family history of bleeding or diagnosed VWD and abnormal laboratory testing for VWD.

Bleeding Assessment Tools

Mild bleeding events may frequently occur also in subjects without a specific bleeding disorder. Furthermore, reporting and interpreting bleeding episodes may be subjective. The development of bleeding assessment tools (BATs) has

allowed to standardize bleeding history collection and to assign a quantitative score to bleeding symptoms.[44] These questionnaires generate a cumulative score of different bleeding symptoms according to their severity and frequency. The ISTH-BAT is designed for both pediatric and adult subjects and it evaluates 12 different bleeding symptoms, scoring from −1 (eg, no bleeding after invasive procedures) to +4 (eg, blood transfusion required after invasive procedures) according to the presence and severity of a specific symptom. An ISTH-BAT score ≥3 in children, ≥4 in males, ≥6 in females is considered positive for the presence of a significant bleeding history.[45] BAT is an important mean to gather a detailed and standardized bleeding history thus selecting patients requiring additional hemostatic evaluation.[46]

Laboratory Testing for VWD

Among the hemostatic screening tests, the aPTT (activated partial thromboplastin time) and PFA-100 (Platelet Function Analyzer) may be used as an initial screen for possible VWD patients. However, the aPTT may be prolonged only in those patients in whom FVIII plasma level is reduced (Type 3 or Type 2N VWD). The PFA-100 mimics in vivo primary hemostasis after small vessel wall injury and has a high sensitivity (90%) when VWF is significantly reduced (<20–25 U/dL) and thus most patients with type 2 are also identified.[47] However, PFA-100 is normal in type 2N and in many patients with VWF levels only slightly reduced. Furthermore, a complete blood count is also required to assess the presence of thrombocytopenia which may suggest type 2B VWD.

The appropriate VWD diagnosis is based on several laboratory assays, which explore the different functions of VWF[14] (Table 3). Laboratory testing for VWD initially includes VWF antigen determination (VWF:Ag), a VWF-platelet binding test, usually VWF-ristocetin cofactor activity assay (VWF:RCo) or less widely used newer tests (VWF:GPIbM, VWF:GPIbR), and the measurement of coagulant activity of FVIII (FVIII:C).

Type 1 and 3 VWD are characterized by equally low or absent VWF:Ag and VWF-platelet binding activity. The qualitative type 2 variants require additional tests to make an appropriate diagnosis, like VWF:collagen binding activity (VWF:CB), platelet-rich plasma agglutination at different ristocetin concentrations (RIPA), VWF-FVIII binding (VWF:FVIIIB) and VWF propeptide (VWFpp). The assessment of VWF multimer pattern may be also needed.[48] VWD laboratory diagnosis can therefore be

complex not only for the number of tests that may be necessary, but also for the poor reproducibility for some of them. Furthermore, some tests are still limited to a few selected laboratories.

VWF:Ag measurement is usually carried out with automated ELISA or latex immunoassays (LIA).[49] Although these tests have a high reproducibility, they are of limited value for type 2 VWD in which sometimes they could be normal and must be used with other functional assays.

VWF:RCo activity is the time-honored assay for assessing the interaction of VWF with its platelet GpIb receptor. Ristocetin induces a conformational change in VWF, thus exposing the A1 domain which binds to GpIb and produces platelet agglutination proportional to the function of VWF. A VWF:RCo/VWF:Ag ratio <0.6 suggests the presence of a qualitatively abnormal VWF (type 2 VWD).[24] However, traditional VWF:RCo assays using normal fresh or formalin-fixed platelets have low inter-laboratories reproducibility,[50] high coefficient of variation and poor sensitivity for very low VWF values. The optimal ristocetin concentration may be another limitation of the test,[51] as well as also the erroneous low levels in presence with p. P1467S and p.D1472H VWF polymorphisms.[52]

The search for assays with less variability and increased sensitivity than VWF:RCo has prompted the development of new tests. VWF:GPIbR uses recombinant GPIbα fragments (rGPIb) adhered to microparticles, eliminating the use of whole platelets.[53] Instead, VWF:GPIbM uses recombinant GPIbα fragments containing two gain-of-function variants to induce spontaneous binding of VWF without ristocetin,[54] but introducing a non-physiologic mutant GPIb receptor. Both assays have an excellent coefficient of variation, a more sensitive lower limit of detection, and excellent correlation with VWF:RCo,[55] besides not being affected by some common *VWF* variants.[56]

FVIII:C assay must also be performed because VWF is the carrier for FVIII and usually a reduction of VWF causes also a variable reduction of FVIII. FVIII:C/VWF:Ag ratio is around 1 in normal subjects while in type 2N it is decreased (<0.5). In type 3 patients, FVIII:C is typically <10 U/dL.

The VWF multimer pattern analysis is useful to identify type 2 cases, although the traditional procedure with agarose gel electrophoresis is technically difficult and time consuming.[57] Recently a semiautomated rapid, and sensitive method[58] has been made available. All size multimers are present in Type 1 VWD, although reduced in concentration, while they are absent in type 3. The absence of HMW

Table 3 Laboratory Pattern in Von Willebrand Disease

Laboratory Assay	Type 1	Type 2A	Type 2B	Type 2M	Type 2N	Type 3
APTT	Prolonged or normal	Prolonged or normal	Normal or prolonged	Normal or prolonged	Prolonged or normal	Prolonged
Platelet count	Normal	Normal	Low or normal	Normal	Normal	Normal
PFA-100 (closure time; CT)	Prolonged or normal	Prolonged, no closure	Prolonged, no closure	Prolonged, no closure	Normal	Prolonged, no closure
FVIII:C	Low or normal	Low or normal	Low or normal	Normal or low	Low	Low (<10 U/dL)
VWF:Ag	Low (<50 U/dL)	Low or normal	Low or normal	Normal or low	Normal or low	Very low (<3 U/dL)
VWF:RCo VWF:GPIbR VWF:GPIbM	Low, rarely normal	Low (<30 U/dL)	Low, rarely normal	Low or normal	Normal or low	Very low (<3 IU/dL)
VWF:CB	Low, rarely normal	Very low (<15 U/dL)	Low (<40 U/dL)	Low or normal	Normal or low	Very low (<3 U/dL)
VWF:RCo/ VWF:Ag ratio	Normal (>0.7)	Low (<0.7)	Low (<0.7)	Low or normal	Normal (>0.7)	Variable
RIPA using patient platelets	Reduced or normal	Reduced or normal	Increased	Reduced or normal	Normal	Absent
VWF multimer pattern	Normal pattern, VWF reduced	Large to intermediate multimers lacking	Large multimers missing	Normal VWF multimer distribution (but with possible abnormal bands)	Normal	Multimers absent
VWFpp/VWF: Ag ratio	Normal, Increased in type 1C	Normal or increased	Increased	Normal	Normal	Absent

Abbreviations: APTT, activated partial thromboplastin time; FVIII:C, factor VIII coagulant; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor; VWF:GPIbR, VWF recombinant GPIbα fragments; VWF:GPIbM, VWF recombinant GPIbα fragments with two mutations; VWF:CB, VWF collagen binding; RIPA, ristocetin-induced platelet agglutination; VWFpp, VWF propeptide.

multimers is observed in type 2A and 2B VWD, while a normal pattern is present in type 2M and 2N.[59]

VWF:CB measures the VWF ability to bind to exposed collagen at the site of vascular injury. This ability correlates with the presence of HMW multimers, and patients with type 2A or 2B VWD have reduced VWF:CB.[60] In Type 1, VWF:CB/VWF:Ag ratio is approximately 1, while a VWF:CB/VWF:Ag ratio <0.6 suggests the deficiency of HMW multimers as in type 2A or more rarely a specific collagen binding defect without loss of HMW multimers. The ratio is normal in type 2 M which has a normal VWF multimer pattern.

VWF:CB is usually tested by an ELISA but recently an automated and rapid chemiluminescence method has become available.[61] Typically, a mixture of type I or type III collagen is used because it increases specificity.[62] The A3 VWF domain binds type I and type III collagen, while the A1 domain binds type IV and type VI collagen, but assays testing this latter interaction are not widely available.[63]

The VWF-platelet binding assessed in platelet-rich-plasma at different ristocetin concentrations (RIPA) may help in distinguishing type 2B from type 2A because in type 2 B (and platelet-type VWD) platelet aggregation

occurs even at low-dose ristocetin concentration (≤0.6 mg/m).[14]

The VWF-FVIII binding (VWF:FVIIIB) test is required to diagnose type 2N, characterized by an abnormal VWF-FVIII binding.[64] The ELISA microplate assay explores the capacity of patient's plasma VWF to bind to recombinant FVIII.

The VWF propeptide (VWFpp) is in a 1:1 ratio with VWF in plasma.[65] Its measurement is used as a surrogate marker of VWF synthesis and secretion. An increased VWFpp/VWF:Ag ratio identifies those patients with a shortened VWF half-life from plasma, commonly referred as Type 1 C (clearance), like the Vicenza variant. VWFpp/VWF:Ag ratio is usually slightly elevated increased in type 2A and 2B. The assay is still not widely performed.

Finally, genetic analysis for VWD diagnosis is not common practice because of the large size of the VWF gene and the time required. Furthermore, VWF is highly polymorphic.[14,66] Therefore, it is used in specific cases, where it may have implications for diagnosis, patient's management or counseling.[14] Genetic testing in type 1 VWD is poorly informative, because an average of 62% of subjects shows a sequence variant in *VWF* gene. In particular, when VWF is <30 U/dL, the detection rate of identifiable sequence variants is around 80%[67] and in patients with VWF:Ag >30 U/dL, mutations in VWF gene are not consistently identified.[40,68] Mutations are more detectable in type 2 and type 3 VWD. Mutations causing type 2A usually are located in A2 domain, near the site of ADAMTS13 cleavage, or in the N or C terminal multimerization domains.[69] In type 2B and 2M, mutations are mainly identified in the A1 domain and in type 2N mutations are located in the VWF D' and D3 domains. Identifying mutations responsible for type 2 B rule out the possible diagnosis of platelet-type VWD. Identifying
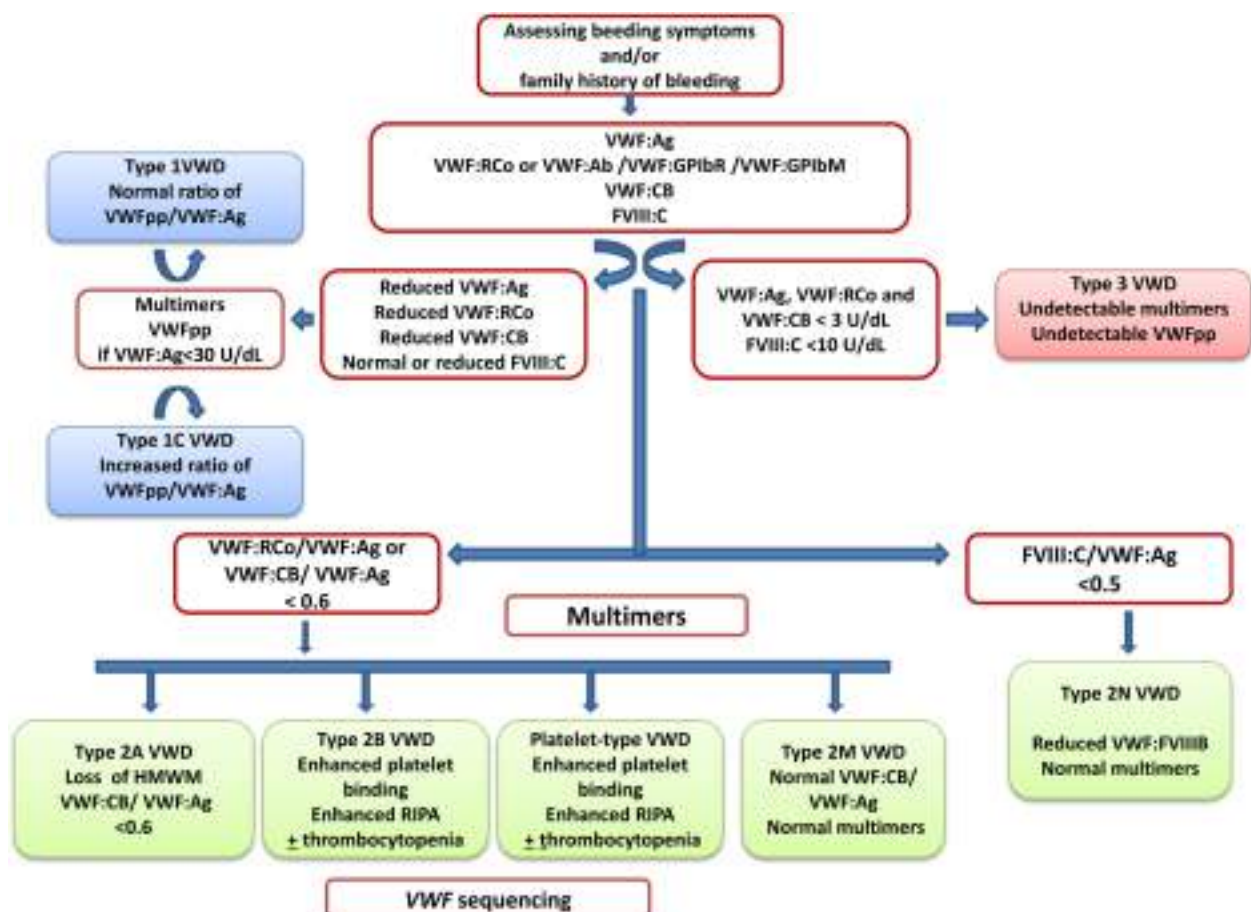
Figure 1 Algorithm for laboratory diagnosis of von Willebrand disease, modified with permission of Nancy International Ltd Subsidiary AME Publishing Company, from Von Willebrand disease in the United States: perspective from the Zimmerman program, Flood VH, Abshire TC, Christopherson PA, et al, volume 3, 2018]; permission conveyed through Copyright Clearance Center, Inc.[54]

mutations responsible for type 2 N is helpful to exclude the possible diagnosis of mild/moderate hemophilia A. Mutations responsible for type 1 and 3 are scattered over the entire VWF gene. In type 3 VWD, genetic analysis may help to determine the risk of alloantibodies development with possible anaphylactic reactions upon treatment, a complication more common in presence of homozygous large gene deletions.[70] The online ISTH VWF database includes most of the mutations and polymorphisms so far identified (http://www.vwf.group.shef.ac.uk).[16]

Diagnostic Flow-Chart and Therapeutic Management

VWD is a frequent and very heterogeneous bleeding disorder. Bleeding severity increases from type 1 to 3 and treatment differs. Several laboratory assays are available to properly diagnose VWD. In clinical practice, a flow-chart can be very useful to support a laboratory diagnosis.[71]

This algorithm starts with the evaluation of bleeding history in the patient and take advantage of the different laboratory assays used in different steps for a possible diagnosis of VWD (Figure 1).

When not all laboratory assays are available for a definitive VWD diagnosis, minimal diagnostic criteria may be considered an increased BS and VWF:RCo <40 U/dL. Based on the significance of these two criteria, an adequate VWD management may be provided (Figure 2).[72]

Conclusion

VWD is a common inherited bleeding disorder; however, its early diagnosis still may be complex. Several reasons are responsible for these diagnostic and consequently therapeutic difficulties. The first difficulty lies in understanding the significance of the reported bleeding. Mild bleeding symptoms may occur also in normal subjects and interpretation of hemorrhagic events erratic. For this
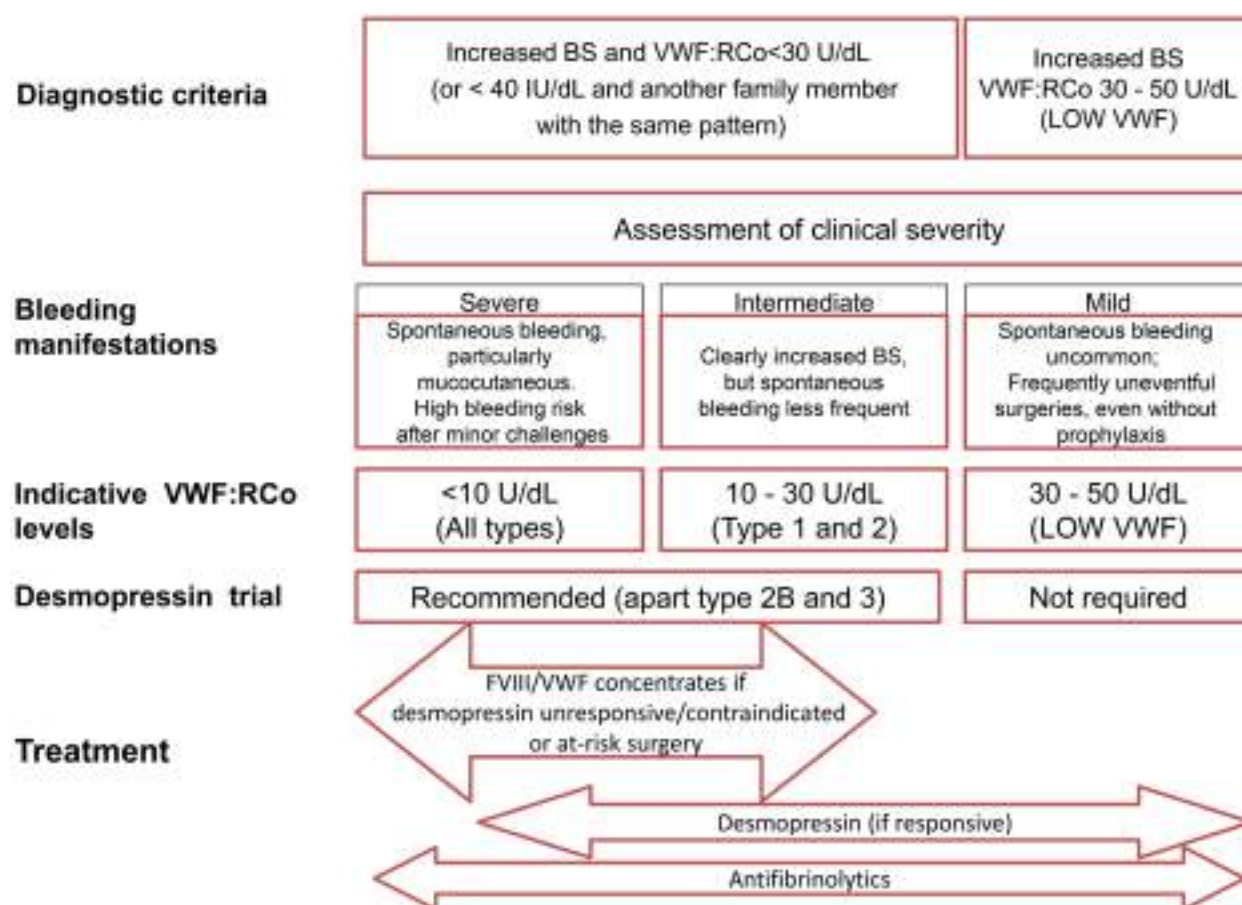
Figure 2 Clinical spectrum of VWD: implications for management.
Notes: Copyright ©2019. Taylor & Francis Online. Adapted from Castaman G, Linari S. Advances in diagnosis of VWD. *Expert Opinion on Orphan Drugs*. 2019;7(4):147-155.[59]

reason, the use of BATs helps to standardize bleeding history collection and to quantify bleeding frequency and severity. ISTH-BAT represents a valid tool to identify patients for further laboratory evaluation. An important issue in early VWD diagnosis is represented by those individuals with slightly reduced VWF levels, which may vary over time and be significantly influenced by several physiological variables. The definition "low VWF" should be restricted to subjects with consistent VWF levels ranging from 30 to 50 U/dL and personal and/or family evidence of bleeding symptoms. Therefore, again a careful evaluation of bleeding history is of utmost significance in the diagnostic and prognostic work-up of a patient with suspected VWD or low VWF.

Severe quantitative VWF deficiencies are easy to be identified diagnose, while type 2 qualitative abnormalities are more challenging from the diagnostic point of view. Therefore, several assays s are required to evaluate the whole spectrum of VWF activities and interactions with specialized cellular receptors. These assays are becoming more and more specific, accurate and available also for non-specialized laboratories. The first step is to assess the ability of VWF to bind platelets. New tests are now increasingly available, which improve sensitivity and avoid laboratory artifacts. However, all the necessary assays are not widely available and VWF:RCo still represents the standard for measuring VWF activity, despite it does not reflect the range of physiologic VWF activities.

The correct typing of VWD allows the identification of the most appropriate treatment for the patient. For this purpose, DDAVP trial is also useful to identify the most suitable patient candidates. Type 1 patients are the ideal candidates for its use which could be considered also in some type 2 M and 2 N cases on the basis of the results of the infusion test. Type 2 B cases should not be treated with the compound because of the risk of thrombocytopenia. Replacement therapy is the cornerstone of treatment for type 2 A and B and 3 patients. Genetic testing is particularly indicated when the identification of a specific mutation guides clinical and laboratory monitoring after treatment (eg, assessing risk for alloantibody with use of factor concentrates), or for genetic counseling (eg, discriminating between mild/moderate hemophilia A versus type 2N VWD). Laboratory methods are being developed to permit rapid testing and more accurate measurements improving sensitivity, but some of them are still not widely used. Therefore, bleeding history still represents the crucial key in order to induce an early diagnosis and consequent optimal treatment of affected patients.

Disclosure

References

1. De Meyer SF, Deckmyn H, Vanhoorelbeke K. von Willebrand factor to the rescue. *Blood*. 2009;113:5049–5057. doi:10.1182/blood-2008-10-165621
2. Yee A, Kretz CA. Von Willebrand factor: form for function. *Semin Thromb Hemost*. 2014;40:17–27. doi:10.1055/s-0033-1363155
3. De Jong A, Eikenboom J. Developments in the diagnostic procedures for von Willebrand disease. *J Thromb Haemost*. 2016;14:449–460. doi:10.1111/jth.13243
4. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69:454–459. doi:10.1182/blood.V69.2.454.454
5. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. 1998;67:395–424. doi:10.1146/annurev.biochem.67.1.395
6. Wagner DD, Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells. Identification of a large precursor polypeptide chain. *J Biol Chem*. 1983;258:2065–2067. doi:10.1016/S0021-9258(18)32879-5
7. Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. *J Clin Invest*. 1985;76:1102–1106. doi:10.1172/JCI112064
8. Mancuso DJ, Tuley EA, Westfield LA, et al. Structure of the gene for human von Willebrand factor. *J Biol Chem*. 1989;264:19514–19527. doi:10.1016/S0021-9258(19)47144-5
9. Mancuso DJ, Tuley EA, Westfield LA, et al. Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochemistry*. 1991;30:253–269. doi:10.1021/bi00215a036
10. Zhou YF, Eng ET, Zhu J, et al. Sequence and structure relationships within von Willebrand factor. *Blood*. 2012;120:449–458. doi:10.1182/blood-2012-01-405134
11. Haberichter SL. Biosynthesis and organization of von Willebrand factor. In: Federici AB, Lee CA, Berntorp EE, editors. *Von Willebrand Disease: Basic and Clinical Aspects*. Oxford: Wiley-Blackwell; 2011:7–29.
12. Dong JF. Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. *J Thromb Haemost*. 2005;3:1710–1716. doi:10.1111/j.1538-7836.2005.01360.x
13. Sadler JE. Low von Willebrand factor: sometimes a risk factor and sometimes a disease. *Hematology Am Soc Hematol Educ Program*. 2009;2009:106–112. doi:10.1182/asheducation-2009.1.106
14. Castaman G, Goodeve A, Eikenboom JC; on behalf of the European Group on von Willebrand disease (EUVWD). Principles of care for diagnosis and treatment of von Willebrand disease. *Haematologica*. 2013;98:667–674. doi:10.3324/haematol.2012.077263
15. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the subcommittee on von Willebrand factor. *J Thromb Haemost*. 2006;4:2103–2114. doi:10.1111/j.1538-7836.2006.02146.x
16. Hampshire DJ, Goodeve AC. The International Society on Thrombosis and Haemostasis von Willebrand disease database: an update. *Semin Thromb Hemost*. 2011;37:470–479. doi:10.1055/s-0031-1281031

17. Favaloro EJ. Von Willebrand disease: local diagnosis and management of a globally distributed bleeding disorder. *Semin Thromb Hemost*. 2011;37:425–426. doi:10.1055/s-0031-1280567

18. Rodeghiero F, Castaman G, Tosetto A. How I treat von Willebrand disease. *Blood*. 2009;114(6):1158–1165. doi:10.1182/blood-2009-01-153296

19. Leissinger C, Carcao M, Gill JC, Journeycake J, Singleton T, Valentino L. Desmopressin (DDAVP) in the management of patients with congenital bleeding disorders. *Haemophilia*. 2014;20 (2):158–167. doi:10.1111/hae.12254

20. Castaman G, Lethagen S, Federici AB, et al. Response to desmopressin is influenced by the genotype and the phenotype in type 1 von Willebrand disease (VWD): results from the European study MCMDM-1 VWD. *Blood*. 2008;111:3531–3539. doi:10.1182/blood-2007-08-109231

21. Federici AB, Mannucci PM, Castaman G, et al. Clinical and molecular predictors of thrombocytopenia and risk of bleeding in patients with von Willebrand disease type 2B: a cohort study of 67 patients. *Blood*. 2009;113(3):526–534. doi:10.1182/blood-2008-04-152280

22. Mannucci PM. New therapies for von Willebrand disease. *Hematology Am Soc Hematol Educ Program*. 2019;2019:590–595. doi:10.1182/hematology.2019000368

23. Batlle J, Lopez-Fernandez MF, Fraga EL, et al. Von Willebrand factor/factor VIII concentrates in the treatment of von Willebrand disease. *Blood Coagul Fibrinolysis*. 2009;20:89–100. doi:10.1097/MBC.0b013e3283254570

24. Nichols WL, Hultin MB, James AH, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171–232. doi:10.1111/j.1365-2516.2007.01643.x

25. Laffan MA, Lester W, O'Donnell JS, et al. The diagnosis and management of von Willebrand disease: a United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology. *Br J Haematol*. 2014;167 (4):453–465. doi:10.1111/bjh.13064

26. Franchini M. Surgical prophylaxis in von Willebrand's disease: a difficult balance to manage. *Blood Transfus*. 2008;6(Suppl 2):33–38.

27. O'Donnell JS, Lavin M. Perioperative management of patients with von Willebrand disease. *Hematology Am Soc Hematol Educ Program*. 2019;2019:604–609. doi:10.1182/hematology.2019000065

28. Coppola A, Franchini M, Makris M, Santagostino E, Di Minno G, Pannucci PM. Thrombotic adverse events to coagulation factor concentrates for treatment of patients with haemophilia and von Willebrand disease: a systematic review of prospective studies. *Haemophilia*. 2012;18(3):e173–e187. doi:10.1111/j.1365-2516.2012.02758.x

29. Borel-Derlon A, Federici AB, Roussel-Robert V, et al. Pharmacokinetic studies on Wilfactin, a von Willebrand factor concentrate with a low FVIII content treated with three virus-inactivation/removal methods. *J Thromb Haemost*. 2005;3 (10):2219–2227. doi:10.1111/j.1538-7836.2005.01435.x

30. Turecek PL, Mitterer A, Matthiessen HP, et al. Development of a plasma- and albumin-free recombinant von Willebrand factor. *Hamostaseologie*. 2009;29(Suppl 1):S32–S38. doi:10.1055/s-0037-1617202

31. Stockschlaeder M, Schneppenheim R, Budde U. Update on von Willebrand factor multimers: focus on high-molecular-weight multimers and their role in hemostasis. *Blood Coagul Fibrinolysis*. 2014;25:206–216. doi:10.1097/MBC.0000000000000065

32. Mannucci PM, Kempton C, Millar C, et al. Pharmacokinetics and safety of a novel recombinant human von Willebrand factor manufactured with a plasma-free method: a prospective trial. *Blood*. 2013;122(5):648–657. doi:10.1182/blood-2013-01-479527

33. Gill JC, Castaman G, Windyga J, et al. Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. *Blood*. 2015;126(17):2038–2046. doi:10.1182/blood-2015-02-629873

34. Peyvandi F, Mamaev A, Wang JD, et al. Phase 3 study of recombinant von Willebrand factor in patients with severe von Willebrand disease who are undergoing elective surgery. *J Thromb Haemost*. 2019;17(1):52–62. doi:10.1111/jth.14313

35. Albanez S, Ogiwara K, Michels A, et al. Aging and ABO blood type influence von Willebrand factor and factor VIII levels through inter-related mechanisms. *J Thromb Haemost*. 2016;14:953–963. doi:10.1111/jth.13294

36. Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in 0 blood group subjects explains how AB0 determinants influence plasma von Willebrand factor. *Blood*. 2008;111:3540–3545. doi:10.1182/blood-2007-11-122945

37. Bowen DJ. An influence of AB0 group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost*. 2003;1:33–40. doi:10.1046/j.1538-7836.2003.00007.x

38. Sadler JE. Slippery criteria for von Willebrand disease type 1. *J Thromb Haemost*. 2004;2:1720–1723. doi:10.1111/j.1538-7836.2004.00933.x

39. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4:766–773. doi:10.1111/j.1538-7836.2006.01847.x

40. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease(MCMDM-1VWD). *Blood*. 2007;109:112–121. doi:10.1182/blood-2006-05-020784

41. Flood VH, Christopherson PA, Gill JC, et al. Clinical and laboratory variability in a cohort of patients diagnosed with type 1 VWD in the United States. *Blood*. 2016;127:2481–2488. doi:10.1182/blood-2015-10-673681

42. Lavin M, Aguila S, Schneppenheim S, et al. Novel insights into the clinical phenotype and pathophysiology underlying low VWF levels. *Blood*. 2017;130:2344–2353. doi:10.1182/blood-2017-05-786699

43. Lavin M, O'Donnell JS. How I treat low von Willebrand factor levels. *Blood*. 2019;133:795–804. doi:10.1182/blood-2018-10-844936

44. Rydz N, James PD. The evolution and value of bleeding assessment tools: the evolution and value of bleeding assessment tools. *J Thromb Haemost*. 2012;10:2223–2229. doi:10.1111/j.1538-7836.2012.04923.x

45. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders: ISTH/SSC bleeding assessment tool. *J Thromb Haemost*. 2010;8:2063–2065. doi:10.1111/j.1538-7836.2010.03975.x

46. Spradbrow J, Letourneau S, Grabell J, et al. Bleeding assessment tools to predict von Willebrand disease: utility of individual bleeding symptoms. *Res Pract Thromb Haemost*. 2020;4:92–99. doi:10.1002/rth2.12256

47. Favaloro EJ. The Platelet Function Analyser (PFA)-100 and von Willebrand disease: a story well over 16 years in the making. *Haemophilia*. 2015;21:642–645. doi:10.1111/hae.12710

48. Sharma R, Haberichter SL. New advances in the diagnosis of von Willebrand disease. *Hematology Am Soc Hematol Educ Program*. 2019;2019:596–600. doi:10.1182/hematology.2019000064

49. Castaman G, Tosetto A, Cappelletti A, et al. Validation of a rapid test (VWF-LIA) for the quantitative determination of the von Willebrand factor antigen in type 1 von Willebrand disease diagnosis within the European multicenter study MCMDM-1VWD. *Thromb Res*. 2010;126:227–231. doi:10.1016/j.thromres.2010.06.013

50. Meijer P, Haverkate F. An external quality assessment program for von Willebrand factor laboratory analysis: an overview from the European concerted action on thrombosis and disabilities foundation. *Semin Thromb Haemost*. 2006;32:485–491. doi:10.1055/s-2006-947862

51. Vanhoorelke K, Cauwenberghs N, Vauterin S, et al. A reliable and reproducible ELISA method to measure ristocetin cofactor activity of von Willebrand factor. *Thromb Haemost*. 2000;83:107–113. doi:10.1055/s-0037-1613765

52. Flood VH, Gill JC, Morateck PA, et al. Common VWF exon 28 polymorphisms in African Americans affecting the VWF activity assay by ristocetin cofactor. *Blood*. 2010;116:280–286. doi:10.1182/blood-2009-10-249102

53. Verfaillie CJ, De Witte E, Devreese KM. Validation of a new panel of automated chemiluminescence assays for von Willebrand factor antigen and activity in the screening for von Willebrand disease. *Int J Lab Hematol*. 2013;35:555–565. doi:10.1111/ijlh.12087

54. Flood VH, Abshire TC, Christopherson PA, et al. Von Willebrand disease in the United States: perspective from the Zimmerman program. *Ann Blood*. 2018;3:7. doi:10.21037/aob.2017.12.05

55. Bodò I, Eikenboom J, Montgomery R, Patzke J, Schneppenheim R, Di Paola J. von Willebrand factor Subcommittee of the Standardization and Scientific Committee of the International Society for Thrombosis and Haemostasis. Platelet-dependent von Willebrand factor activity. Nomenclature and methodology: communication from the SSC of the ISTH. *J Thromb Haemost*. 2015;13 (7):1345–1350. doi:10.1111/jth.12964

56. Boender J, Eikenboom J, van der Bom JC, et al. Clinically relevant differences between assays for von Willebrand factor activity. *J Thromb Haemost*. 2018;16(12):2413–2424.

57. Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of FVIII/von Willebrand factor in plasma and platelets. *J Clin Invest*. 1980;65:1318–1325. doi:10.1172/JCI109795

58. Bowyer AE, Goodfellow KJ, Seidel H, et al. Evaluation of a semiautomated von Willebrand factor multimer assay, the hydragel von Willebrand multimer, by two European Centers. *Res Pract Thromb Haemost*. 2018;2:790–799. doi:10.1002/rth2.12141

59. Castaman G, Linari S. Advances in diagnosis of von Willebrand disease. *Exp Opin Orphan Drugs*. 2019;7:147–155. doi:10.1080/21678707.2019.1609352

60. Favaloro EJ, Grispo L, Exner T, et al. Development of a simple collagen based ELISA assay aids in the diagnosis of, and permits sensitive discrimination between type I and II, von Willebrand's disease. *Blood Coagul Fibrinolysis*. 1991;2:285–291. doi:10.1097/00001721-199104000-00011

61. Jousselme E, Jourdy Y, Rugeri L, et al. Comparison of an automated chemiluminescent assay to a manual ELISA assay for determination of von Willebrand Factor collagen binding activity on VWD plasma patients previously diagnosed through molecular analysis of VWF. *Int J Lab Hematol*. 2018;40:77–83. doi:10.1111/ijlh.12743

62. Keesler DA, Flood VH. Current issues in diagnosis and treatment of von Willebrand disease. *Res Pract Thromb Haemost*. 2018;2:34–41. doi:10.1002/rth2.12064

63. Flood VH, Gill JC, Christopherson PA, et al. Critical von Willebrand factor A1 domain residues influence type VI collagen binding. *J Thromb Haemost*. 2012;10:1417–1424. doi:10.1111/j.1538-7836.2012.04746.x

64. Nishino M, Girma JP, Rothschild C, et al. New variant of von Willebrand disease with defective binding to factor VIII. *Blood*. 1989;74:1591–1599. doi:10.1182/blood.V74.5.1591.1591

65. Haberichter SL, Balistreri M, Christopherson R, et al. Assay of the von Willlebrand factor (VWF) propeptide to identify type 1 von Willebrand disease patients with decreased VWF survival. *Blood*. 2006;108:3344–3351. doi:10.1182/blood-2006-04-015065

66. Bellissimo DB, Christopherson PA, Flood VH, et al. VWF mutations and new sequence variations identified in healthy controls are more frequent in the African-American population. *Blood*. 2012;119:2135–2140. doi:10.1182/blood-2011-10-384610

67. Sharma R, Flood VH. Advances in the diagnosis and treatment of von Willebrand disease. *Hematology Am Soc Hematol Educ Program*. 2017;2017(1):379–384. doi:10.1182/asheducation-2017.1.379

68. James PD, Notley C, Hegardon C, et al. The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood*. 2007;109:145–154. doi:10.1182/blood-2006-05-021105

69. Goodeve AC. The genetic basis of von Willebrand disease. *Blood Rev*. 2010;24:123–134. doi:10.1016/j.blre.2010.03.003

70. Christopherson PA, Perry CL, Bellissimo DB, et al. Genotype-phenotype relationship and the role of alloantibodies in type 3 VWD in the Zimmerman Program. *Blood*. 2017;130(suppl 1):19.

71. Boender J, Eikenboom J, van der Bom JG, et al. Clinically relevant differences between assays for von Willebrand factor activity. *J Thromb Haemost*. 2018;16:2413–2424.

72. Castaman G. How I treat von Willebrand disease. *Thromb Res*. 2020;196:618–625. doi:10.1016/j.thromres.2020.07.051

Complement in Sickle Cell Disease: Are We Ready for Prime Time?

Christos Varelas, Athina Tampaki, Ioanna Sakellari, Achilles Anagnostopoulos, Eleni Gavriilaki & Efthymia Vlachaki

Published online: 23 Mar 2021.

Submit your article to this journal

Article views: 232

View related articles

View Crossmark data

Citing articles: 11 View citing articles

REVIEW

Complement in Sickle Cell Disease: Are We Ready for Prime Time?

Christos Varelas[1]
Athina Tampaki [2]
Ioanna Sakellari[1]
Achilles Anagnostopoulos[1]
Eleni Gavriilaki[1,*]
Efthymia Vlachaki [2,*]

[1]Hematology Department – BMT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece; [2]Adults Thalassemia Unit, 2nd Department of Internal Medicine, Hippokration Hospital, Thessaloniki, Greece

*These authors contributed equally to this work

Abstract: Sickle cell disease (SCD) is a widely spread inherited hemoglobinopathy that includes a group of congenital hemolytic anemias, all characterized by the predominance of sickle hemoglobin (HbS). Its features are anemia, predisposal to bacterial infections and complications such as vaso-occlusive crisis (VOC) or delayed hemolytic transfusion reaction (DHTR), which lead to increased rate of morbidity and mortality even in the era of hydroxyurea. The interaction between sickle cells, neutrophils, platelets or endothelial cells in small vessels results in hemolysis and has been considered the disease's main pathophysiological mechanism. Complement activation has been reported in small cohorts of SCD patients, but the governing mechanism has not been fully elucidated. This will be important to predict the patient group that would benefit from complement inhibition. Until now, eculizumab-mediated complement inhibition has shown beneficial effects in DHTR, with limited reports in patients with VOC. In the meantime, several innovative agents are under clinical development Our state-of-the-art review summarizes current data on 1) complement activation in SCD both in steady state and crisis, 2) underlying mechanisms of complement over-activation for the clinician in the context of SCD, 3) actions of hydroxyurea and new therapeutic approaches including indirect involvement in complement activation, and 4) novel paradigms in complement inhibition.

Keywords: sickle cell disease, complement system, eculizumab, complement inhibition

Introduction

Sickle cell disease (SCD) still remains a devastating and dire condition with subsequent increased rates of morbidity and mortality in the era of hydroxyurea.[1] It is a genetic, autosomal recessive condition caused by a single β-globin gene mutation on chromosome 11, leading to an amino-acid substitution (Glutamine -> Valine, β^A -> β^s), thus resulting in the formation of the abnormal hemoglobin S (HbS) tetramer.[2] HbS is a tetramer with abnormal physicochemical properties that will polymerize under hypoxic stress, leading into the sickling of circulating red blood cells (RBCs).[3]

Our current understanding of the disease's pathophysiology has mostly focused on the interaction between red blood cells and neutrophils, platelets or endothelial cells in small blood vessels.[4] More recently, the effects of red blood cell adhesion and hemolysis that result in vaso-occlusive crisis (VOC) have also been investigated.[5] A rather neglected entity in SCD that seems to be a key component of this pathophysiological mechanism may be complement activation. In this context, increased interest has been shown in the identification of the innate immune system's pivotal role in the promotion of inflammation in SCD.[6] The

Correspondence: Eleni Gavriilaki
Hematology Department - BMT Unit, G. Papanicolaou Hospital, Exochi, Thessaloniki, 57010, Greece
Email elenicelli@yahoo.gr

activation of the complement cascade is one of the hallmarks in this inflammatory process.[7] In general, systemic complement dysregulation induces host tissue damage.[8] Biomarkers of complement activation in the serum of SCD patients were revealed in various clinical studies, along with increased levels of C5b-9 – which is the definitive marker of complement activation - and other surface-bound C3 fragments not only on patients' erythrocytes, but also in skin and kidney biopsies.[10–14] Additionally, further experience about the role of complement activation and inhibition has been gained in the context of other disorders. Findings of complement activation in β-thalassemia major, thrombotic microangiopathies (TMAs), antiphospholipid antibody syndrome, HELLP syndrome and malaria enhance our efforts in understanding complement activation and its role in the pathophysiology of SCD.[15–21]

Our review originated from the aspiration to provide further evidence in the investigation of complement activation in SCD. This regards a summary of current data on complement activation both in steady state and crisis, probable elemental mechanisms of complement activation in the frame of SCD, actions of hydroxyurea, novel therapeutic approaches including indirect involvement in complement activation and novel endeavours of complement inhibition; all under the prism of a clinician's point of view.

Complement Activation in SCD

Scientific effort to explain increased rates of bacterial infections and mortality in SCD patients[1,22,23] led to the investigation of a possible involvement of the complement system in the disease's pathophysiology, with the hypothesis that this excessive sensitivity to infections was a result of defective opsonization.[9,24,25] Complement components, such as C3b and iC3b, are key ingredients of innate immune system and not only opsonize pathogens but also generate sequential adaptive immune responses. As a result, the complement system was placed at the center of scientific community's research.

Francis and Womack were the first investigators who reported remarkable complement activity in the serum of SCD patients in 1967.[26] Six years later, Johnston et al followed the same hypothesis and confirmed complement over-activation through the AP pathway in SCD.[27] In 1976, Wilson et al revealed total AP function (AP50) in abnormally low levels, along with significantly depressed levels of factor B in serum of SCD patients compared to normal controls.[28] Generally, initial surveys' results with the depiction of depressed levels of Factor B, functional deficiency, and C3 inferred to the assumption that defective AP activation was responsible for defective opsonization. However, the possible underlying mechanisms were not further investigated.[27–31]

Later studies managed to measure Bb fragments and C3, P (properdin) complexes in serum from SCD patients in crisis.[9] This study revealed elevated concentrations of both factors and was consistent with the hypothesis that irreversibly sickled red cells, or membrane spicules and vesicles are the site of increased AP activation. Furthermore, complement over-activation markers such as Bb, C5a, soluble sC5b9 were measured in patients' serum or plasma and associated with SCD in steady state or in crisis.[10,12–14,32] Additional results reported over-activation of the AP pathway in painful crisis periods, in contrast with asymptomatic circumstances.

On the other hand, more measurable molecules of the complement system such as factors D, I and H were found to be within normal range or even reduced but not statistically significantly in SCD patients who exhibit complement activation.[28,32,33] Moreover, a defective regulation mechanism of soluble C5b-9 has been correlated to a major increase in lysis of sickled cells.[34] In addition, elevated soluble c5b-9 and sC5b-9 vascular deposition were revealed.[11,12,14]

Other studies showed that regulation of C5b-9 (membrane attack complex/MAC) formation in sickle erythrocytes was defective, especially in the densest cells. The defect is characterized by increased binding of C5b-7 and of C9 to more dense sickle cells, and as a result, sickle cells are more susceptible to C5b-9-mediated (reactive) lysis. Among the dense sickle cells, irreversibly sickled cells (ISC) are those that are more sensitive to lysis.[34] Interestingly, dense ISC have the ability to directly activate the AP.[9] Their density propagates an increased AP activation when compared to sickled RBCs or control RBCs. Defective modulation from surface-anchored complement regulators on dense sickled RBCs, along with an increased amount of C3 are potent complement activators.[10,12]

Since C3a and C3b fragments can be produced by either pathway, whereas Bb is specific to the AP and C4b both to the classical pathway (CP) and the lectin pathway (LP), several surveys used additional methods to block CP activation, in order to achieve only AP to be activated.[9,27,32] Collectively, these data are conclusive in

that complement activation is a major characteristic of SCD and sickled RBC's death in vivo or in vitro is AP mediated. This mechanism of complement over-activation needs further exploration. However, numerous observational studies, mouse model data and experiments have attempted to shed a light in this obscure area of SCD pathophysiology.

Underlying Mechanisms of Complement Activation in SCD for the Clinician

Complement system and its role in the pathophysiology of SCD is considered a complex and vast entity. The key aspects that bind complement and SCD are cell-free heme and hemolysis-derived molecules, C5a together with P-selectin, the surface complement regulatory protein, the hypercoagulant state in SCD, the amendment of the bilayer membrane structure in sickle RBCs and the very essence of SCD pathobiology: the vicious cycle of ischemia/reperfusion (I/R). These mechanisms can forward complement activation through different, distinct pathways.[32,35,36] Circulating microvesicles (MVs) of damaged SCD erythrocytes during oxygenation–deoxygenation sickling cycles have been identified with the ability to activate the complement system, and as a result, they can be used as biomarkers.[35,37,38]

AP activation due to alterations in the organization of membrane phospholipids of sickled RBCs is a speculated adjunctive mechanism. Early studies made reports of in vivo AP activation in the circulations of SCD patients during painful crisis[32] and subsequent studies further evidenced an abnormal translocation of the sickled RBC membrane phospholipids, which transpires as a potential locale of AP activation in SCD.[14,39]

Chronic intravascular hemolysis is identified as an essential characteristic of SCD.[40] Free extracellular heme interacts with toll-like receptor 4 (TLR-4) leading to the production of cytokines and adhesion molecules that promote inflammatory response and ultimately VOC. Endothelial TLR-4 signaling is rather critical for SCD VOC and recent data suggest that targeted inhibition of heme-induced TLR-4 signaling can actually reduce VOC.[41] Moreover, free heme is released in circulation due to hemoglobin degradation during hemolysis. Counter protective mechanisms are not sufficient to prevent hemolysis from accumulating free heme in plasma.[42] Recent studies exhibit increased plasma free

heme (PFH), along with MVs, in many SCD patients and recognized their devastating effects – most notably intense kidney VOC, acute chest syndrome (ACS) not only in mouse models but also pediatric population.[43–45] However, a recent single-cell RNA study reported that liver macrophages, are turned into "erythrophagocytes" that dominate the macrophage population and provide an on-demand adaptation to hemolytic driven oxidative stress.[46] Recent studies report that loss.

C5a anaphylatoxin, an inflammatory molecule, has the ability to propagate P-selectin-mediated vaso-occlusion. A recent study by Vercelloti et al demonstrated that C5a alone when given to SS mice promotes VOC. Another finding was that C5a up-regulates P-selectin and vWF in vitro and in vivo in the same population, inducing P-selectin mediated VOC.[36] In line with these findings, another study established that AP opsonins, anchored on sickled RBCs, serve actually as adhesion sites on vascular endothelium, resulting in the irregular trajectory of these cells by involvement of P-selectin/Mac-1.[14] Other studies revealed the capacity of P-selectin to bind C3b and C3 (H2O)-like, promoting complement activation.[47,48]

Early evidence suggests that abnormal expression of surface complement regulatory proteins is not just a key element of paroxysmal nocturnal hemoglobinuria (PNH). These proteins propagate complement-mediated cell lysis and their deficiency or dysfunction has been associated with the pathophysiology of other diseases as well.[49–53] Most studies have focused on surface-bound complement regulators – CD55, CD59, CD46 and CR1 -. In the frame of SCD, some studies revealed decreased CD55 and CD59 on sickled erythrocytes, in comparison with control subjects.[34,54] Other surveys showed difference in the expression of these regulators on dense vs non-dense RBC but not in the total population of RBC between controls and SCD patients.[12]

A growing consensus has been achieved between the complement and coagulation/fibrinolysis pathways in the context of clinically significant tissue damage and activation of inflammatory process. Thrombin effectively catalyzes the cleavage of both C3 and C5 acting as convertase, producing active anaphylatoxins and other complement sub-products.[55] Interestingly, MVs propagate complement activation through a thrombin-mediated signal route both in vitro and in vivo, independently of CP or AP activation.[56] Another noteworthy finding was that thrombin acts as an alternative molecule for C5-convertase in the absence of C3.[57]

Last but not least, I/R underlies the pathophysiology of SCD. The paradox of this cycle is that tissue damage is caused not only by ischemia but also by the following reperfusion-mediated reentry of oxygen[58] I/R results in complement activation and neutrophilic stimulation, along with rapid oxygen production and ultimately contributes to tissue damage in various organs.[59] Significant interest has been risen for the role of the LP and its role in inflammatory responses during I/R injury. There have been reports of "bypass" activation events that lead to lectin-mediated cleavage of C3, and as a result, I/R tissue damage is caused. Moreover, certain deficiencies of mannose-binding lectin (MBL) have been positively associated with better outcomes in experimental models of renal reperfusion injury after transplantation.[60] More recent observations report that inhibition of mannose-binding lectin-associated serine protease (MASP2), the effector enzyme of LP, may be beneficial in conditions such as thrombotic microangiopathy (TMA) post allogeneic hematopoietic stem cell transplantation (alloHSCT).[61]

In general, all these proposed mechanisms share in common the dominant role of complement activation – especially through the AP pathway – in the pathophysiology of SCD. Some studies revealed elevated biomarkers of complement activation in both steady state and crisis of SCD patients or mouse models. In other cases, despite preliminary data, no complement activation was reported in association with the factors studied. Nevertheless, there are still many elements of complement deregulation that have yet to be explored. Importantly, new therapeutic perspective is needed, since despite the fact that SCD is the first genetic disease described, treatment evolution seems to be stuck in the era of hydroxyurea.

Hydroxyurea and New Therapeutic Approaches

Hydroxyurea was the first drug approved by the Food and Drug Administration (FDA) to be administered in adult SCD patients and it remains the main therapeutic choice for more than 30 years.[62] It has many features that make it an ideal drug for SCD and can provide multiple benefits through several mechanisms of action. Over the past 30 years, substantial experience has been earned in matters of safety and efficacy for SCD patients.

Its main effect – through a mechanism still not fully understood – is the induction of fetal hemoglobin (HbF), high levels of which reduce significantly SCD severity.

Hydroxyurea improves erythrocyte deformation, decreases the population of circulating neutrophils and reticulocytes and reduces the expression of adhesion receptors.[63] Early findings of principle studies were followed by prospective Phase 1/2 trials that demonstrated efficacy first in affected adults, adolescents and children, and more recently even in infants and toddlers.[64]

In a double-blind, randomized clinical trial, hydroxyurea was proved to be effective and efficient in reducing the frequency of painful crises in adults who had a history of three or more such crises per year. A mean follow-up of 21 months was reported at the end of the trial.[65] This study reported that patients assigned to hydroxyurea had lower annual rates of crises, and fewer of them suffered from acute chest syndrome or underwent transfusion in comparison with patients that received the placebo.[65] Hydroxyurea treatment did not cause any severe adverse effects.

Hydroxyurea has proven its clinical efficacy and become acknowledged as the main therapeutic option for many SCD patients. It reduces number of painful crises and hospitalizations[66] and is useful to treat acute VOCs both in adults and children. Infants receiving hydroxyurea preserve their splenic function.[67] Furthermore, hydroxyurea has been correlated to fine growth and development,[68] along with no delays on sexual maturation, including menarche.[69]

Interestingly, a recent study by Roumenina et al revealed novel evidence of an indirect effect of hydroxyurea in complement activation. This study revealed that although complement activation is a rather common event in the pathophysiology of SCD that is associated with the development of dense RBCs and hemolysis, treatment with hydroxyurea may partly alleviate this effect.[12] More specifically, complement activation was shown by sC5b-9 concentration and upregulation of CD46 and it was reported substantially reduced in SCD patients treated with hydroxyurea.

However, hydroxyurea has many contraindications as well. Pregnancy or unwillingness to use contraception, history of severe hydroxyurea toxicity or hypersensitivity and history of significant non-compliance with recommended medical care, deprive patients of the benefits of this myelosuppressive agent.[70] All in all, despite its multiple benefits, an important number of patients do not achieve satisfactory clinical response.

Due to the fact that hydroxyurea along with anticoagulants remain the sole treatment for SCD for over 30 years,

several novel treatments are currently under advanced clinical development in SCD. L-Glutamine is a fundamental amino acid in the process of pyridine synthesis of nucleotides, including nicotinamide adenine dinucleotide (NAD) and glutathione, as well as glutamate, which becomes essential during exposure to oxidative stress. Thus, glutamine availability is important in SCD.[71] Phase II and III randomized, double-blind, controlled trials of L-glutamine 0.6 g/kg/day compared with placebo in children and adults with SCD and at least 2 episodes of crisis during the last year provided evidence that L-glutamine is safe and associated with a reduction in painful episodes and in hospitalizations.[72]

Another novel agent is voxelotor. It is an orally administered drug that increases Hb's affinity to oxygen and inhibits the sickling of RBCs. Several clinical trials have reported its benefits.[73] Crizanlizumab is a new, FDA-approved drug for the prevention of VOC. It is a humanized IgG2 kappa monoclonal antibody that binds to P-selectin and blocks interactions with its ligands including P-selectin glycoprotein ligand 1. Binding P-selectin on the surface of the activated endothelium and platelets blocks interactions between endothelial cells, platelets, RBCs, and leukocytes.[74] Baseline analysis from systematic literature review and network meta-analysis proved that this monoclonal antibody can reduce crises and hospitalization days in comparison with placebo or other therapeutic agents with an acceptable adverse event profile in adult and adolescent SCD patients.[75] Furthermore, binding of P-selectin by crizanlizumab also inhibits C5-a, which may be beneficial for SCD patients.[36]

Hematopoietic cell transplantation (HCT) is a potential definitive cure for SCD. The goal is to eliminate the sickle erythrocyte and its cellular progenitors and replace them with donor hematopoietic pluripotent stem cells that will produce mature erythrocytes which will not express sickle hemoglobin (Hb S), thereby reducing Hb S levels to those associated with the trait condition.[76] However, the risk of post-transplantation severe adverse events must be balanced against SCD's own serious complications which are known for causing morbidity and even death.[77]

Genetic modulation of phenotype may also have curative potential for SCD patients. SCD has different phenotypes due to differences in the genetic makeup of the affected patient. Although the exact genes are still under extensive study, further advances in our understanding of the pathophysiology imply that genes involved in numerous mechanisms might have epistatic potential in SCD.[78]

This may actually lead to gene therapy with the aim to replace the defective gene with a normal one.

Additional novel agents are being tested that target abnormal interactions between RBCs and their surrounding micro-environment (specifically the endothelium, neutrophils and platelets), as well as the inflammatory and prothrombotic setting. Some of these drugs may indirectly interact with the complement system. A recent review reported 20 Phase 1 studies, 10 Phase 2 and 3 Phase 3.[79] Of all these studies, eight investigate drugs which are applied during an acute VOC, while the others are aimed at the reduction of VOC or adjustment of biomarkers. Table 1 summarizes novel approved and under development therapies in SCD.

As our understanding in the pathophysiology of sickling is constantly improving, many potential novel therapeutic agents are being in development. This is indeed an exciting and optimistic time in SCD research. Nevertheless, the multifactorial and vast nature of the disease is more likely to require combination therapies, and some single agents are doomed to be unsuccessful. More studies are needed in this effort to unravel the mystery of SCD and its pathophysiology.

Novel Paradigms in Complement Inhibition

Complement inhibition has already been tested in Paroxysmal Nocturnal Hemoglobinuria (PNH) with two FDA-approved drugs: eculizumab since 2007 and ravulizumab since 2019. They are both monoclonal antibodies that block terminal complement activation.[80,81] Ravulizumab has a longer half-life, thus providing sustained C5 inhibition,[82,83] while eculizumab has already been used in other diseases and was found to be safe and have long-term efficacy.[84] In order to overcome eculizumab's own limitations, several other novel inhibitors are currently in advanced clinical development.[49] Novel proximal inhibitors are being tested aiming at proteins within the early stages of the complement cascade, such as C3, factor B and factor D.[85,86] Figure 1 summarizes the complement cascade, and inhibitors' target points which could provide potential benefits in SCD.

Despite our better knowledge about complement's role in SCD, advanced options for new treatment plans are not extensively explored. Eculizumab has been used with success as a salvage therapy in SCD patients presenting with delayed hemolytic transfusion reaction (DHTR), as it has

Table 1 Summary of Novel Approved and Under Development Therapies in SCD

Mechanism	Clinical Trials' Results	Status
1) Selectin inhibitors		
Crizalinzumab (humanized anti-P selectin mAB)	Reduction in number of pain crisis	FDA approved
Rivapansel (pan-selectin inhibitor)	Reduction in opioid use during VOC	Pending
Sevuparin (anti P-selectin heparin derivative)	Possible preventive use	Pending
2) Adenosine and invariant NK T cells		
Regadenoson (A2A receptor agonist)	Decreased activation of invariant NK T Cells	Pending
3) Leukotrienes		
Zileuton (5 lipo-oxygenase inhibitor)	Well tolerated both in children and adults	Pending
Mometasone (inhaled corticosteroid)	Reduction in pain scores	Pending
3) Antioxidants		
L-Glutamine (NADH production)	Reduction in VOC events	FDA approved
N-acetylcysteine (enhances glutathione, weakens oxidative stress)	Diary data suggest no impact on pain scores - lab measures of VWF activity under evaluation	Pending
4) Enhanced nitric oxide bioavailability		
L-Arginine (NO substrate)	Reduction in opioid use and pain scores	Pending
IMR-687 (PDE9 inhibitor)	Reduction in VOC and opioid use	FDA has granted rare pediatric disease designation
5) Other agents		
Voxelotor	Inhibition of red cell sickling – reduction in hemolysis	FDA Approved

been documented in some case reports.[87–91] Patients received the monoclonal antibody after multiple lines of treatments and after confirmation of complement activation by analyses. It managed to overcome and reverse complement-mediated hemolysis and its devastating effects.[87,89] It has also been found safe to be used during pregnancy as revealed in at least one case report.[92] More recently, a study summarized the results of 18 SCD patients with DHTR receiving eculizumab.[93] Most recent guidelines by the American Society of Hematology (ASH) recommend complement inhibition in patients with DHTR and ongoing hyperhemolysis.[94]

In addition, eculizumab has also been used in patients suffering with SCD crisis. These patients were considerably benefited by the reduction of heme-induced thrombo-inflammation through C5 inhibition.[95] Importantly, eculizumab was found to be effective even in one case of bone marrow necrosis of a young adult SCD patient, where other lines of treatment failed.[96] It has also found further use in patients with hemoglobinopathies who develop TMA after alloHSCT.[97] Stress factors during transplantation process along with their constant underlying hemolytic condition puts these patients at high risk for complement-mediated hemolysis. These patients seem to benefit from inhibition of complement activation, thus keeping TMA activity biomarkers under careful monitoring can help establish the right moment for applying treatment with eculizumab.[98] Moreover, inhibition of proximal complement activators, such as C3 inhibition with Compstatin or its analog AMY-101, is currently under investigation. Compstatin analogs have been used in preclinical and clinical studies for a plethora of disease models, such as age-related macular degeneration, sepsis, PNH, hemodialysis-induced inflammation and transplantation.[99]

Microvascular thrombosis and endothelial dysfunction are major characteristics of SCD and its complications. Our experience from severe coronavirus/COVID-19 infection may prove to be useful in SCD management.[100] Undeniably, early reports indicate that severe COVID-19 infection bears many similarities to complement-mediated TMA.[101] We have the knowledge from previous studies of other coronaviruses that inhibition of C3 activation results in weakening the lung-directed proinflammatory conditions. The genetic absence of C3 and the blockade of downstream complement effectors as well, have given rise to significant optimism.[102,103] In line with these findings, AMY-101 – a C3 inhibitor – has been successfully administrated in a patient suffering from severe COVID-19 infection.[100] Eculizumab was proven to be successful in such cases.[104] As a matter of fact, such encouraging results led to ongoing clinical trials aiming at results that could not only shape future COVID-19 management but also affect the rest of complement-mediated diseases.
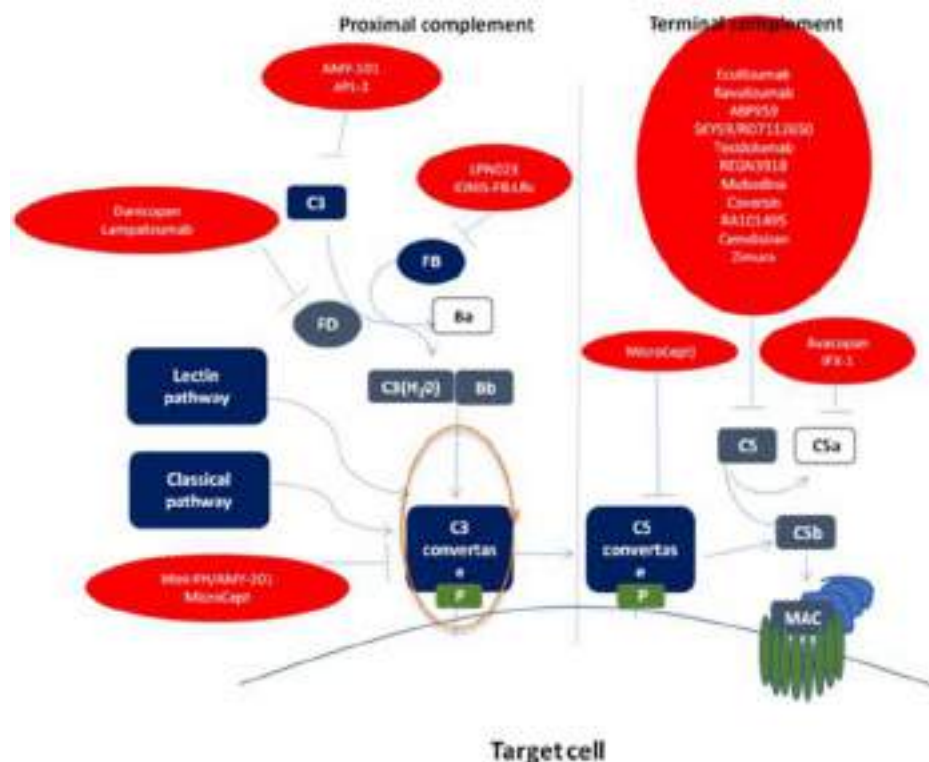
Figure 1 Complement activation and inhibition. The complement cascade has been traditionally considered to be activated by the classical, alternative and lectin pathway. The alternative pathway serves as an amplification loop for the lectin and classical pathway accounting for almost 80% of complement activation products. Spontaneous hydrolysis of C3 resulting from different triggers allows propagation of C3 convertase (C3bBb) and as a result the alternative pathway is constantly "on". C3 convertase sustains the amplification loop together with factors B and D. Terminal complement pathway begins with propagation of C5 convertase which in turn cleaves C5 into C5a, a potent inflammatory mediator, and C5b; C5b together with C6-9 form C5b-9 (membrane attack complex/MAC), a cytolytic complex. Eculizumab, ravulizumab, ABP959, SKY59/RO7112650, tesidolumab, REGN3918, mubodina, coversin, RA101495, cemdisiran and zimura inhibit C5; AMY-101 and APL-2 inhibit C3 and C3 convertase activity; mini-FH/AMY-201 inhibits alternative pathway C3 convertase; LPN023 and IONIS-FB-LRx inhibit factor B; danicopan and lampalizumab inhibit factor D; mirococept inhibits C3 and C5 convertases; avacopan inhibits C5a receptor and IFX-1 C5a.

However, special attention should be paid in complement inhibition in the context of SCD. The biggest barrier in complement inhibition seems to be the increased risk of infections. These patients are prone to infection for a number of reasons including splenic dysfunction, defective opsonization and impaired adaptive immunity.[105] Atypical viruses and encapsulated bacteria are at the front line. Use of eculizumab is associated with a 1000-fold to 2000-fold increased incidence of meningococcal disease despite preventive vaccination.[106] Therefore, SCD patients are even at a higher risk for invasive infections because of probable complement inhibition.

Conclusions and Future Considerations

Our comprehension of complement activation and its role in the pathophysiology of SCD has expanded over the course of the past few decades. Of note, complement regulation and its mechanisms require further interpretation in the context of the disease. Current results are more than encouraging. However, improvements need to be made in complement biomarkers monitoring, in order to achieve a sufficient, modern approach to SCD management.

This review presents data that underline the expanding need for supplementary prospective studies – with more sensitive biomarkers and clinical assays included – so as to evaluate complement activation in SCD and determine selection of patients with clinical characteristics that are potentially predisposed to complement activation. Strong criteria are needed for early recognition of patients that may be benefited by complement inhibition. Additionally, should complement inhibition utilize in SCD other questions arise about the way they would be administered. These inhibitors will probably be able to stop a crisis or pain even in VOC; however, more clinical studies are needed to determine their place in treatment plans. It

should also not be forgotten that hydroxyurea and other novel treatment options may also have beneficial effects on the vicious cycle that attenuates complement activation.

Acknowledgments

Disclosure

Eleni Gavriilaki reports serving on Alexion and Omeros advisory boards, outside the submitted work, and has consulted for Alexion Pharmaceuticals, Inc and Omeros Pharmaceuticals, Inc. The authors report no other potential conflicts of interest for this work.

References

1. Maitra P, Caughey M, Robinson L, et al. Risk factors for mortality in adult patients with sickle cell disease: a meta-analysis of studies in North America and Europe. *Haematologica*. 2017;102 (4):626–636. doi:10.3324/haematol.2016.153791
2. Sundd P, Gladwin MT, Novelli EM. Pathophysiology of sickle cell disease. *Annu Rev Pathol*. 2019;14(1):263–292. doi:10.1146/ annurev-pathmechdis-012418-012838
3. Stuart MJ, Nagel RL. Sickle-cell disease. *Lancet*. 2004;364 (9442):1343–1360. doi:10.1016/S0140-6736(04)17192-4
4. Kim K, Li J, Barazia A, et al. ARQ 092, an orally-available, selective AKT inhibitor, attenuates neutrophil-platelet interactions in sickle cell disease. *Haematologica*. 2017;102:246–259. doi:10.3324/haematol.2016.151159
5. Lamarre Y, Romana M, Waltz X, et al. Hemorheological risk factors of acute chest syndrome and painful vaso-occlusive crisis in children with sickle cell disease. *Haematologica*. 2012;97:1641–1647. doi:10.3324/haematol.2012.066670
6. Allali S, Maciel TT, Hermine O, de Montalembert M. Innate immune cells, major protagonists of sickle cell disease pathophysiology. *Haematologica*. 2020;105(2):273–283. doi:10.3324/haematol.2019.229989
7. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol*. 2015;6:262.
8. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement system part II: role in immunity. *Front Immunol*. 2015;6:257.
9. Chudwin DS, Papierniak C, Lint TF, Korenblit AD. Activation of the alternative complement pathway by red blood cells from patients with sickle cell disease. *Clin Immunol*. 1994;71 (2):199–202.
10. Mold C, Tamerius JD, Phillips G. Complement activation during painful crisis in sickle cell anemia. *Clin Immunol Immunopathol*. 1995;76(3):314–320. doi:10.1006/clin.1995.1131
11. Gavriilaki E, Mainou M, Christodoulou I, et al. In vitro evidence of complement activation in patients with sickle cell disease. *Haematologica*. 2017;102(12):e481. doi:10.3324/ haematol.2017.174201
12. Roumenina LT, Chadebech P, Bodivit G, et al. Complement activation in sickle cell disease: dependence on cell density, hemolysis and modulation by hydroxyurea therapy. *Am J Hematol*. 2020;95(5):456–464. doi:10.1002/ajh.25742
13. Chudwin DS, Korenblit AD, Kingzette M, Artrip S, Rao S. Increased activation of the alternative complement pathway in sickle cell disease. *Clin Immunol Immunopathol*. 1985;37 (1):93–97. doi:10.1016/0090-1229(85)90139-4
14. Lombardi E, Matte A, Risitano AM, et al. Factor H interferes with the adhesion of sickle red cells to vascular endothelium: a novel disease modulating molecule. *Haematologica*. 2019;104:919–928. doi:10.3324/haematol.2018.198622
15. Vaught AJ, Gavriilaki E, Hueppchen N, et al. Direct evidence of complement activation in HELLP syndrome: a link to atypical hemolytic uremic syndrome. *Exp Hematol*. 2016;44:390–398. doi:10.1016/j.exphem.2016.01.005
16. Brady TM, Pruette C, Loeffler LF, et al. Typical Hus: evidence of acute phase complement activation from a daycare outbreak. *J Clin Exp Nephrol*. 2016;1(2). doi:10.21767/2472-5056.100011
17. Gavriilaki E, Chrysanthopoulou A, Sakellari I, et al. Linking complement activation, coagulation, and neutrophils in transplant-associated thrombotic microangiopathy. *Thromb Haemost*. 2019;119(09):1433–1440. doi:10.1055/s-0039-1692721
18. Chaturvedi S, Braunstein EM, Yuan X, et al. Complement activity and complement regulatory gene mutations are associated with thrombosis in APS and CAPS. *Blood*. 2020;135(4):239–251. doi:10.1182/blood.2019003863
19. Gavriilaki E, Gkaliagkousi E, Grigoriadis S, Anyfanti P, Douma S, Anagnostopoulos A. Hypertension in hematologic malignancies and hematopoietic cell transplantation: an emerging issue with the introduction of novel treatments. *Blood Rev*. 2019;35:51–58. doi:10.1016/j.blre.2019.03.003
20. Gavriilaki E, Touloumenidou T, Sakellari I, et al. Pretransplant genetic susceptibility: clinical relevance in transplant-associated thrombotic microangiopathy. *Thromb Haemost*. 2020;120 (04):638–646. doi:10.1055/s-0040-1702225
21. Gavriilaki E, Christodoulou I, Koravou EE, et al. Pre- and post-transfusion complement activation in transfusion-dependent beta-thalassaemia. *Hemasphere*. 2018;2:e58. doi:10.1097/ HS9.0000000000000058
22. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. 1994;330(23):1639–1644. doi:10.1056/ NEJM199406093302303
23. Manci EA, Culberson DE, Yang YM, et al. Causes of death in sickle cell disease: an autopsy study. *Br J Haematol*. 2003;123:359–365. doi:10.1046/j.1365-2141.2003.04594.x
24. Mold C, Gewurz H, Du Clos TW. Regulation of complement activation by C-reactive protein. *Immunopharmacology*. 1999;42 (1–3):23–30. doi:10.1016/S0162-3109(99)00007-7
25. Neth O, Jack DL, Dodds AW, Holzel H, Klein NJ, Turner MW. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. *Infect Immun*. 2000;68(2):688–693. doi:10.1128/IAI.68.2.688-693.2000
26. Francis WG, Womack CR. Serum complement activity in normal individuals and patients with sickle cell hemoglobin abnormalities. *Am J Med Technol*. 1967;33:77–86.
27. Johnston RB, Newman SL, Struth AG. An abnormality of the alternate pathway of complement activation in sickle-cell disease. *N Engl J Med*. 1973;288(16):803–808. doi:10.1056/ NEJM197304192881601
28. Wilson WA, Hughes GR, Lachmann PJ. Deficiency of factor B of the complement system in sickle cell anaemia. *Br Med J*. 1976;1:367–369. doi:10.1136/bmj.1.6006.367
29. deCiutiis AC, Peterson CM, Polley MJ, Metakis LJ. Alternative pathway activation in sickle cell disease and beta-thalassemia major. *J Natl Med Assoc*. 1978;70:503–506.

30. Larcher VF, Wyke RJ, Davis LR, Stroud CE, Williams R. Defective yeast opsonisation and functional deficiency of complement in sickle cell disease. *Arch Dis Child*. 1982;57:343–346. doi:10.1136/adc.57.5.343

31. Koethe SM, Casper JT, Rodey GE. Alternative complement pathway activity in sera from patients with sickle cell disease. *Clin Exp Immunol*. 1976;23:56–60.

32. Wang RH, Phillips G Jr, Medof ME, Mold C. Activation of the alternative complement pathway by exposure of phosphatidyl-lethanolamine and phosphatidylserine on erythrocytes from sickle cell disease patients. *J Clin Invest*. 1993;92:1326–1335. doi:10.1172/JCI116706

33. Wilson WA, Thomas EJ, Sissons JG. Complement activation in asymptomatic patients with sickle cell anaemia. *Clin Exp Immunol*. 1979;36:130–139.

34. Test ST, Woolworth VS. Defective regulation of complement by the sickle erythrocyte: evidence for a defect in control of membrane attack complex formation. *Blood*. 1994;83(3):842. doi:10.1182/blood.V83.3.842.842

35. Merle NS, Grunenwald A, Rajaratnam H, et al. Intravascular hemolysis activates complement via cell-free heme and heme-loaded microvesicles. *JCI Insight*. 2018;3. doi:10.1172/jci.insight.96910

36. Vercellotti GM, Dalmasso AP, Schaid TR Jr, et al. Critical role of C5a in sickle cell disease. *Am J Hematol*. 2019;94(3):327–337. doi:10.1002/ajh.25384

37. Gkaliagkousi E, Gavriilaki E, Vasileiadis I, et al. Endothelial microvesicles circulating in peripheral and coronary circulation are associated with central blood pressure in coronary artery disease. *Am J Hypertens*. 2019;32(12):1199–1205. doi:10.1093/ajh/hpz116

38. Gkaliagkousi E, Nikolaidou B, Gavriilaki E, et al. Increased erythrocyte- and platelet-derived microvesicles in newly diagnosed type 2 diabetes mellitus. *Diabetes Vascular Dis Res*. 2019;16(5):458–465. doi:10.1177/1479164119844691

39. de Jong K, Larkin SK, Styles LA, Bookchin RM, Kuypers FA. Characterization of the phosphatidylserine-exposing subpopulation of sickle cells. *Blood*. 2001;98(3):860–867. doi:10.1182/blood.V98.3.860

40. Kato GJ, Steinberg MH, Gladwin MT. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J Clin Invest*. 2017;127(3):750–760. doi:10.1172/JCI89741

41. Beckman JD, Abdullah F, Chen C, et al. Endothelial TLR4 expression mediates vaso-occlusive crisis in sickle cell disease. *Front Immunol*. 2021;11:613278. doi:10.3389/fimmu.2020.613278

42. Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8(12):1383–1389. doi:10.1038/nm1202-799

43. Camus SM, De Moraes JA, Bonnin P, et al. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. *Blood*. 2015;125 (24):3805–3814. doi:10.1182/blood-2014-07-589283

44. Ghosh S, Adisa OA, Chappa P, et al. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. *J Clin Invest*. 2013;123(11):4809–4820. doi:10.1172/JCI64578

45. Adisa OA, Hu Y, Ghosh S, Aryee D, Osunkwo I, Ofori-Acquah SF. Association between plasma free haem and incidence of vaso-occlusive episodes and acute chest syndrome in children with sickle cell disease. *Br J Haematol*. 2013;162(5):702–705. doi:10.1111/bjh.12445

46. Pfefferle M, Ingoglia G, Schaer CA, et al. Hemolysis transforms liver macrophages into antiinflammatory erythrophagocytes. *J Clin Invest*. 2020;130(10):5576–5590. doi:10.1172/JCI137282

47. Del Conde I, Crúz MA, Zhang H, López JA, Afshar-Kharghan V. Platelet activation leads to activation and propagation of the complement system. *J Exp Med*. 2005;201(6):871–879. doi:10.1084/jem.20041497

48. Merle NS, Paule R, Leon J, et al. P-selectin drives complement attack on endothelium during intravascular hemolysis in TLR-4/heme-dependent manner. *Proc Natl Acad Sci U S A*. 2019;116 (13):6280–6285. doi:10.1073/pnas.1814797116

49. Ruiz-Delgado GJ, Vazquez-Garza E, Mendez-Ramirez N, Gomez-Almaguer D. Abnormalities in the expression of CD55 and CD59 surface molecules on peripheral blood cells are not specific to paroxysmal nocturnal hemoglobinuria. *Hematology*. 2009;14(1):33–37. doi:10.1179/102453309X385089

50. Asimakopoulos JV, Terpos E, Papageorgiou L, et al. The presence of CD55- and/or CD59-deficient erythrocytic populations in patients with rheumatic diseases reflects an immune-mediated bone-marrow derived phenomenon. *Med Sci Monit*. 2014;20:123–139. doi:10.12659/MSM.889727

51. Sartain SE, Turner NA, Moake JL. TNF regulates essential alternative complement pathway components and impairs activation of protein C in human glomerular endothelial cells. *J Immunol*. 2016;196(2):832–845. doi:10.4049/jimmunol.1500960

52. Salama MA, Sadek NA, Hassab HM, Abadeer AF, Mikhael IL. Erythrocyte autoantibodies and expression of CD59 on the surface of red blood cells of polytransfused patients with beta-thalassaemia major. *Br J Biomed Sci*. 2004;61:88–92. doi:10.1080/09674845.2004.11732650

53. Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell*. 1993;73 (4):703–711. doi:10.1016/0092-8674(93)90250-T

54. Chapin J, Terry HS, Kleinert D, Laurence J. The role of complement activation in thrombosis and hemolytic anemias. *Transfus Apher Sci*. 2016;54(2):191–198. doi:10.1016/j.transci.2016.04.008

55. Amara U, Flierl MA, Rittirsch D, et al. Molecular intercommunication between the complement and coagulation systems. *J Immunol*. 2010;185(9):5628–5636. doi:10.4049/jimmunol.0903678

56. Zecher D, Cumpelik A, Schifferli JA. Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement. *Arterioscler Thromb Vasc Biol*. 2014;34(2):313–320. doi:10.1161/ATVBAHA.113.302378

57. Huber-Lang M, Sarma JV, Zetoune FS, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med*. 2006;12(6):682–687. doi:10.1038/nm1419

58. Hebbel RP, Belcher JD, Vercellotti GM. The multifaceted role of ischemia/reperfusion in sickle cell anemia. *J Clin Invest*. 2020;130(3):1062–1072. doi:10.1172/JCI133639

59. Riedemann NC, Ward PA. Complement in ischemia reperfusion injury. *Am J Pathol*. 2003;162(2):363–367. doi:10.1016/S0002-9440(10)63830-8

60. Farrar CA, Asgari E, Schwaeble WJ, Sacks SH. Which pathways trigger the role of complement in ischaemia/reperfusion injury? *Front Immunol*. 2012;3:341. doi:10.3389/fimmu.2012.00341

61. Elhadad S, Chapin J, Copertino D, Van Besien K, Ahamed J, Laurence J. MASP2 levels are elevated in thrombotic microangiopathies: association with microvascular endothelial cell injury and suppression by anti-MASP2 antibody narsoplimab. *Clin Exp Immunol*. 2021;203(1):96–104. doi:10.1111/cei.13497

62. Rai P, Ataga K. Drug therapies for the management of sickle cell disease. *F1000Research*. 2020;9:592. doi:10.12688/f1000research.22433.1

63. Baliga B, Pace B, Chen H, Shah A, Yang Y. Mechanisms for fetal hemoglobin induction by hydroxyurea in sickle cell erythroid progenitors. *AJH*. 2000;65(3):227–233.

64. Agrawal RK, Patel RK, Shah V, et al. Hydroxyurea in Sickle Cell Disease: drug Review. *Indian J Hematol Blood Transfus*. 2014;30 (2):91–96. doi:10.1007/s12288-013-0261-4

65. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med*. 1995;332(20):1317–1322. doi:10.1056/NEJM199505183322001

66. Jayabose S, Tugal O, Sandoval C, et al. Clinical and hematologic effects of hydroxyurea in children with sickle cell anemia. *J Pediatr*. 1996;129(4):559–565. doi:10.1016/S0022-3476(96)70121-X

67. Wang WC, Wynn LW, Rogers ZR, Scott JP, Lane PA, Ware RE. A two-year pilot trial of hydroxyurea in very young children with sickle-cell anemia. *J Pediatr*. 2001;139(6):790–796. doi:10.1067/mpd.2001.119590

68. Wang WC, Helms RW, Lynn HS, et al. Effect of hydroxyurea on growth in children with sickle cell anemia: results of the HUG-KIDS study. *J Pediatr*. 2002;140(2):225–229. doi:10.1067/mpd.2002.121383

69. Hankins JS, Ware RE, Rogers ZR, et al. Longterm hydroxyurea therapy for infants with sickle cell anemia: the HUSOFT extension study. *Blood*. 2005;106(7):2269–2275. doi:10.1182/blood-2004-12-4973

70. Ryan K, Heeney M. New England pediatric sickle cell consortium. 2004.

71. Sadaf A, Quinn CT. L-glutamine for sickle cell disease: knight or pawn? *Exp Biol Med*. 2020;245(2):146–154. doi:10.1177/1535370219900637

72. Cox SE, Hart E, Kirkham FJ, Stotesbury H. L-Glutamine in sickle cell disease. *Drugs Today*. 2020;56(4):257–268. doi:10.1358/dot.2020.56.4.3110575

73. AlDallal SM. Voxelotor: a ray of hope for sickle disease. *Cureus*. 2020;12(2):e7105.

74. Prescribing information for Adakveo (crizanlizumab): US food and drug administration; 2019. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/761128s000lbl.pdf. Accessed March 16, 2021.

75. Thom H, Jansen J, Shafrin J, et al. Crizanlizumab and comparators for adults with sickle cell disease: a systematic review and network meta-analysis. *BMJ Open*. 2020;10(9):e034147. doi:10.1136/bmjopen-2019-034147

76. Parkman R. The application of bone marrow transplantation to the treatment of genetic diseases. *Science*. 1986;232(4756):1373–1378. doi:10.1126/science.3520819

77. Brodsky RA, DeBaun MR. Are genetic approaches still needed to cure sickle cell disease? *J Clin Invest*. 2020;130(1):7–9. doi:10.1172/JCI133856

78. Ferrone F, Nagel RL, et al. Polymer structure and polymerization of deoxyhemoglobin S. In: Steinberg MH, Forget BG, Higgs DR, editors. *Disorders of Hemoglobin: Genetics, Pathophysiology, Clinical Management*. Cambridge, UK: Cambridge University Press; 2001.

79. Morrone K, Mitchell WB, Manwani D. Novel sickle cell disease therapies: targeting pathways downstream of sickling. *Semin Hematol*. 2018;55(2):68–75. doi:10.1053/j.seminhematol.2018.04.007

80. Hillmen P, Young NS, Schubert J, et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2006;355(12):1233–1243. doi:10.1056/NEJMoa061648

81. Brodsky RA, Young NS, Antonioli E, et al. Multicenter phase 3 study of the complement inhibitor eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2008;111(4):1840–1847. doi:10.1182/blood-2007-06-094136

82. Kulasekararaj AG, Hill A, Rottinghaus ST, et al. Ravulizumab (ALXN1210) vs eculizumab in C5-inhibitor-experienced adult patients with PNH: the 302 study. *Blood*. 2019;133:540–549. doi:10.1182/blood-2018-09-876805

83. Lee JW, Sicre de Fontbrune F, Wong lee lee L, et al. Ravulizumab (ALXN1210) vs eculizumab in adult patients with PNH naive to complement inhibitors: the 301 study. *Blood*. 2019;133(6):530–539. doi:10.1182/blood-2018-09-876136

84. Legendre CM, Licht C, Muus P, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med*. 2013;368:2169–2181. doi:10.1056/NEJMoa1208981

85. Gavriilaki E, Anagnostopoulos A, Mastellos DC. Complement in thrombotic microangiopathies: unraveling ariadne's thread into the labyrinth of complement therapeutics. *Front Immunol*. 2019;10:337. doi:10.3389/fimmu.2019.00337

86. Yuan X, Gavriilaki E, Thanassi JA, et al. Small-molecule factor D inhibitors selectively block the alternative pathway of complement in paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. *Haematologica*. 2017;102(3):466–475. doi:10.3324/haematol.2016.153312

87. Ricklin D, Mastellos DC, Reis ES, Lambris JD. The renaissance of complement therapeutics. *Nat Rev Nephrol*. 2018;14(1):26–47. doi:10.1038/nrneph.2017.156

88. Dumas G, Habibi A, Onimus T, et al. Eculizumab salvage therapy for delayed hemolysis transfusion reaction in sickle cell disease patients. *Blood*. 2016;127(8):1062–1064. doi:10.1182/blood-2015-09-669770

89. Vlachaki E, Gavriilaki E, Kafantari K, et al. Successful outcome of hyperhemolysis in sickle cell disease following multiple lines of treatment: the role of complement inhibition. *Hemoglobin*. 2018;42(5–6):339–341. doi:10.1080/03630269.2018.1540353

90. Boonyasampant M, Weitz IC, Kay B, Boonchalermvichian C, Liebman HA, Shulman IA. Life-threatening delayed hyperhemolytic transfusion reaction in a patient with sickle cell disease: effective treatment with eculizumab followed by rituximab. *Transfusion*. 2015;55(10):2398–2403. doi:10.1111/trf.13144

91. Chonat S, Quarmyne MO, Bennett CM, et al. Contribution of alternative complement pathway to delayed hemolytic transfusion reaction in sickle cell disease. *Haematologica*. 2018;103(10):e483–e485. doi:10.3324/haematol.2018.194670

92. Kirui Chebet L, Scully M, Mcqueen N, Porter J, Elftheriou P. Use of eculizumab for the treatment of hyperhaemolysis in pregnancy in sickle cell disease: a case report. *Blood*. 2018;132(Supplement 1):4922. doi:10.1182/blood-2018-99-118396

93. Floch A, Morel A, Zanchetta-Balint F, et al. Anti-C5 antibody treatment for delayed hemolytic transfusion reactions in sickle cell disease. *Haematologica*. 2020;105(11):2694–2697. doi:10.3324/haematol.2020.253856

94. Chou ST, Alsawas M, Fasano RM, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv*. 2020;4(2):327–355. doi:10.1182/bloodadvances.2019001143

95. Chonat S, Graciaa S, Shin HS, et al. Eculizumab for complement mediated thrombotic microangiopathy in sickle cell disease. *Haematologica*. 2020;105(12).

96. Azul M, Shah S, Williams S, Vercellotti GM, Boucher AA. Evidence for complement-mediated bone marrow necrosis in a young adult with sickle cell disease. *Blood Cells Mol Dis*. 2021;86:102508. doi:10.1016/j.bcmd.2020.102508

97. Jodele S. Double trouble: complement-mediated thrombotic microangiopathy in patients with hemoglobinopathies after stem cell transplantation. *Pediatr Blood Cancer*. 2017;64(9):e26566. doi:10.1002/pbc.26566

98. Chonat S, Chandrakasan S, Kalinyak KA, et al. Atypical haemolytic uraemic syndrome in a patient with sickle cell disease, successfully treated with eculizumab. *Br J Haematol*. 2016;175(4):744–747. doi:10.1111/bjh.13884

99. Mastellos DC, Yancopoulou D, Kokkinos P, et al. Compstatin: a C3-targeted complement inhibitor reaching its prime for bedside intervention. *Eur J Clin Invest*. 2015;45(4):423–440. doi:10.1111/eci.12419

100. Mastaglio S, Ruggeri A, Risitano AM, et al. The first case of COVID-19 treated with the complement C3 inhibitor AMY-101. *Clinical Immunology*. 2020;215:108450.

101. Gavriilaki E, Brodsky RA. Severe COVID-19 infection and thrombotic microangiopathy: success does not come easily. *Br J Haematol*. 2020;189(6). doi:10.1111/bjh.16783

102. Gralinski LE, Sheahan TP, Morrison TE, et al. Complement activation contributes to severe acute respiratory syndrome coronavirus pathogenesis. *mBio*. 2018;9(5). doi:10.1128/mBio.01753-18

103. Jiang Y, Zhao G, Song N, et al. Blockade of the C5a–C5aR axis alleviates lung damage in hDPP4 transgenic mice infected with MERS-CoV. *Emerg Microbes Infect*. 2018;7(77):1–12. doi:10.1038/s41426-018-0063-8

104. Diurno F, Numis FG, Porta G, et al. Facchini, Eculizumab treatment in patients with COVID-19: preliminary results from real life ASL Napoli 2 Nord experience. *Eur Rev Med Pharmacol Sci*. 2020;24:4040–4047. doi:10.26355/eurrev_202004_20875

105. Ochocinski D, Dalal M, Black LV, et al. Life-threatening infectious complications in sickle cell disease: a concise narrative review. *Front Pediatr*. 2020;8:38. doi:10.3389/fped.2020.00038

106. McNamara LA, Topaz N, Wang X, Hariri S, Fox L, MacNeil JR. High risk for invasive meningococcal disease among patients receiving eculizumab (Soliris) despite receipt of meningococcal vaccine. *MMWR Morb Mortal Wkly Rep*. 2017;66(27):734–737. doi:10.15585/mmwr.mm6627e1

Evaluation of Warfarin Anticoagulation at University of Gondar Comprehensive Specialized Hospital, North-West Ethiopia

Nahusenay Masresha, Esileman Abdela Muche, Asmamaw Atnafu & Ousman Abdela

Published online: 23 Mar 2021.

Submit your article to this journal ☑

Article views: 257

View related articles ☑

View Crossmark data ☑

Citing articles: 7 View citing articles ☑

ORIGINAL RESEARCH

Evaluation of Warfarin Anticoagulation at University of Gondar Comprehensive Specialized Hospital, North-West Ethiopia

Nahusenay Masresha[1]
Esileman Abdela Muche [ID][2]
Asmamaw Atnafu [ID][3]
Ousman Abdela [ID][2]

[1]Hospital Pharmacy, University of Gondar Comprehensive and Specialized Hospital, Gondar, Ethiopia; [2]Department of Clinical Pharmacy, School of Pharmacy, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia; [3]Department of Health Systems and Policy, Institute of Public Health, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia

Purpose: To assess the quality of warfarin anticoagulation and its clinical outcomes on patients treated with warfarin at the University of Gondar comprehensive specialized hospital, North-west Ethiopia.

Methods: We reviewed medical records of patients treated with warfarin between June 1, 2016, and May 30, 2018, at the University of Gondar comprehensive specialized hospital. The quality of anticoagulation was evaluated using the percentage of time spent in the therapeutic range. Data were entered into Statistical Product and Service Solutions (SPSS), version 20. Descriptive statistics were used to describe the socio-demographic and clinical characteristics of study participants. Multivariable logistic regression analysis was performed to identify independent predictors of quality of anticoagulation. Statistical significance was declared when the p-value was less than 0.05 at 95% confidence interval (CI).

Results: From a total of 202 patients' charts reviewed, women accounted for 134 (67.3%). The mean participants' age was 44.33 years (±17.05years SD). The median time spent in the therapeutic range was 37.91 with an IQR of (0.00–65.86). More than two-third (143, (70.8%)) of participants had poor anticoagulation quality (time spent in the therapeutic range is less than 65%). Twenty-seven patients (13.4%) experienced adverse medication events of bleeding and thromboembolic events. Logistic regression analysis showed that potential medication interaction [p= 0.003 95% CI Adjusted odds ratio (AOR): 0.32 (0.-152–0.689)] and presence of co-morbidity [p= 0.037 95% CI AOR: 0.70 (1.046–4.105)] were significantly associated with quality of anticoagulation.

Conclusion: The quality of warfarin anticoagulation at the University of Gondar comprehensive specialized hospital was poor. A strong effort is needed to improve the quality of anticoagulation. Patients who had other co-morbidity conditions and potentially interacting medication need special attention.

Keywords: warfarin, anticoagulation, University of Gondar, TTR, Ethiopia

Introduction

Cardiovascular disease (CVD) and stroke produce enormous health and economic burdens worldwide.[1] CVD is becoming the second common cause of death in most African countries following infectious disease, estimated to account for 20% of total deaths in 2020.[2]

Anticoagulants are prescribed for the prevention and treatment of deep vein thrombosis, pulmonary embolism, atrial fibrillation, myocardial infarction, unstable angina, rheumatic heart disease, etc. They decrease the burden of CVD.[3,4]

Correspondence: Ousman Abdela
Tel +251-912-02-3692
Fax +251-58-114 1240
Email ousmy2009@gmail.com

Warfarin acts as an anticoagulant by reducing the synthesis of functional vitamin K-dependent clotting factors.[5] It is thought to interfere with clotting factors by inhibiting the C1 subunit of the vitamin K epoxide reductase.[6]

Warfarin is underutilized in clinical care as it is related to adverse medication events.[7,8] Some medications including clopidogrel or aspirin may increase the risk of bleeding complications if co-administered.[9] Food supplements that are rich in vitamin k such as garlic, ginger, and ginkgo have interactions with warfarin.[10,11] Inter-individual differences in medication response, and its narrow therapeutic range makes warfarin a problematic medication.[10,12,13]

Time in therapeutic range (TTR) is commonly used to evaluate the quality of warfarin therapy. It is defined as the percentage of time the patients INR was within the target range.[6] There are three different methods for calculating TTR; Percent of INRs in the therapeutic range, the cross-section of the file's method, and the Roosendaal linear interpolation method.[14] Among these, the Roosendaal linear interpolated method has become a widely accepted measure of the quality of anticoagulation.[7]

Assessing TTR allows physicians to estimate the success of warfarin therapy.[15] The quality of warfarin therapy is good if the average time of the INR in the therapeutic range is high and exceeds 65%.[16]

A time therapeutic range above 65% has been reported to be beneficial against stroke and vascular events among patients treated with warfarin. On the other hand, TTRs values of less than 40% are not associated with any significant mortality benefit.[16–18] On contrary, patients with TTR <65% were found to have a 2.6-fold higher risk of stroke, 1.5-fold higher risk of major bleeding, and 2.4-fold higher risk of all-cause mortality.[19]

In general, assessing TTR is important to evaluate the current standard of anticoagulation.[2] Studies addressing the quality of anticoagulation in Ethiopia are scarce. This study aimed to evaluate the quality of anticoagulation and associated factors among patients treated with warfarin at the chronic outpatient department of the University of Gondar comprehensive and specialized Hospital (UoGCSH), North-West Ethiopia.

Methods
Study Area and Period
The study was conducted at the University of Gondar comprehensive specialized hospital (UoGCSH) from June 1st to July 30th, 2018. The hospital is located in Gondar city, North-West of Ethiopia. It is one of the tertiary care hospitals in the Amhara regional state, and it is offering outpatient, inpatient, and emergency services. The hospital provides services for about seven million populations in the catchment areas. The hospital has currently about 2000 medical and non-medical staff and has more than 598 beds.

Study Design
A retrospective cross-sectional study was conducted on adult patients treated with warfarin at UoGCSH North-West Ethiopia.

Population
Source population
All adult patients who were treated with warfarin and had follow-up at the chronic outpatient department of UoGCSH.

Study Population
All adult patients who were treated with warfarin and had attended their follow-up in the chronic outpatient department of UoGCSH from June 1st, 2016 to May 30th 2018.

Study Variables
Independent Variables
- Age, Sex, Residence, Duration of anticoagulation, Number of concomitant medications, presence of potentially interacting Medications, presence of co-morbidity, and presence of adverse drug event.

Dependent Variables
- Quality of anticoagulation.

Sampling Technique and Sample Size Determination
Sampling Technique
All adult patients who attended the chronic outpatient department of UoGCSH and received warfarin from June 1st, 2016 to May 30th, 2018, and fulfilled the inclusion criteria were included.

Inclusion and Exclusion Criteria
Inclusion Criteria
- All adult patients treated with warfarin for more than one month and on follow-up between June 1st, 2016 and May 30th, 2018 at the chronic outpatient department of UoGCSH.

Exclusion Criteria

- Patients with less than 2 INR measurements, INR value discrepancy between hematology laboratory record and chart data were excluded.

Data Collection Process

Patients' charts were retrieved from the outpatient record office based on chart numbers on the chronic outpatient log book. Then, laboratory INR records of included charts were reviewed from the hematology laboratory office. A pre-designed structured data collection format was utilized to extract necessary data from patients' medical files. Information on socio-demographics of participants, indications, and duration of warfarin therapy, comorbidities, concomitant medications, and international normalized ratio values, and the dates of the tests were extracted. Trained nurses who were working at the chronic outpatient department at the time of the data collection period filled the data abstraction form.

Data Quality Assurance

A pre-test was done on 5% of the sample size in another tertiary care hospital in Amhara regional state. Data were then collected, reviewed, and checked for completeness and relevance by the principal investigator each day.

Data Analysis and Interpretation

The extracted data were entered and analyzed by SPSS version 20. We used the Roosendaal linear interpolation method to calculate the TTR. The %TTR was calculated using excel. Those whose INR records out of range and in the range were given a %TTR value of 0% and 100% respectively. The predictor variables were checked by the chi-square test. Those variables that had full filled the assumption underwent bivariate analysis, and variables with P-value <0.20 were taken and analyzed using multivariable logistic regression. P-values less than 0.05 with 95% CI was taken to be statistically significant.

Ethical Consideration

The study was conducted after getting an ethical clearance letter from an ethical review committee of the school of pharmacy, University of Gondar. Letter of cooperation was obtained from the hospital clinical director and head of chronic outpatient department offices. Data were collected anonymously and kept confidential. This study was conducted in accordance with the Declaration of Helsinki.

Operational Definitions

Good quality anticoagulation: The time spent in the therapeutic range (TTR) is greater than or equal to 65%.

Poor quality anticoagulation: The time spent in the therapeutic range is less than 65%.

Therapeutic: The time they spent in the therapeutic range (INR between 2 & 3 and including them).

Subtherapeutic: The time they spent in the subtherapeutic range (INR<2)

Supra therapeutic: The time they spent in supratherapeutic range (INR>3)

Definition of Terms

Major bleeding: are non-surgical bleedings that result in death, are life-threatening, cause chronic sequelae or consume major health care resources. All non-major bleeds will be considered minor bleedings.

Thromboembolism: Formation in a blood vessel of a clot (thrombus) that breaks loose and is carried by the blood stream to plug another vessel.

Results

Socio-Demographic and Clinical Characteristics of Participants

A total of 202 patients' charts fulfilled the inclusion criteria and hence studied. Slightly higher than two-third of the studied subjects (134, (67.3%)) were females. Most of the participants (140, (69.3%)) had taken warfarin for less than one-year duration. Hundred and twenty-six (62.67%) patients had co-morbid conditions; heart failure (45 (22.3%)), hyperthyroidism (23, (11.4%)), and hypertension (20 (9.9%)) being the three most frequent co-morbidities (see Table 1).

Clinical Outcomes of Participants

Participants' mean time spent during their anticoagulation therapy was 41% in the therapeutic range, 42% of their time in the sub-therapeutic range, and 17% of their time in the supra-therapeutic range. Twenty-seven patients experienced adverse medication events; 9 patients (4.5%) had bleeding events, 15 patients (7.4%) had thromboembolic events, and 3 (1.5%) had emergency hospital visits (see Table 2).

Warfarin Indications

Warfarin was prescribed for treatment ofthe following diseases: Atrial fibrillation (131, (39.8%)),

Table 1 Socio-Demographic and Clinical Characteristics of Participants at UoGCSH from June 2016 to June 2018 (n=202)

Patient Characteristics	Frequency (%)
Age	
18–30years	65 (32.2)
31–45 years	44 (21.8)
46–60years	58 (28.7)
Above 60years	35 (17.3)
Mean age ± SD	44.33 years ±17.05
Sex	
Female	134 (66.3)
Male	68 (33.7)
Residence	
Urban	141 (69.8)
Rural	61 (30.2)
Duration of anticoagulation	
1–3 month	45 (22.3)
4–12month	95 (47)
>12 month	62 (30.7)
Co morbidities	
Congestive heart failure	45 (22.3)
Hypertension	20 (9.9)
Hyperthyroidism	23 (11.4)
Diabetes	18 (8.9)
HIV/AIDS	9 (4.5)
Ischemic heart disease.	4 (2)
Epilepsy	6 (3)
0steomyletis	2 (1)
Others*	8 (4)
Quality of anticoagulation	
Good quality (TTR>65%)	59 (29.2)
Poor quality (TTR<65%)	143 (70.8)

Notes: *Asthma 1 (0.5%), chronic pulmonary obstructive disease1 (0.5%), chronic liver disease1 (0.5%), chronic kidney disease1 (0.5%), pneumonia1 (0.5%), rheumatoid heart disease 1 (0.5%), chronic disease 1(0.5%), peripheral arthritis 1 (0.5).

valvular heart diseases (127, (38.6%)), deep venous thrombosis (49 (14.9%)), and Pulmonary embolism (PE) (22, (6.7%))

Number of Concomitant Medications

Most study participants (178 (88.1%)) had at least one medication besides warfarin. The mean number of concomitant medications was 2.08 (SD ± 1.4), and 53.5% of participants had more than four medications (Table 3). A total of 703 INR measurements were analyzed. The mean INR measurement frequency per patient was 3.48 ±1.26 SD. The median time-frequency of INR measurement was 20.875 days IQR (25–16.27). The median time spent in the therapeutic range was 37.91 with an IQR of (0.00–65.86)

Associated Factor for Quality of Anticoagulation

Determinants of poor quality of anticoagulation on multi-variable logistic regression analysis were potential medication interaction (p= 0.003; AOR with 95% CI = 0.32 (0.15–0.69)) and presence of co-morbidity condition (p= 0.037; AOR with 95% CI = 0.70 (1.05–4.11)) (see Table 4).

Discussion

The current study revealed that warfarin prescription for more than two-thirds (70.8%) of participants was of poor quality. The mean time spent in the therapeutic range is 41%. This figure is lower compared to the report from South Africa (48.5%) 13, USA (56.7%) 27, Spain (63.8%) 26, Iran (54.9%) 18, Portugal (60.3%) 19, Sweden (77%)

Table 2 Clinical Outcome of Patients on Warfarin in COPD at UoGCSH from June 2016 to June 30th 2018 (N= 202)

Age Classification		Clinical Outcome			Total
		Thromboembolism	Emergency Department Visit	Bleeding	
18–30 years		7	2	0	9
31–45 years		3	0	3	6
46–60 years		4	0	4	8
Above 60 years		1	1	2	4
Total		15	3	9	27
Percentage of TTR	≥65% good quality	4	0	2	
	<65% poor quality	11	3	7	
Sex	Male	6	1	1	
	Female	9	2	8	

Table 3 Frequency and Number of Concomitant Medications Among Patients on Warfarin COPD at UoGCSH from June 2016 to June 2018 (N=202)

Number of Concomitant Medication		Frequency	Percent
	0	24	11.9
	1	11	5.4
	2	27	13.4
	3	32	15.8
	>4	108	53.5
	Total	202	100.0

21, Finland and Australia%28. The reason behind this difference may be their standard of care is much better than the current setting.[20–22]

In the current study, participants spent more than half of the time (59%) out of the therapeutic range. They spent the majority of their time (42%) in the sub-therapeutic range. Time spent in the therapeutics range of patients is related to mortality benefit. More than half of study participants had a TTR of less than 40%. Therefore; they may not get mortality benefits from warfarin treatment. This is evidenced by a meta-analysis done by Mearns ES et al which showed patients having %TTR less than 40% do not have mortality benefit.[20]

The current study showed that male participants had a better quality of anticoagulation compared to their female counterparts. This finding is supported by findings from Sweden and South Africa which reported males to have better coagulation outcomes than females. Their food habit of eating more green vegetables which have high Vit K was indicated as the reason behind the vulnerability of females for poor coagulation.[12]

Table 4 Predictors of Quality of Anticoagulation Among Patients Treated with Warfarin at UoGCSH from June 2016 to June 2018 (N=202)

Variables	Quality of Anticoagulation		Crude Odds Ratio	P-value 95% CI	Adjusted Odds Ratio	P-value 95% CI
	Poor <65%	Good ≥65%				
Patients gender						
Female	97	37	0.80 (0.42–1.50)	0.484		
Male	46	22	1			
Presence of co-morbidity						
Yes	73	41	0.46 (0.24–0.87)	0.017*	0.48 (0.24–0.96)	0.037**
No	70	18	1			
Residence						
Urban	98	43	0.81 (0.41–1.59)	0.541		
Rural	45	16	1			
Number of medications						
0	17	7	1			
1	9	2	0.98 (0.37–2.59)	0.964		
2	19	8	1.81 (0.37–8.87)	0.463		
3	21	11	0.60 (0.38–2.41)	0.924		
≥4	77	31	0.77 (0.33–1.78)	0.539		
Age of patients						
18–30 years	53	12	1			
30–45years	29	15	2.30 (0.90–5.89)	0.081*	1.67 (0.62–4.50)	0.310
46–60 years	38	20	1.01 (0.40–2.57)	0.986*	1.01 (0.38–2.67)	0.981
>60 years	23	12	0.99 (0.41–2.40)	0.985*	1.00 (0.40–2.50)	0.998
Presence of potentially interacting medication with warfarin						
Yes	60	11	3.15 (1.51–6.58)	0.002*	3.09 (1.45–6.59)	0.003**
No	83	48	1			

Notes: *p<0.20. **P<0.05.

The current study revealed the presence of co-morbidity to be a predictor of poor quality of anticoagulation (p= 0.037), AOR=0.70 (1.05–4.11) which means 29.8% less chance of getting a good quality of anti-coagulation than patients without the co-morbid condition. This is in line with the report from Israel,[23] and Saudi Arabia.[24] The study reported by Melamed et al and Khan et al also showed that poor quality of anticoagulation had been associated with populations affected by co morbidities.[23,24]

More than fifty percent of our patients had greater than four medications co-prescribed with warfarin. A study done in Iran by Farsad et al identified that the number of medications more than four has significant predictors of poor quality of anticoagulation this may be due to poor adherence.[15]

The current study also revealed that participants who have been prescribed warfarin with another medication(s) with the known potential of interaction with warfarin were found to have 67.6% less chance of good quality of anti-coagulation (p=0.003), AOR =0.32 (0.15–0.69). This finding is supported by reports from Canada which showed that patients who had taken warfarin with interacting medications had spent less time in the therapeutic range compared with those without concomitant medications.[25] There are pieces of evidence that the medications commonly co-prescribed in the current study participants including digoxin, Aspirin, and NSAIDs have pharmaco-dynamic interaction with warfarin leading to poor quality of anticoagulation. A case report from Britania stated that loss of warfarin anticoagulation effect with prolonged INR > 10 was recorded on a patient who was on warfarin and digoxin.[26]

In this study, thromboembolic events were the most prevalent clinical outcome followed by bleeding events. This is in line with the report from the United States of America.[20] The reason why the thromboembolic event was more prevalent in our study might be because the current study participants spent most of their time in the sub-therapeutic range.

Limitations of the Study

The study design was retrospective crossectional. Due to incompleteness of data, variables that may potentially affect the quality of anti-coagulation including, educational status, income, diet, use of over-the-counter medications, adherence, and others were not studied. In addition, patients might seek health service in other hospital or clinic and had other co-morbid conditions and medications that were not recorded. Otherwise, the study tried to show the quality of anticoagulation in resource limited settings.

Abbreviations

AOR, Adjusted Odds Ratio; CVD, Cardio Vascular Disease; DVT, Deep Venous Thrombosis; INR, International Normalization Ratio; ISI, International Sensitivity Index; MI, Myocardial Infarction; OR, Odds Ratio; PE, Pulmonary Embolism; SD, Standard Deviation; TTR, Time in Therapeutic Range; VKA, Vitamin K Antagonist; VTE, Venous Thrombo Embolism; UoGCSH, University of Gondar comprehensive Specialized Hospital.

Data Sharing Statement

The datasets during and/or analyzed during the current study are available from the author on reasonable request.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart disease and stroke statistics—2016 update: a report from the American Heart Association. *Circulation*. 2016;133(4):e38–e360. doi:10.1161/CIR.00 00000000000350
2. Mbewu A. The burden of cardiovascular disease in sub-Saharan Africa: burden of cardiovascular disease. *SA Heart J*. 2009;6(1):4–10.
3. Geerts WH, Pineo GF, Heit JA, et al. Prevention of venous thrombo-boembolism: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest*. 2004;126(3):338S–400S.
4. Wadelius M, Pirmohamed M. Pharmacogenetics of warfarin: current status and future challenges. *Pharmacogenomics J*. 2007;7(2):99. doi:10.1038/sj.tpj.6500417
5. Barnes G, Ageno W, Ansell J, Kaatz S. Recommendation on the nomenclature for oral anticoagulants: communication from the SSC of the ISTH. *J Thromb Haemost*. 2015;13(6):1154–1156. doi:10.1111/jth.12969
6. Ansell J, Hirsh J, Hylek E, Jacobson A, Crowther M, Palareti G. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest*. 2008;133(6):160S–98S.

7. Ageno W, Gallus AS, Wittkowsky A, Crowther M, Hylek EM, Palareti G. Oral anticoagulant therapy: antithrombotic therapy and prevention of thrombosis: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest*. 2012;141(2): e44S–e88S.

8. Kuruvilla M, Gurk-Turner C. A review of warfarin dosing and monitoring. *Proc (Bayl Univ Med Cent)*. 2001;14(3):305. doi:10. 1080/08998280.2001.11927781

9. Drugs.com. Warfarin drug interactions; February 2, 2021. https:// www.drugs.com/drug-interactions/warfarin.html. Accessed February 18, 2021.

10. Nutescu EA, Shapiro NL, Ibrahim S, West P. Warfarin and its interactions with foods, herbs and other dietary supplements. *Expert Opin Med Saf*. 2006;5(3):433–451. doi:10.1517/147403 38.5.3.433

11. Holbrook AM, Pereira JA, Labiris R, et al. Systematic overview of warfarin and its medication and food interactions. *Arch Intern Med*. 2005;165(10):1095–1106. doi:10.1001/archinte.165.10.1095

12. Sonuga BO, Hellenberg DA, Cupido CS, Jaeger C. Profile and antic-oagulation outcomes of patients on warfarin therapy in an urban hospital in Cape Town, South Africa. *Afr J Prim Health Care Fam Med*. 2016;8(1):1–8. doi:10.4102/phcfm.v8i1.1032

13. Teles JS, Fukuda EY, Feder D. Warfarin: pharmacological profile and medication interactions with antidepressants. *Einstein (Sao Paulo)*. 2012;10(1):110–115. doi:10.1590/S1679-45082012000100 024

14. Karuri SW Quality of oral anticoagulation management among patients on follow up at Kenyatta national hospital. University of Nairobi; 2016.

15. Farsad B-F, Abbasinazari M, Dabagh A, Bakshandeh H. Evaluation of Time in Therapeutic Range (TTR) in patients with non-valvular atrial fibrillation receiving treatment with Warfarin in Tehran, Iran: a cross-sectional study. *J Clin Diagn Res*. 2016;10(9):FC04. doi:10. 7860/JCDR/2016/21955.8457

16. Caldeira D, Cruz I, Morgado G, et al. Evaluation of time in ther-apeutic range in anticoagulated patients: a single-center, retrospec-tive, observational study. *BMC Res Notes*. 2014;7(1):891. doi:10. 1186/1756-0500-7-891

17. Morgan CL, McEwan P, Tukiendorf A, Robinson PA, Clemens A, Plumb JM. Warfarin treatment in patients with atrial fibrillation: observing outcomes associated with varying levels of INR control. *Thromb Res*. 2009;124(1):37–41. doi:10.1016/j.thromres.2008.09.016

18. Connolly SJ, Pogue J, Eikelboom J, et al. Benefit of oral anticoagulant over antiplatelet therapy in atrial fibrillation depends on the quality of international normalized ratio control achieved by centers and countries as measured by time in therapeutic range. *Circulation*. 2008;118 (20):2029–2037. doi:10.1161/CIRCULATIONAHA.107.750000

19. Haas S, Ten Cate H, Accetta G, et al. Quality of vitamin K antagonist control and 1-year outcomes in patients with atrial fibrillation: a global perspective from the GARFIELD-AF registry. *PLoS One*. 2016;11(10):e0164076. doi:10.1371/journal.pone.0164076

20. Mearns ES, White CM, Kohn CG, et al. Quality of vitamin K antagonist control and outcomes in atrial fibrillation patients: a meta-analysis and meta-regression. *Thromb J*. 2014;12(1):14. doi:10.1186/1477-9560-12-14

21. Steffel J, Verhamme P, Potpara TS, et al. The 2018 European Heart Rhythm Association practical guide on the use of non-vitamin K antagonist oral anticoagulants in patients with atrial fibrillation. *Eur Heart J*. 2018;39(16):1330–1393.

22. McDonald KM; UCSF-Stanford E. Making health care safer a critical analysis of patient safety practices. *Am J Health Syst Pharm*. 1995;52:2543–2549. doi:10.1093/ajhp/52.22.2543

23. Khan TI, Kamali F, Kesteven P, Avery P, Wynne H. The value of education and self-monitoring in the management of warfarin therapy in older patients with unstable control of anticoagulation. *Br J Haematol*. 2004;126(4):557–564. doi:10.1111/j.1365-2141.2004.05074.x

24. Melamed OC, Horowitz G, Elhayany A, Vinker S. Quality of antic-oagulation control among patients with atrial fibrillation. *Am J Manag Care*. 2011;17(3):232–237.

25. Verhovsek M, Motlagh B, Crowther MA, et al. Quality of antic-oagulation and use of warfarin-interacting medications in long-term care: a chart review. *BMC Geriatr*. 2008;8(1):13. doi:10.1186/1471-2318-8-13

26. Bhattacharyya A, Bhavnani M, Tymms DJ. Serious interaction between digoxin and warfarin. *Br J Cardiol*. 2002;9(6):356–357.

Possible Role of CD11a in Primary Immune Thrombocytopenia Patients on Immunosuppressive Therapy

Muhamad R Abdel Hameed, Howiada A Nafady, Mona Ibrahim Mostafa, Douaa Sayed & Ahmad A Obiedallah

Published online: 25 Mar 2021.

Submit your article to this journal 🗗

Article views: 142

View related articles 🗗

View Crossmark data 🗗

Citing articles: 3 View citing articles 🗗

ORIGINAL RESEARCH

Possible Role of CD11a in Primary Immune Thrombocytopenia Patients on Immunosuppressive Therapy

Muhamad R Abdel Hameed [1,2]
Howiada A Nafady[1,2]
Mona Ibrahim Mostafa[3]
Douaa Sayed[4]
Ahmad A Obiedallah [5]

[1]Hematology Unit, Department of Internal Medicine, Assiut University Hospitals, Assiut University, Assiut, Egypt; [2]Bone Marrow Transplantation Unit, South Egypt Cancer Institute, Assiut University, Assiut, Egypt; [3]Department of Internal Medicine, Assiut General Hospital, Assiut, Egypt; [4]Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut, Egypt; [5]Critical Care Unit, Department of Internal Medicine, Assiut University Hospitals, Assiut University, Assiut, Egypt

Background and Objectives: Immune thrombocytopenia (ITP) is one of the autoimmune diseases that presented by thrombocytopenia and increased risk of bleeding. Etiology of immune thrombocytopenia (ITP) is very complex. Lymphocyte function associated antigen-1 (LFA-1) plays important role in ITP. The aim of this study was evaluation of expression of CD11a on lymphocytes to explore its possible role in primary ITP patients also, regarding severity and response to immunosuppressive treatment.

Patients and Methods: This is a cross-sectional case-control study. Forty adult patients aged (18:58) years, 29 females and 11 males were enrolled as newly diagnosed primary ITP. Forty age and sex matched control subjects were randomly selected. The expression of CD11a on lymphocyte subpopulations (CD3+ T cells, CD3+CD4+ T cells and CD19+ B cells) was analyzed by flowcytometry at the start of the study and after 6 months of follow-up.

Results: The mean fluorescence intensity (MFI) of CD11a on CD3+ T and CD19+ B lymphocytes was significantly highly increased in ITP patients compared to healthy controls while MFI of CD11a on CD3+ CD4+Tclls was non-significant. MFI of CD11a on CD3+ and CD19+ B lymphocytes showed non-significant elevation with platelet count or bleeding score. MFI of CD11a on CD3+ showed significant highly increased level in refractory ITP compared with responder cases.

Conclusion: CD11a had possible role in the pathogenesis of ITP. Immunosuppressive therapy in ITP did not affect the level of CD11a expression on T and B lymphocytes. Levels of CD11a do not reflect the severity of ITP neither platelet count nor bleeding score. Increased MFI of CD11a in CD3+T lymphocytes of ITP patients may cause resistance to immunosuppressive therapy.

Keywords: Primary immune thrombocytopenia, immunosuppressive therapy, LFA-1, CD11a, B cells, T cells

Background

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by thrombocytopenia with or without mucocutaneous bleeding.[1] Antibody coated platelets in ITP, are destroyed by macrophages of the reticuloendothelial-system.[2]

Auto reactive B lymphocytes producing antiplatelet antibodies are the main etiology of ITP. Autoantibodies were not found in 30–40% of ITP patients. The antiplatelet autoantibodies are under the control of T cells. Although there are multiple drugs used in the treatment of ITP, still there are many refractory cases even after splenectomy, so

Correspondence: Muhamad R Abdel Hameed
Hematology Unit, Department of Internal Medicine, Assiut University Hospitals, Assiut University, Assiut, 71515, Egypt
Tel +20 1097510010
Email dr.muhamadramadan@yahoo.com

there is a need to understand more about the pathogenesis of ITP to find other lines of treatment.[3]

Immune thrombocytopenia (ITP) is a significant clinical problem due to chronicity, treatment cost, occurrence mainly in, young, and relatively poorer quality of life than in people with other chronic diseases such as hypertension, arthritis, and cancer subjects.[4] Most of the current therapeutic agents for ITP do not solve the fundamental problems that are responsible for the beginning and progression of the autoimmune process. Treatment strategy for ITP has focused on improving platelet life (either by immunosuppressive treatment or splenectomy) and increase platelet production.[5]

Abnormalities in the regulation of the immune system play an important role in the initiation and perpetuation of ITP.[6]

Lymphocyte function associated antigen-1 (LFA-1) is belonging to the integrin family and consists of alpha chain CD11a and beta chain CD18 dimers, and expressed on the surface of T lymphocytes, B lymphocytes, macrophages, neutrophils, and monocytes. Its major ligand, intercellular adhesion molecule-1 (ICAM-1) is belonging to the immunoglobulin superfamily, on the surface of antigen-presenting cells (APCs).[7]

The combination of LFA-1 and ICAM-1 provide a coordinated signal and initiates lymphocyte activation, proliferation, and differentiation. Interaction of T cells with antigen-presenting cells (APCs), LFA-1, and ICAM-1 participate in the formation of immunological synapse that promotes T cell proliferation and cytotoxicity.[8]

LFA-1 is similar to receptors such as CD4 and CD8. Disruption of LFA-1 activity strongly affects immune stability.[9] CD11a is important for the generation of lymphoid progenitors in the BM that is essential to normal lymphocyte development.[10]

Patients and Methods

The current study is a cross-sectional case-control study conducted at Assiut University Hospital, Assiut, Egypt, from December 2017 to October 2019. Research Ethics Medical Review Board of Assiut University has approved the protocol (number 17200350). This study was conducted in accordance with the Declaration of Helsinki. An informed written consent was obtained from all subjects who participated in the study.

Forty newly diagnosed primary ITP patients aged 18:58 years (29 females and 11 males) attended the Clinical Hematology unit of Internal Medicine Department, Assiut University Hospital, Assiut, Egypt were enrolled in the study. Diagnosis was done according to American Society of Hematology (ASH) guidelines.[11,12]

Exclusion criteria including:

1- Secondary cause of ITP as systemic lupus erythematous (SLE), viral infections (HIV, hepatitis B or C infections)

2-Underlying medical diseases that may cause thrombocytopenia as malignancy, megaloblastic anemia, aplastic anemia, lymphoproliferative disorders, liver disease, renal impairment, or pregnancy.

3-Organomegally and/or lymphadenopathy.

All patients in the study had full history taking, clinical examination, and assessment of bleeding according to ITP bleeding score (IBLS). All investigation for exclusions of secondary thrombocytopenia or other autoimmune diseases were done including rheumatoid factor, ANA and anti-double stranded DNA antibodies, Hepatitis B &C, and HIV markers, liver function test, renal function, CMV IgM & IgG, and Monospot test for infectious mononucleosis.

Expression of CD11a on lymphocyte subpopulations (CD3+ T cells, CD3+CD4+ T cells, and CD19+ B cells) were analyzed by flow cytometry at time of diagnosis.

Forty (age and sex matched) control subjects were randomly selected; they had no evidence of bleeding or autoimmune diseases. Females included in control group are non-pregnant. Informed consents were taken from all subjects.

All studied patients received initial treatment, dexamethasone 40 mg/day for 4 days.[12]

During follow-up 8 patients showed insufficient elevation of platelet count and moderate bleeding continued after 4 weeks, we added Azathioprine (150 mg/day).

Five patients using rituximab (375 mg/m^2 administered once weekly) with stopping steroid due to intolerable side effects after 9 weeks of using steroid including stress hyperglycemia and uncontrollable elevated blood pressure. One patient received cyclosporine (3–5 mg/kg/day) after stopping steroid due to patient preference.

After 6 months all patients re-evaluated to detect response to therapy. The responders to treatment will comprise subjects with complete response (CR) or response (R). A platelet count of $\geq 100 \times 10^9$/L was interpreted as a complete response (CR), while response (R) was interpreted when platelets ranging from 30×10^9/L to less than 100×10^9/L with the duplication of the pretreatment platelet count.[13]

Second estimation for The expression of CD11a on lymphocyte subpopulations (CD3+ T cells, CD3+CD4+ T cells, and CD19+ B cells) were analyzed by flow cytometry in patients how showed response to immunosuppressive therapy.

Flow Cytometry Analysis

The whole peripheral blood samples (anticoagulated with EDTA) were stained with the following antibodies; Fluorescein isothiocyanate FITC (Exbio) conjugated anti-CD11a, CD3 V450, CD19 APC and CD4 PerCP Cy5.5 (B. D bioscience). A minimum of 10,000 events were acquired and analyzed by BD FACS Canto system (BD, San jose, CA). Appropriate isotype-matched controls were included in the experiments to identify positive populations. Data were analyzed with FASCDIVA software. The B (CD19+) and T (CD3+) lymphocytes were gated, expression of CD11a was detected on B and T-lymphocytes, total T-lymphocytes CD3+CD4+ (T-helper) cells were gated and expression of CD11a on them was detected before treatment (Figure 1) and after 6 months follow-up in those respond to treatment (Figure 2).

Statistical Analysis

Data were analyzed by version 24 of SPSS software (Chicago, IL, USA). The Shapiro–Wilk test for continuous variables was used as a measure of normality. Mean ± standard deviation (SD) was used to express parametric variables, independent t-test was used to compare means of level of CD11a between patients and control and also used to compare means between patients who respond and who did not respond to immunosuppressive therapy while paired t-test was used to compare means of level of CD11a in patients who respond (before and after treatment) and Pearson's correlation coefficients were calculated to explore associations between level of CD11a in patients and severity of disease at presentation according to platelet count and IBLS (ITP bleeding score). P value < 0.05 was considered significant.

Results

Ages of included patients ranged from 18 to 58 years with a mean ±SD = 31.70 ± 10.96 years. Female patients were 29 (72.5%) and 11 patients (27.5%) were males.

The platelet count of patients at presentation was 2 ×10^9/L: 30 ×10^9/L. All patients showed skin bleeding (purpura and ecchymosis), 2 patients (5%) were presented by skin bleeding only while 4 patients (10%) were presented by skin and nasal bleeding (epistaxis) and 2 patients (5%) were presented by skin, nasal and oral bleeding. Vaginal bleeding was the main presentation, in 20 patients (50% of all patients (68.96% of female patients)) and was associated with other types of bleeding. Vaginal bleeding was associated with skin bleeding in 11 patients (27.5%) and was associated with skin and oral bleeding in 4 patients (10%) while associated with skin and nasal bleeding in 5 patients (12.5%). Urinary bleeding associated with skin bleeding in 1 (2.5%) patient and associated with skin and oral bleeding in 2 patients (5%), while rectal bleeding was reported in 2 patients (5%)(Figure 3). The severity of bleeding was variable and presented by ITP bleeding score ranged between 2 and 11.

All patients in the study received immunosuppressive therapy which included steroid alone (26 patients 65%), steroid + azathioprine (8 patients 20%), rituximab after stopping steroid (5 patients 12.5%) and cyclosporine (1 patient 2.5%).

Follow-up after 6 months we reported that thirty patients respond to immunosuppressive therapy (75%) while 10 patients (25%) were refractory for treatment. The platelets count follow-up after 6 months in patients responding to treatment was 190.50 ±59.19×110^9/L. While platelets count before treatment was 15.46 ± 8.17×110^9/L with highly significant (p value= 0.000). Platelets count before treatment in Patients non-responder to treatment was 19.30± 7.81×110^9/L. While, it was 28.88 ±8.94×110^9/L after treatment with significant (p value= 0.02) (Table 1).

Forty healthy control were included in the study, their ages were ranged between 18 and 51 years old with a mean ± SD =33.92 ± 10.30, Females control were 30 (75%) and none of them were pregnant at the time of the study. Males control were 10 (25%) All control had normal platelet count ranged from 150 to 387 ×110^9/L.

The mean fluorescence intensity (MFI) of CD11a was detected by flowcytometry in T-lymphocytes (CD3+ cells), T helper (CD3+CD4+ cells), and B-lymphocytes (CD19+) in patients and control (Table 2). There was a statistically highly significant difference between cases and control in MFI of CD11a in CD3+ cells (MFI in patients 70.15 +23.88 while in control 46.85 + 17.50) with more expression in patients than control. Also, there was a statistically highly significant difference between cases and control in MFI of CD11a in CD19+ cells (MFI in patients 65.20 ±32.36 while in control 44.54 ±21.02 with p value= 0.001) with more expression in patients than control.
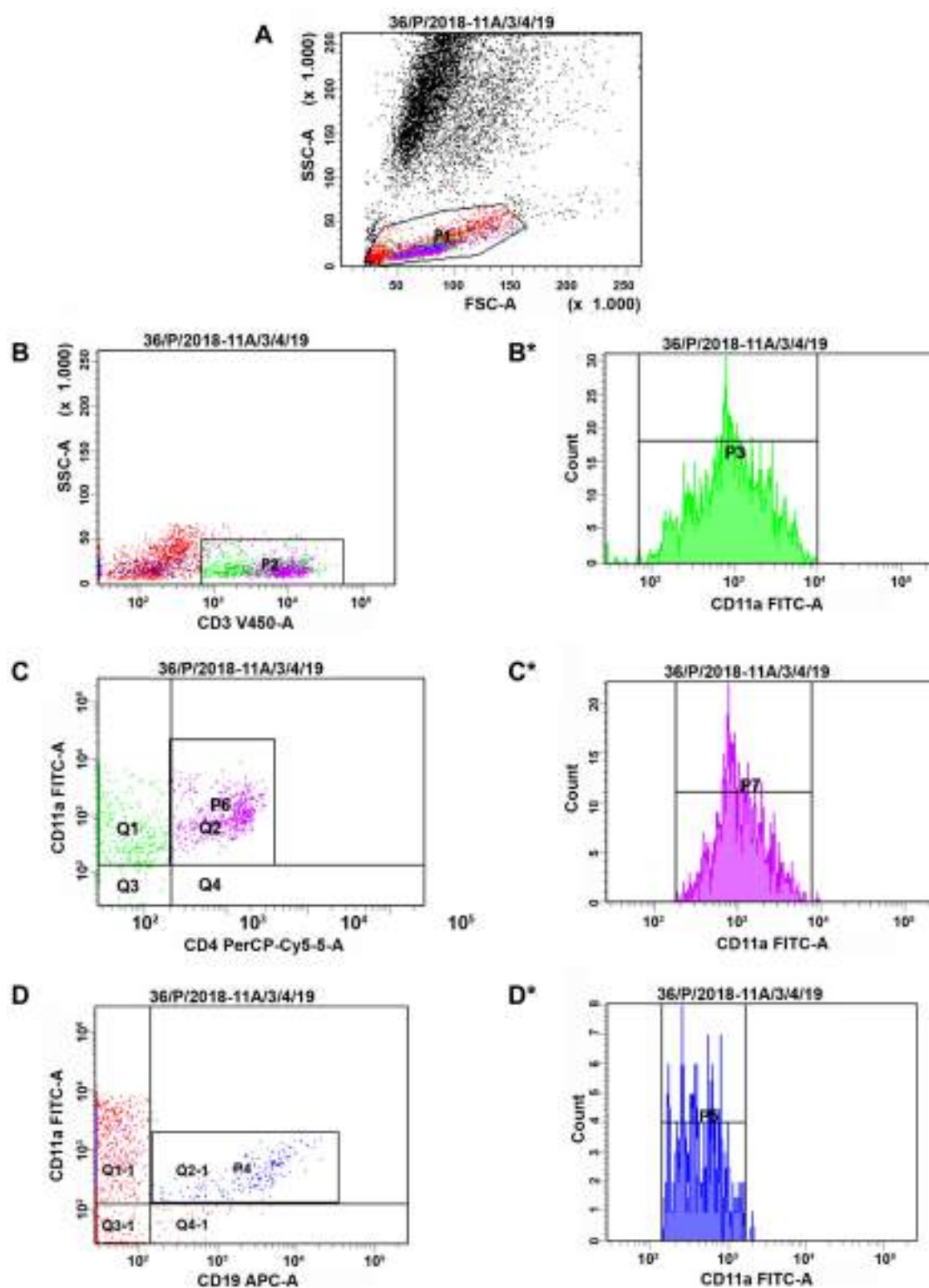
Figure 1 The expression of CD11a on lymphocyte subsets in ITP patient before treatment. (**A**) Lymphocytes were gated by flow cytometry (P1). (**B**) CD3+ cells gated by flow cytometry (P2). (**B***) MFI of CD11a gated on CD3+ cells (P3). (**C**) Expression of CD11a on CD3+CD4+ T cells (P6). (**C***) MFI of CD11a gated on CD3+CD4+ T cells (P7). (**D**) Expression of CD11a on CD19+ cells (P4). (**D***) MFI of CD11a gated on CD19+ cells (P5).
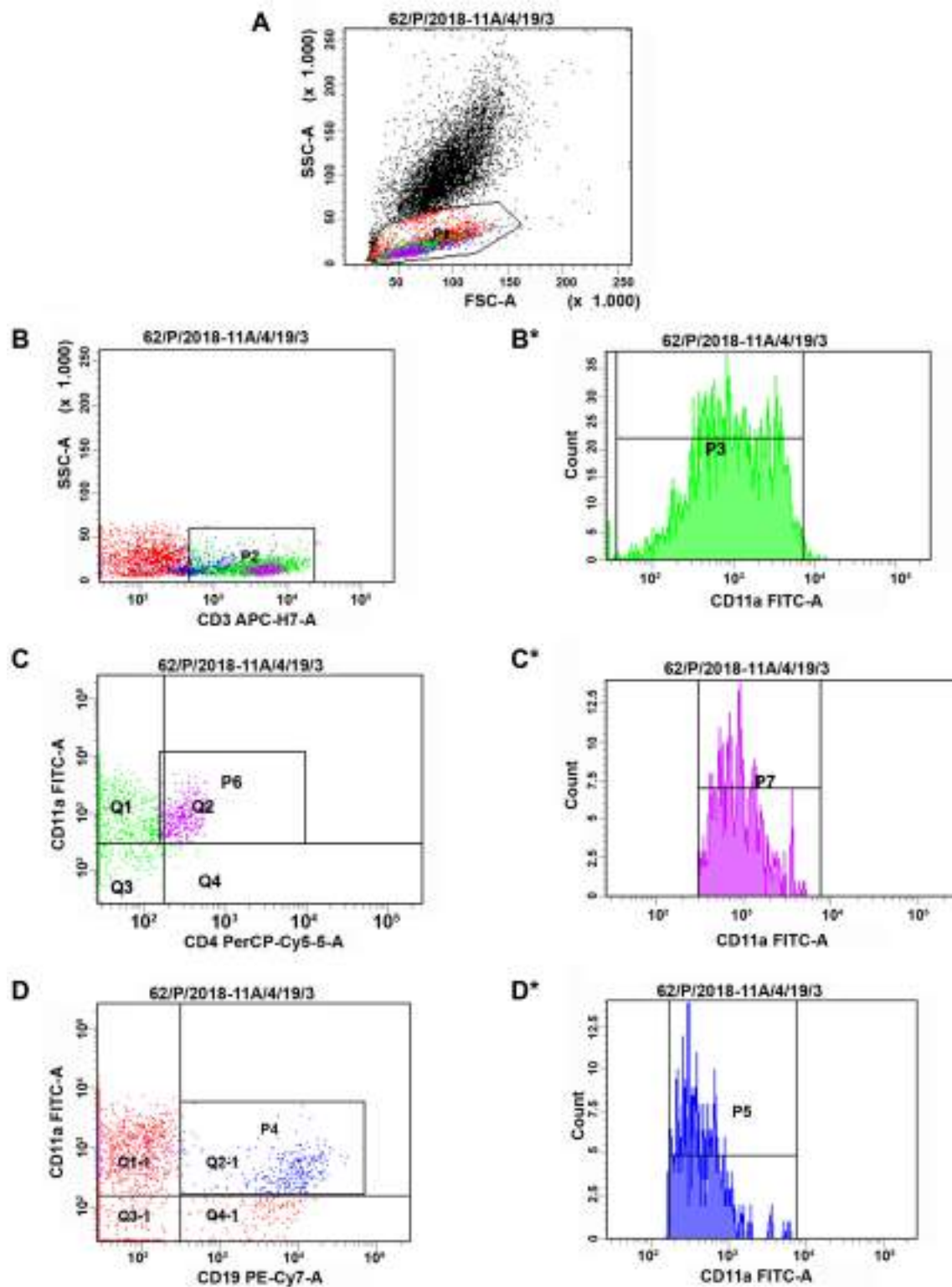
Figure 2 The expression of CD11a on lymphocyte subsets after 6 months follow-up in ITP patient whose response to treatment. (**A**) Lymphocytes were gated by flow cytometry (P1). (**B**) CD3+ cells gated by flow cytometry (P2). (**B***) MFI of CD11a gated on CD3+ cells (P3). (**C**) Expression of CD11a on CD3+CD4+ T cells (P6). (**C***) MFI of CD11a gated on CD3+CD4+ T cells (P7). (**D**) Expression of CD11a on CD19+ cells (P4). (**D***) MFI of CD11a gated on CD19+ cells (P5).
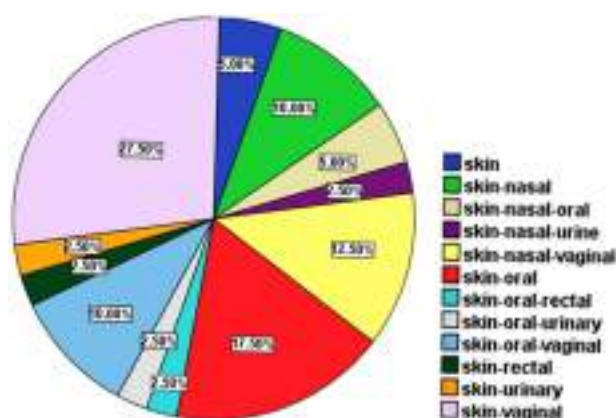
Figure 3 Pattern of bleeding in ITP patients at presentation.

There was no significant difference in the MFI of CD11a in CD3+CD4+ cells between patients and control (78.56 ±34.04 and 70.13 +22.45, respectively) with *p* -value = 0.19.

At presentation, no significant correlation between MFI of CD11a in CD3+ cells with platelet count (p value = 0.61, r=0.07) or with bleeding score (p =0.79, r=0.02). Also there was no significant correlation between MFI of

CD11a in CD19+ cells with platelet count (p value = 0.54, r=17) or with bleeding score (p = 0.71, r=0.04) (Table 3).

All patients in the study received immunosuppressive therapy and after follow-up for 6 months we found that 30 patients respond to immunosuppressive therapy (75%) while 10 patients (25%) were refractory for treatment.

There was no statistically significant difference in the MFI of CD11a in CD19+ cells between patients who responded and not responded to immunosuppressive therapy (*p*=0.50) while there was a highly significant difference in MFI of CD11a in CD3+ cells between patients who responded and not responded to therapy (*p*=0.000) with higher MFI in patients who did not respond to immunosuppressive therapy (Table 4).

There was no significant difference in the MFI of CD11a in CD19+ and CD3+ cells between patients before and after response to treatment with *p*- value = 0.16 and 0.19, respectively (Table 5).

Discussion

In this study 40 patients diagnosed as primary ITP were included after exclusion of secondary causes for

Table 1 Platelet Count Changes Before and After Treatment

Group	Platelet Count Before Treatment Mean + SD	Platelet Count After 6 Month of Treatment Mean + SD	P value
Responders	15.46 + 8.17	190.50 + 59.19	0.000***
Non-responders	19.30 + 7.81	28.88 + 8.94	0.02**
P value	0.2*	0.000***	

Notes: *Non-significant. **Significant. ***Highly significant.

Table 2 Difference Between Mean of CD11a Level in Patients and Control

	Case Mean + SD	Control Mean + SD	Significant (2-Tailed)
CD 11a MFI in CD3+ cells	70.15± 23.88	46.85 ± 17.50	0.000***
CD 11a MFI in CD19+ cells	65.20 ± 32.36	44.54± 21.02	0.001***
CD 11a MFI in CD3+CD4+ cells	78.56 ± 34.04	70.13 ± 22.45	0.19*

Notes: *Non-significant (P value > 0.05). ***Highly significant (P value < 0.005).

Table 3 Correlation of CD11a with Platelet Count and Bleeding Score

Correlation	PLT Count	Bleeding Score
CD 11a in CD19+ cells	P = 0.54* r=0. 17	P = 0.71* r=0.04
CD 11a in CD3+ cells	P = 0.61* r=0.07	P = 0.79* r=0.02

Note: *Non-significant (P value > 0.05).

Table 4 Response of Patients to Immunosuppressive Therapy

	N	CD 11a in CD3+ Cells	CD 11a in CD19+ Cells
Refractory	10 (25%)	79.47 ± 18.25	71.33 ± 32.53
Response	30 (75%) P value	42.18 ±15.43 0.000**	63.16 ± 32.60 0.50*

Notes: *Non-significant (P value > 0.05). **Highly significant (P value <0.005).

Table 5 Difference Between Level of CD11a Before and After Treatment in Patients Who Respond to Treatment

Group	CD 11a in CD3+ Cells Mean + SD	CD 11a in CD19+ Cells Mean + SD
Before treatment	79.47 ± 18.25	63.16 ± 32.60
After treatment	69.44 ± 24.55	43.59 ± 21.26
P value	0.19*	0.16*

Note: *Non-significant (P value > 0.05).

thrombocytopenia. Most of the patients were females. Other studies showed that ITP is more common in females.[14,15]

The bleeding pattern of patients in this study was bleeding from different body orifices and skin but without dangerous bleeding, this confirming that ITP is rarely associated with life-threatening bleeding events as stated by Michel and his colleagues.[16]

The results of the present study provide insight into the possible role of (LFA-1) that represented by CD11a in the pathogenesis of ITP by comparing its MFI on T and B lymphocytes and T-helper cells between newly diagnosed ITP patients and healthy control and results showed that there was high statistically significant difference between cases and control in the MFI of CD11a in T-cells (CD3+ cells) (p value=0.000) with more expression in patients than control confirming LFA-1 plays an important role on T cell activation and increased CD11a expression on T cells may induce auto reactivity in ITP. Also there was a highly significant difference in the level of CD11a in CD19+ cells (p value= 0.001) with more expression in patients than control so we suspected that increase expression of CD11a on CD19+ cells in ITP could result in sufficient B cell stimulatory signals that lead to activation of B cells to produce autoantibodies. Results of this study were confirming the theory of Carrasco and his colleagues, that LFA-1/ICAMs interaction leads to biochemical signals for T-cell-dependent B cell activation and antibody production and decreases the threshold of B cell activation caused by facilitating B cell adhesion and synapse formation.[17]

Results of this study were agreed with the study of Sela who detect an important role of LFA-1/ICAMs interaction in the pathogenesis of SLE. Using LFA-1 monoclonal antibodies in lupus mice reduce the production of auto-antibodies; reduce the development of autoimmune reaction, and the symptoms of lupus nephritis improved.[18]

In the current study, there was no significant difference between MFI of CD11a on T-helper lymphocytes (CD3 +CD4+ cells) and this may indicate that T-helper may have no role in the pathogenesis of ITP. There was no significant correlation between level of CD11a in CD3+ and CD19+ and platelet count and bleeding score so the CD11a expressions on B and T lymphocyte were not correlated with the platelet counts or bleeding score in ITP, indicating that the expression levels of CD11a on lymphocyte cells can not reflect the severity of the disease. These previous results agreed with the result Chinese study by Liu.[19]

Patients with primary ITP in this study received immunosuppressive therapy including, steroid, azathioprine, rituximab, and cyclosporine according to the international consensus report and evidence-based practice guideline by the American Society of Hematology, the standard therapy for primary ITP includes corticosteroids as the first-line agent.[20]

Follow-up after 6months, response achieved in 30 patients while the other 10 patients were refractory to immunosuppressive therapy and need other lines of treatment. Comparing means of level of CD11a in CD3+ and CD19+ cells between patients according to response to immunosuppressive therapy showed no significant

difference between responders and refractory patients in the MFI of CD11a in CD19+ cells while there was a highly significant difference in expression of CD11a in CD3+ cells between patients with more expression in patients who could not achieve response to immunosuppressive therapy, this reflect that excessive expression of CD11a in T-lymphocytes can increase the possibility of resistance to immunosuppressive therapy so, the level of CD11a in T-lymphocytes in ITP patients can be used to expect a response to treatment. Within responders to treatment, comparing means of level of CD11a in CD3+ and CD19+ cells before and after response to immunosuppressive therapy showed no significant difference which indicates that immunosuppressive therapy did not affect LFA-1/ICAM-1 pathway and using LFA-1 monoclonal antibodies may provide different mechanism for controlling ITP. A study by Sela showed that using LFA-1 monoclonal antibodies in lupus mice leading to decrease the production of autoantibodies and stopping the development of autoimmune reaction, and the symptoms of lupus nephritis resolved.[18] A study by Stephen and his colleagues detected the possible role of LFA-1/ICAM-1 interaction in the pathophysiology of dry eye disease (DED) as one of the autoimmune diseases. LFA-1/ICAM-1 interaction is a rational therapeutic target in DED. Inhibition of LFA-1/ICAM-1 interaction represents a rational targeted approach in treating DED.[21]

Conclusions

LFA-1 and ICAM-1 play a role in the pathogenesis of ITP. Immunosuppressive therapy in ITP did not affect the level of CD11a expression on T and B lymphocytes. Levels of CD11a do not reflect the severity of ITP neither platelet count nor bleeding score. Increased MFI of CD11a in CD3 +T-lymphocytes of ITP patients may cause resistance to immunosuppressive therapy.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

All authors declare that they have no conflicts of interest for this work.

References

1. McMillan R. Immune-mediated thrombocytopenias focus on chronic immune thrombocytopenic purpura. *Semin Hematol*. 2007;44:1–2. doi:10.1053/j.seminhematol.2007.11.001
2. Ahn YS, Horstman LL. Idiopathic thrombocytopenic purpura: pathophysiology and management. *Int J Hematol*. 2002;76(2):123–131. doi:10.1007/BF03165102
3. Kawana M, Ikeda Y. The role of autoreactive T Cells in the pathogenesis of idiopathic thrombocytopenic purpura. *Int J Hematol*. 2005;81:106–112. doi:10.1532/IJH97.04176
4. Kim SY, Solomon DH. Tumor necrosis factor blockade and the risk of viral infections. *Nat Rev Rheumatol*. 2010;6(3):165–174. doi:10.1038/nrrheum.2009.279
5. Targownik LE, Bernstein CN. Infectious and malignant complications of TNF inhibitor therapy in IBD. *Am J Gastroenterol*. 2013;108 (12):1835–1842. doi:10.1038/ajg.2013.294
6. McKenzie CG, Guo L, Freedman J, Semple JW. Cellular immune dysfunction in immune thrombocytopenia (ITP). *Br J Hematol*. 2013;163:10–23. doi:10.1111/bjh.12480
7. Ghislin S, Obino D, Middendorp S, Boggetto N, Alcaide-Loridan C, Deshayes FLFA. 1 and ICAM-1 expression induced during melano-maendothelial cell co-culture favors the transendothelial migration of melanoma cell lines in vitro. *BMC Cancer*. 2012;12:455. doi:10.1186/1471-2407-12-455
8. Parameswaran N, Suresh R, Bal V, Rath S, George A. Lack ofICAM-1 on APCs during T cell priming leads to poor generation of central memory cells. *J Immunol*. 2005;175:2201–2211. doi:10.4049/jimmunol.175.4.2201
9. Pribila JT, Quale AC, Mueller KL, Shimizu Y. Integrins and T cell mediated immunity. *Annu Rev Immunol*. 2004;22:157–180. doi:10.1146/annurev.immunol.22.012703.104649
10. Bose TO, Colpitts SL, Pham QM, Puddington L, Lefrançois L. CD11a is essential for normal development of hematopoietic intermediates. *J Immunol*. 2014;193:2863–2872. doi:10.4049/jimmunol.1301820
11. Neunert C, Lim W, Crowther M. The American Society of Hematology 2011 evidence-based practice, guidelines for immune thrombocytopenia. *Blood*. 2011;117:4190–4207. doi:10.1182/blood-2010-08-302984
12. Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. *Blood Adv*. 2019;10(23):3829–3866. doi:10.1182/bloodadvances.2019000966
13. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113:2386–2393. doi:10.1182/blood-2008-07-162503.
14. Abbasi SY, Milhem M, Zaru L. A positive antinuclear antibody test predicts for a poor response to initial steroid therapy in adults with idiopathic thrombocytopenic purpura. *Ann Hematol*. 2008;87(6):459–462. doi:10.1007/s00277-008-0448-1
15. Grimaldi-Bensouda L, Nordon C, Michel M, et al. Immune thrombocytopenia in adults: a prospective cohort study of clinical features and predictors of outcome. *Haematologica*. 2016;101(9):1039–1045. doi:10.3324/haematol.2016.146373.

16. Rosenblum MD, Remedios KA, Abbas AK. Mechanisms of human autoimmunity. *J Clin Invest*. 2015;125(6):2228–2233.

17. Carrasco YR, Fleire SJ, Cameron T, Dustin ML, Batista FD. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. *Immunity*. 2004;20:589–599. doi:10.1016/S1074-7613(04)00105-0

18. Sela U, Mauermann N, Hershkoviz R, et al. The inhibition of autoreactive T cell functions by a peptide based on the CDR1 of an anti-DNA autoantibody is via TGF-beta mediated suppression of LFA-1 and CD44 expression and function. *J Immunol*. 2005;175:7255–7263. doi:10.4049/jimmunol.175.11.7255

19. Liu YX, Zhang F, Yao QM, Yuan T, Xu J, Zhu XJ. Expression of CD11a in lymphocyte subpopulation in immune thrombocytopenia. *Int J Clin Exp Pathol*. 2015;8(12):15642–15651.

20. Provan D, D M A, J B B, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *J Blood Adv*. 2019;3:3780–3817.

21. Stephen C, Pflugfelder MS, Steven Z, Amir SLFA. 1/ICAM-1 interaction as a therapeutic target in dry eye disease. *J Ocular Pharmacol Ther*. 2017;33(1). doi:10.1089/jop.2016.0105

Thalassemia Minor Presenting with Vitamin B$_{12}$ Deficiency, Paraparesis, and Microcytosis [Corrigendum]

CORRIGENDUM

Thalassemia Minor Presenting with Vitamin B$_{12}$ Deficiency, Paraparesis, and Microcytosis [Corrigendum]

Lardhi A, Alhaj Ali R, Ali R, Mohammed T. *J Blood Med.* 2018;9:141—144.

The authors have advised there is an error in the author list on page 141. The author name "Tarek Mohammed" should read "Tarek Mohamed".

The authors apologize for this error.

Asymptomatic Joint Bleeding and Joint Health in Hemophilia: A Review of Variables, Methods, and Biomarkers

Richard Gooding, Jecko Thachil, Jayanthi Alamelu, Jayashree Motwani & Pratima Chowdary

REVIEW

Asymptomatic Joint Bleeding and Joint Health in Hemophilia: A Review of Variables, Methods, and Biomarkers

Richard Gooding[1]
Jecko Thachil[2]
Jayanthi Alamelu[3]
Jayashree Motwani[4]
Pratima Chowdary[5]

[1]Leicester Haemophilia Centre, Haemostasis & Thrombosis Unit, Leicester Royal Infirmary, Leicester, UK; [2]Haematology Department, Manchester Royal Infirmary, Manchester, UK; [3]Haemophilia Department, Evelina Children's Hospital – St Thomas' Hospital, London, UK; [4]Haemophilia Comprehensive Care Centre, Birmingham Children's Hospital, Birmingham, UK; [5]Katharine Dormandy Haemophilia Centre, Royal Free Hospital, London, UK

Abstract: Joint health is a key contributor to quality of life in patients with hemophilia. However, variables that impact long-term joint outcomes have not been comprehensively defined. A systematic literature search identified publications relating to joint health in patients with hemophilia. Studies clearly show that early, sustained prophylaxis with factor replacements improves long-term joint outcomes. However, a subset of patients appear to develop arthropathy despite maintaining excellent bleeding outcomes, which suggests possible recurrent asymptomatic bleeding into the joints in these patients. Furthermore, limited data are available on how long-acting factor VIII and factor IX replacement therapies could impact long-term joint outcomes. Many variables were identified as potential indicators that a patient may develop hemophilic arthropathy, including genetic mutations, endogenous factor VIII and IX levels, bone health, and physical activity levels. Tools for the diagnosis and monitoring of hemophilic arthropathy are critical to detect early joint damage, so that management can be adjusted accordingly. Imaging techniques, particularly magnetic resonance imaging, can detect synovial changes, a strong predictor for the future development of hemophilic arthropathy. In addition, several biomarkers associated with cartilage and bone formation, vascularization, and angiogenesis could potentially identify the onset and progression of early joint damage. Since the development of hemophilic arthropathy is complex, a comprehensive therapeutic approach is necessary for the effective prevention of arthropathy in patients with hemophilia.

Keywords: hemophilia, asymptomatic bleeding, joint disease, hemarthrosis, hemophilic arthropathy, hemarthropathy

Introduction

Joint bleeds can lead to inflammation and destruction of the joint, which ultimately results in arthropathy, a significant morbidity in patients with hemophilia. Recurrent hemarthrosis results in the accumulation of hemosiderin, synovial inflammatory changes, cartilage degradation and, in late stages, joint destruction.[1,2] Hemophilic arthropathy is associated with increased pain, loss of employment, school absenteeism, and a reduction in physical wellbeing and quality of life.[3–7]

Effective prevention of joint deterioration is limited by the relatively limited knowledge of the pathogenesis of hemophilic arthropathy.[1] In addition, although commonly associated with severe disease, it is not currently possible to accurately predict which patients will develop hemophilic arthropathy as patients may develop arthropathy with few or no joint bleeds.[8–12] In patients with established joint

Correspondence: Richard Gooding
Leicester Haemophilia Centre, Haemostasis & Thrombosis Unit, Level 2, Osborne Building, The Leicester Royal Infirmary, Infirmary Square, Leicester, LE1 5WW, UK
Tel +44 116 258 6500
Fax +44 116 258 6120
Email richard.gooding@uhl-tr.nhs.uk

damage, joint deterioration often progresses even if few or no further joint bleeds occur.[13] These observations suggest the occurrence of bleeding that is not detected by the patients, and this recurrent asymptomatic joint bleeding might contribute to the development of hemophilic arthropathy.[9,11,13]

Early prophylaxis with factor replacement concentrates can prevent joint bleeding, and thus the development of arthropathy.[12,14] Furthermore, identifying the variables that influence the progression of hemophilic arthropathy, including asymptomatic joint bleeding, may highlight modifiable aspects that could aid patients, or help to identify patients who may benefit from more intensive therapy in the future. Here, we firstly present the findings from a systematic literature review to identify variables that influence joint deterioration in patients with hemophilia. Secondly, we discuss methods to help identify patients with hemophilia who are at risk of joint deterioration and we consider the impact of long-acting factor replacement therapies on joint outcomes. We also consider which biomarkers may be associated with bone health in these patients. Lastly, we consider future research and practice directions for the prevention of arthropathy in patients with hemophilia.

Joint Outcomes in Hemophilia: A Systematic Literature Review
Methods

A systematic literature search was conducted in both EMBASE and PubMed, according to PRISMA guidelines,[15] on November 2018 and updated in July 2020 to identify variables that could influence the development of arthropathy, as assessed by joint scores, in patients with hemophilia. Search terms were designed to select publications according to the patient population, treatment administered, and outcomes reported. The search was limited to articles published in English, with no specified date range for PubMed and 1947 to present for EMBASE.

The following search term was used: Hemophilia AND (Magnetic Resonance Imaging OR Diagnostic imaging OR Ultrasonography OR Radiography OR Arthroscopy OR Synovectomy OR Arthralgia OR Hemarthrosis OR synovitis OR Synovial Fluid OR Synovial Membrane OR Hyperalgesia OR Brain Infarction OR Cerebral Hemorrhage OR Subclinical OR Silent OR Cartilage OR Hemochromatosis OR Iron OR Joint OR Articular OR

Table 1 Systematic Literature Review Inclusion and Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
Original article	Review article or case study
Congenital hemophilia	Duplicate results
Included quantifiable	
information on joint health	Not in English
MRI scoring	Included joint interventions – such as
HEAD US scoring	physiotherapy, surgery or
Gilbert	radiosynovitis
Pettersson	Non-relevant disease model
ROM	Absence of hemophilia patients
HJHS/HJHS 2.1	
Arthropathy diagnosis code	
Orthopedic score (WFH)	
Petrini score	

Abbreviations: HEAD US, Hemophilia Early Arthropathy Detection with Ultrasound; HJHS, Hemophilia Joint Health Score; MRI, magnetic resonance imaging; ROM, range of motion; WFH, World Federation of Hemophilia.

Hemosiderin). The search terms were derived from an exploded MeSH term analysis that identified related terms.

All publications retrieved by this search strategy were individually assessed against pre-defined inclusion and exclusion criteria (Table 1). The aim was to identify original articles that looked at joint health outcomes assessed by joint scores. Publications underwent an initial screen based on the title and abstract using these inclusion and exclusion criteria. Potentially relevant publications then underwent a second screen based on the full text of the article. The relevant data from all eligible publications were collected and aggregated to allow further analysis.

Results

The initial search identified 5,380 results. Following the initial abstract and title screen, 861 articles were identified that met the initial screening criteria. The full-text screen identified 256 publications that measured arthropathy and recorded additional data on patients (Figure 1). Due to the wide variability in the outcomes considered, formal study quality assessment and meta-analytic evaluation were not performed.

Variables Influencing Joint Outcomes

The key variables that may influence joint outcomes highlighted by the literature search results were disease severity, gene mutations, bone mineral density (BMD), physical activity, and prophylaxis with factor replacement concentrates.
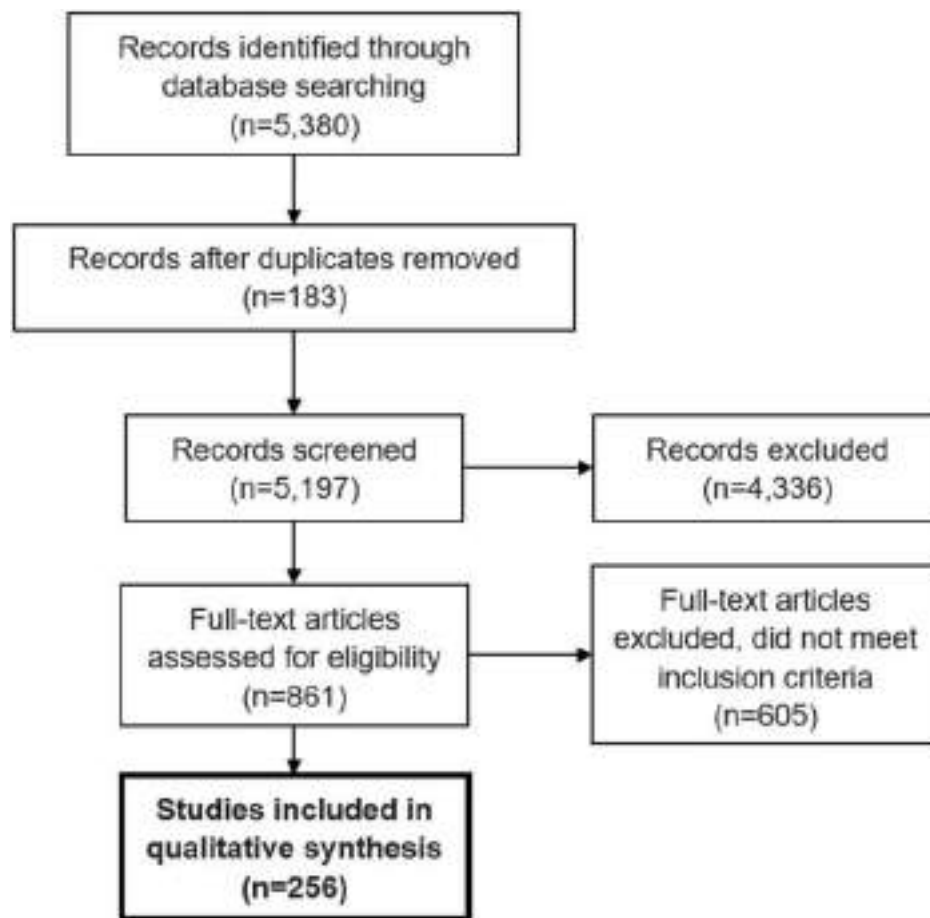
Figure I PRISMA systematic literature review flow chart.

Disease Severity

Low endogenous FVIII/FIX activity levels (~1 IU/dl, 1% of normal) are known to be associated with higher rates of arthropathy due to the increased susceptibility to bleeding.[16–21] Patients with severe hemophilia (<1 IU/dl, <1% of normal) are more prone to joint damage than patients with mild or moderate hemophilia.[17] However, patients with moderate (1–5 IU/dl, 1–5% of normal) and mild (5–40 IU/dl, 5–40% of normal) hemophilia are still susceptible to developing arthropathy.[16,20,22,23] Factor levels of 1–3 IU/dl are now considered insufficient to fully prevent bleeding in all patients with hemophilia.[12] Even though the majority of patients with moderate hemophilia have fewer joint bleeds than patients with severe hemophilia, a subset of these patients (~30% of patients) experience bleeding and long-term joint damage.[16,22] Patients with mild hemophilia have a higher likelihood of developing chronic arthropathy than the general population and asymptomatic bleeding has been suggested as

its potential cause.[23] It is also notable that hemophilia carriers with factor activity levels >40 IU/dl (40% of normal) can experience decreases in joint range of motion (ROM) and structural joint changes.[18,19,21]

Gene Mutations

Several studies have correlated gene mutations with joint outcomes in patients with hemophilia (Table 2).[24–30] An investigation conducted in 14 hemophilia treatment centers in the United States found that a total of 613 single nucleotide polymorphism (SNP) markers were significantly correlated with ROM scores on at least two joint pairs.[24] This study also identified multiple inflammatory- or immune-related genes that were associated with arthropathy; SNPs in the NOD2 (associated with musculoskeletal manifestations in inflammatory bowel disease) and TLR10 (role in pathogen recognition and activation of innate immunity) genes were potentially strongly associated with arthropathy, but only a small number of patients carried the variant

Table 2 Gene Mutations and Genotypes Associated with Joint Health in Patients with Hemophilia

Gene/Genotype	Mutation Type	Joint Health
Inflammatory and immune genes		
NOD2	Nonsynonymous	Increased risk of ROM abnormalities[24]
TLR10	Missense	Increased risk of ROM abnormalities[24]
HLA B27		Greater risk of developing chronic synovitis[27,28]
MTHFR 677TT		Higher number of affected joints[30]
MTHFR 1298AC		Higher degree of effusion[30]
TNFα-308GA		Increased number of subchondral cysts[30]
Genes encoding coagulation factors		
FVIII	Missense, nonsense, frameshift, intron 22 inversion	No association with HJHS or Gilbert score[25]
	Inversion, deletion, insertion, nonsense	Increased risk for a severe phenotype (>10 Pettersson score)[26]
FIX	Inversion, deletion, insertion, nonsense	Increased risk for a severe phenotype (>10 Pettersson score)[26]
Gene encoding homeostatic iron regulator protein		
HFE	C282Y, H63D	Increased number of hemarthrosis per year and number of affected joints[29]

Abbreviations: HJHS, Hemophilia Joint Health Score; ROM, range of motion.

alleles.[24] In another study, the underlying factor VIII (FVIII) mutation type (missense, nonsense, frameshift, intron 22 inversion) was not found to be significantly associated with joint deterioration in a cohort of hemophilia A patients with minimal access to hemostatic treatment.[25] In contrast, a study from India found that severe molecular defects of the FVIII or factor IX (FIX) gene were associated with disease severity, including poor joint outcomes, as patients with inversions, deletions, insertions and nonsense mutations had an approximate four-fold increased risk of having a severe disease phenotype.[26] Additionally, functional polymorphisms in the FVIII gene also affect the

phenotype; for example, patients with an FVII353Q allele had an increased risk of a severe phenotype.[26] However, this study did not identify any significant association with inflammatory or immunoregulatory cytokines. Two publications noted that HLA B27 mutations (associated with seronegative spondyloarthritis) were correlated with synovitis and arthropathy[27,28] and another reported that HFE mutations (associated with hemochromatosis), resulting in reduced iron absorption, contributed to the development of arthropathy.[29] A small study reported that patients with the MTHFR variants MTHFR 677TT or MTHFR 1298AC had a higher number of affected joints or a higher degree of effusion, respectively, compared to patients with other genotypes.[30] Furthermore, this study also found a positive association between genetic biomarkers related to inflammation and number of subchondral cysts.

Prophylaxis

Publications largely agreed that prophylaxis with factor replacement therapy reduces the rate of arthropathy development compared to episodic treatment.[8,14,31–38] Several publications showed that an early start to prophylaxis (before 2–3 years of age) reduces the risk of developing arthropathy.[33,35,36,39–42] However, it has been reported that early prophylaxis is not sufficient to completely prevent joint damage.[8–10,42] Prophylaxis initiated later in a patient's life has been shown to still provide benefits over episodic treatment with lower bleeding rates and improved joint health over time.[14,34,35] However, no reduction in structural arthropathy progression was observed, suggesting that pre-existing joint arthropathy may be irreversible,[14] further emphasizing the importance of primary prophylaxis. Continued prophylaxis also appears to be important; patients who stopped prophylaxis in adulthood showed similar bleeding rates to those on prophylaxis but had significantly higher rates of arthropathy over time.[43,44]

Bone Mineral Density and Physical Activity

Several studies found that low BMD correlated with arthropathy.[45–55] It has been suggested that the chronic pain associated with arthropathy leads to reduced mobility and an avoidance of weight-bearing activity, ultimately resulting in decreased BMD.[45–53] Overall, studies indicated that physical activity did not negatively affect joint health and may even improve joint health in patients with

hemophilia.[56–59] This may be due to improved muscle strength, balance and co-ordination seen in patients who maintain physical activity.

Identification of Patients with Hemophilia at Risk of Joint Deterioration

Imaging

Magnetic resonance imaging (MRI) is considered the most sensitive measure for joint assessment.[13] MRI provides detailed joint images and can detect early damage (ie, synovial hypertrophy) and soft tissue changes in joints as well as bleeds (Table 3).[13,60–62] MRI can also identify whether bleeds have occurred in a joint by detecting hemosiderin in the joint.[13,60,63,64] However, MRI can be expensive, time-consuming and difficult to access, and often requires sedation in children.[13,60] Ultrasound is less sensitive to early joint changes than MRI and is not able to

discriminate between soft tissue types,[60,64] but is a cheaper imaging modality, more widely available, and easy to access.[13,64] Ultrasound can be used to detect synovial hypertrophy, and cartilage and bone surface abnormalities (Table 3).[13,60,61,63,65] In addition, it can also be used to rapidly assess multiple joints at once.[61] Patients only need to remain still for a short timeframe, which can be valuable when studying children's joints, and ultrasound can be incorporated into routine check-ups.[60,61,63,64] X-rays can also be used to assess joint deterioration and have demonstrated a good correlation with ultrasound and physical assessments, but low correlation with MRI findings.[66–68] X-rays show only late (ie, osteochondral) joint changes,[13] and when radiographic changes are detected, arthropathy is frequently already advanced.[13,62,65]

Clinical Joint Scores

Physical examination scales such as the Hemophilia Joint Health Score (HJHS) have been successfully used to assess joint outcomes, without requiring imaging techniques.[69–72] However, studies have found that the HJHS is not as sensitive as MRI or ultrasound, and thus assessment methods should be combined to generate a more detailed analysis of joints, until more accurate methods of assessment and scoring are available.[65,67] Smaller studies have also looked at using thermal imaging for inflammation, surface electromyography for muscle function and balance analysis for the lower limbs to detect early signs of joint deterioration.[73–75] These methods are not well characterized and are not routinely used for assessing joint outcomes.

Importantly, several studies have reported that bleeding rates do not or only weakly correlate with other outcomes such as MRI or HJHS,[8,67,71,76,77] and that joints deemed as "normal" according to physical examination or x-ray show abnormalities on MRI.[65,68,71] Therefore, joints that appear normal may be affected by asymptomatic bleeding. MRI is highly sensitive and can detect early joint damage, even before clinical symptoms manifest, and these MRI changes have been shown to strongly predict future development of arthropathy.[62] However, because MRI is not easily accessible, ultrasound may be a good affordable alternative.[13] Imaging techniques used in combination with thorough physical examination could provide accurate joint outcomes assessment tools for early diagnosis and subsequent monitoring of joint damage. For instance, MRI can be used to monitor normal joints in patients with hemophilia, including patients with mild hemophilia, in order to detect

Table 3 Properties of Magnetic Resonance Imaging (MRI) and Ultrasound

Properties	MRI	Ultrasound
Physiology and anatomy		
Detection of hemosiderin deposition	✓	✗
Detection of synovial inflammation	✓	✓
Detection of synovial hypertrophy and hyperplasia	✓	✓
Detection of soft tissue changes	✓	✓
Detection of osteochondral changes	✓	✓
Detection of joint effusions/hemarthrosis	✓	✓
Detection of cartilage and bone surface abnormalities	✓	✓
Detection of osteopenia	✓	✓
Methodology		
Visualization of internal joint structure and soft tissue types	✓	✗
Detection of different signal intensities	✓	✗
High spatial resolution	✓	✗
Not operator-dependent	✓	✗
Possible to scan multiple joints at once	✗	✓
Patient perspective		
No need for sedation in children	✗	✓
Fast	✗	✓
Health care systems		
Economical	✗	✓
Routine use	✗	✓
Readily available	✗	✓

Abbreviation: MRI, magnetic resonance imaging.

early arthropathic changes, which could allow for treatment adjustment and potentially prevent further joint deterioration, irreversible functional impairment, and future need for orthopedic surgery. Similarly, patients with mild damage could be monitored by ultrasound and patients with established disease could be monitored by joint scores and targeted imaging.

Impact of Long-Acting Factor Replacement Therapy on Joint Outcomes

Prophylaxis with long-acting factor replacement therapies in both hemophilia A and B has been associated with a reduced number of joint bleeds and resolution of target joints during clinical trials.[78] Recently, Zanon et al studied whether adherence to prophylaxis impacted on joint outcomes (HJHS) and involvement in physical activity.[79] The study reported a decrease in HJHS and the number of total target joints and an increase in physical activity levels in patients who had high adherence to prophylaxis compared to patients with no or low adherence.[79] Since poor adherence to prophylaxis might result in worsened joint outcomes, the use of long-acting products may help to avoid the low trough levels of FVIII and FIX that put patients at higher risk of bleeds (and then lead to joint problems).

The majority of the studies discussed in the systematic literature review that specifically investigated joint health and prophylaxis used standard-acting products. Recently, Malec et al compared joint health (HJHS) in patients with severe hemophilia A and B receiving standard-acting and long-acting products.[80] The study reported no differences in mean HJHS for either group of patients receiving standard-acting compared to those receiving long-acting products. However, joint health data were collected at a single time point, and thus the study did not assess the time of switch to long-acting products nor the status of joints over time after switching to long-acting products. Further data on arthropathy development in patients on long-acting products, as well as on patients switching from standard-acting to long-acting products, are needed.

Biomarkers and Bone Health in Patients with Hemophilia

Recurrent bleeding into the joints causes the synovium to hypertrophy in order to clear blood from the joint space, the synovium is overwhelmed and iron (hemosiderin) accumulates leading to synovial angiogenesis and

inflammation.[1] The synovium produces pro-inflammatory cytokines and proteases, and this chronic proliferation of inflammatory cells causes the cartilage to breakdown. Furthermore, the constant presence of blood in the joint leads to bone changes, bone remodeling, loss of BMD and osteoporosis.[1] Consequently, biomarkers of inflammation, and cartilage, bone and synovium changes could provide a prospective method to detect and monitor asymptomatic joint bleeding as well as early joint damage.

Several studies have identified biomarkers in the blood or urine that could be indicative of joint deterioration. Table 4 provides an overview of these potential biomarkers of bone health. Proteins associated with inflammation (CRPM, hsCRP), cartilage destruction (C2M, CTX-II, COMP, ADAMTS5) and bone turnover (PINP, CTX-I) have been detected in hemophilia patients with joint disease.[81] Even though not all these biomarkers were correlated with radiological or physical joint assessments, the combination of C2M, CRPM and ADAMTS5 was able to distinguish hemophilia patients with joint disease from healthy controls with high accuracy.[81] Another study reported that several biomarkers of cartilage deterioration (CTX-II, C1,2C, CS-846 and COMP) correlated with radiographic joint damage in patients with hemophilic arthropathy, but bone biomarkers (CTX-I, C1,2C) did not.[82] One study found that serum levels of the key bone turnover markers sRANKL and OPG were significantly lower in patients with hemophilia than in healthy controls ($p<0.05$ and $p<0.001$, respectively), and these had an inverse correlation with joint outcomes (MRI and ultrasound).[83] The levels of serum sclerostin, another key regulator of bone formation, were significantly elevated in children with hemophilia versus healthy controls ($p=0.028$) and had a positive correlation with joint damage (HJHS); elevated levels of serum sclerostin in patients with hemophilia might be indicative of a high risk for developing osteoporosis.[52]

In another study, serum levels of the proinflammatory cytokine TNF-α were significantly elevated in patients with hemophilic arthropathy compared to healthy controls ($p<0.0001$).[84] Moreover, TNF-α levels were positively correlated with the number of joint bleeds, degree of synovial hypertrophy, and clinical and ultrasound joint outcomes. The authors suggest that TNF-α is involved in the progression of hemophilic arthropathy and could be a suitable biomarker to detect joint deterioration in patients with hemophilia.[84] However, TNF-α is an acute phase inflammatory marker that is elevated in a range of conditions, including rheumatoid arthritis, and as such is not specific to hemophilic

Table 4 Potential Biomarkers of Bone Health

Biomarkers	Decreased Levels in Patients with Hemophilia vs Healthy Controls	Increased Levels in Patients with Hemophilia vs Healthy Controls	Significant Correlation with Joint Scores
Inflammation	CRPM[81]	hsCRP[81] TNF-α[84]	TNF-α[84]
Cartilage destruction	ADAMTS5[81]	C2M[81] CTX-II[81] COMP[81]	CTX-II[81,82] C1,2C[82] CS-846[82] COMP[82]
Bone turnover	PINP[81] sRANKL[83] OPG[83]	CTX-I[81] Sclerostin[52]	sRANKL[83] OPG[83] Sclerostin[52]
Vascularization and angiogenesis		Microvascular density[88] VEGF expression[87,88] VEGFA[87] SDF-1α[87] MMP-9[87] HIF-1α[87] VEGFR1/CD11b[87] CD34/VEGFR1[87] VEGF/CD68[87] VEGFR2/AC133[87]	
Others	VitaminD[90–92]		Iron accumulation in cartilage[86] VitaminD[90–92]

arthropathy.[85] Iron accumulation in cartilage is another biomarker that has been shown to correlate with joint damage and progression of hemophilic arthropathy, further it has the advantage of being easily detected with MRI T2* sequences.[86] The development and validation of iron quantification MRI methods could assist in detecting asymptomatic joint bleeding, and provide a tool to evaluate and adjust treatment in patients with hemophilia.[86]

Vascularization and angiogenesis have been found to be increased in patients with joint damage. One study found that proangiogenic factors and proangiogenic macrophage/monocyte cells were up-regulated in patients with joint disease and expression of VEGFR2/AC133 endothelial progenitor cells and CD34/VEGFR1 hematopoietic progenitor cells were increased.[87] Sera from patients with joint damage induced an angiogenic response in endothelial cells, while peripheral blood mononuclear cells from these subjects induced synovial cell proliferation.[87] In another study, microvascular density and VEGF expression were significantly increased (p<0.005 and p=0.02, respectively) in synovial tissue from patients with hemophilic arthropathy compared to healthy controls.[88]

As mentioned above, there is evidence of an association between hemophilic arthropathy and low BMD. Furthermore, it has been shown that FVIII or FIX deficiency results in reduced BMD, with a quarter of patients with hemophilia having osteoporosis.[89] Low vitamin D levels have also been associated with decreased BMD, physical activity, quality of life and poor joint health.[90–92] These studies suggest that routine assessment of serum levels of vitamin D could guide early diagnosis of joint damage and treatment in patients with hemophilia, particularly in children. In patients with hemophilia, prevention of poor bone health should include primary prophylaxis, a diet rich in calcium and vitamin D, physiotherapy, and weight-bearing physical activities.[52,89–91]

Prevention of Arthropathy in Patients with Hemophilia: Future Directions in Research and Practice

There is robust evidence that prophylaxis with factor replacement therapies reduces the number of joint bleeds

and rate of arthropathy development; however, the optimal trough level to protect patients from joint damage has not been determined yet. Furthermore, factor replacement alone may not be sufficient to prevent joint damage. A recent study by Zhou et al reported that joint bleeding events were not associated with time spent below certain clotting factor thresholds, and that vascularity changes also played a role in joint bleeding.[93] Currently, an ongoing clinical trial (NCT03358836) is studying whether a long-acting FIX product with an intended trough level of >10% could provide better joint protection in patients with severe hemophilia B than the standard trough of 1% FIX. This study will also evaluate the early diagnosis of joint damage using ultrasound. The results from this study will be important, particularly since recent World Federation of Hemophilia guidelines have deemed factor trough levels of 1–3% insufficient to fully prevent bleeding in all patients with hemophilia.[12] However, results are not expected until 2027.

As previously discussed, several studies have identified potential targets for the prevention and treatment of arthropathy in patients with hemophilia, including cartilage iron, inflammation, bone remodeling, cartilage regeneration and vascular remodeling. Accordingly, a pilot study of the safety and efficacy of local anti-VEGF therapy with intra-articular bevacizumab (Avastin®) for prevention of recurrent hemarthroses at target joints is currently ongoing in Taiwan in patients with chronic hemophilic synovitis and was due for completion in late 2020 (NCT02060305).

Furthermore, it is not yet known what level of joint protection emergent non-factor replacement therapies and gene therapy may provide. Some of these products are still in development and it will be some time until their effects on joint health, and prevention and treatment of hemophilic arthropathy can be analyzed.

Finally, physiotherapists play a valuable role in assessing patients' joint status and in aiding recovery of function after a joint bleed, but many centers lack a dedicated hemophilia physiotherapist. Dedicated hemophilia 'joint assessors' could play an important role in providing dedicated time for thorough physical examination and joint scores, and in monitoring the progress of joint deterioration. Currently, home-based physiotherapy programs are being evaluated in people with hemophilia as an inexpensive accessible intervention with the potential to improve joint health.[94,95] Individualized home-based exercise programs, with access to online/virtual tools and support from physiotherapists, have been positively received with

encouraging results.[94–96] Future randomized controlled clinical trials will be able to provide evidence of the clinical efficacy of such programs.

Conclusions

Currently, the best treatment option for patients with hemophilia is prophylaxis, particularly in young patients for whom primary prophylaxis is vital for the prevention of bleeding and to promote healthy joints. However, maintaining factor levels over a certain threshold does not fully prevent joint bleeds. Joint abnormalities and reduction of ROM have been reported even in patients who started prophylaxis early, in patients with mild hemophilia and in hemophilia carriers. In these cases, the occurrence of asymptomatic joint bleeding might be undetected for a significant amount of time before it is identified, and intervention can be provided. Therefore, early detection of changes in "normal" joints becomes crucial. To this end, sensitive diagnostic methods such as MRI or ultrasound are critical for early diagnosis of joint damage, monitoring of deterioration and treatment guidance, particularly in patients with normal physical and x-ray assessments. Although specific biomarkers of joint damage are still lacking, a few candidates such as cartilage iron, and inflammatory, cartilage destruction and bone formation factors have the potential to become novel targets for early diagnosis, prevention and treatment of joint damage in patients with hemophilia. The future of treatment of patients with hemophilia may be a therapeutic approach consisting of primary prophylaxis with factor replacement, calcium and vitamin D supplementation, physiotherapy and weight-bearing exercise programs, together with routine monitoring of cartilage iron, vitamin D, and key inflammatory and bone formation biomarker levels, as well as joint status with sensitive imaging and physical assessment tools.

Abbreviations

BMD, bone mineral density; FVIII, factor VIII; FIX, factor IX; HJHS, Hemophilia Joint Health Score; MRI, magnetic resonance imaging; ROM, range of motion; SNP, single nucleotide polymorphism.

Acknowledgments

Funding

Disclosure

Dr Richard Gooding reports personal fees from CSL Behring, during the conduct of the study. Dr Jecko Thachil reports personal fees from CSL Behring, Takeda, Shire, Roche Chugai, and Sobi, during the conduct of the study. Professor Pratima Chowdary reports non-financial support from CSL Behring, during the conduct of the study; grants from and advisory committee for Pfizer, Bayer, CSL Behring, Freeline, Novo Nordisk, Sobi, Chugai, Roche, Takeda, Sanofi, and Spark; personal fees from BioMarin and UniQure, outside the submitted work. The authors report no other conflicts of interest in this work.

References

1. van Vulpen LFD, Holstein K, Martinoli C. Joint disease in haemophilia: pathophysiology, pain and imaging. *Haemophilia*. 2018;24 (S6):44–49. doi:10.1111/hae.13449

2. Rodriguez-Merchan EC. Musculo-skeletal manifestations of haemophilia. *Blood Rev*. 2016;30(5):401–409. doi:10.1016/j.blre.20 16.04.008

3. Chen CM, Huang KC, Chen CC, et al. The impact of joint range of motion limitations on health-related quality of life in patients with haemophilia A: a prospective study. *Haemophilia*. 2015;21(3):e176–e184. doi:10.1111/hae.12644

4. Holstein K, von Mackensen S, Bokemeyer C, Langer F. The impact of social factors on outcomes in patients with bleeding disorders. *Haemophilia*. 2016;22(1):46–53. doi:10.1111/hae.12760

5. Khair K, Holland M, Bladen M, Griffioen A, McLaughlin P, von Mackensen S. Study of physical function in adolescents with haemophilia: the SO-FIT study. *Haemophilia*. 2017;23(6):918–925. doi:10. 1111/hae.13323

6. O'Hara J, Walsh S, Camp C, et al. The impact of severe haemophilia and the presence of target joints on health-related quality-of-life. *Health Qual Life Outcomes*. 2018;16(1):84. doi:10.1186/s12955-018-0908-9

7. Rodriguez-Merchan EC. Treatment of musculo-skeletal pain in haemophilia. *Blood Rev*. 2018;32(2):116–121. doi:10.1016/j.blre.20 17.09.004

8. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535–544. doi:10.1056/NEJMoa067659

9. Olivieri M, Kurnik K, Pfluger T, Bidlingmaier C. Identification and long-term observation of early joint damage by magnetic resonance imaging in clinically asymptomatic joints in patients with haemophilia A or B despite prophylaxis. *Haemophilia*. 2012;18(3):369–374. doi:10.1111/j.1365-2516.2011.02682.x

10. Kraft J, Blanchette V, Babyn P, et al. Magnetic resonance imaging and joint outcomes in boys with severe hemophilia A treated with tailored primary prophylaxis in Canada. *J Thromb Haemost*. 2012;10 (12):2494–2502. doi:10.1111/jth.12025

11. Di Minno MND, Iervolino S, Soscia E, et al. Magnetic resonance imaging and ultrasound evaluation of "healthy" joints in young subjects with severe haemophilia A. *Haemophilia*. 2013;19(3):e167–e173. doi:10.1111/hae.12107

12. Srivastava A, Santagostino E, Dougall A, et al. WFH guidelines for the management of hemophilia, 3rd edition. *Haemophilia*. 2020;26 (Suppl 6):1–158. doi:10.1111/hae.14046

13. Rodriguez-Merchan EC, Jimenez-Yuste V, Aznar JA, et al. Joint protection in haemophilia. *Haemophilia*. 2011;17(S2):1–23. doi:10.1111/j.1365-2516.2011.02615.x

14. Manco-Johnson MJ, Lundin B, Funk S, et al. Effect of late prophylaxis in hemophilia on joint status: a randomized trial. *J Thromb Haemost*. 2017;15(11):2115–2124. doi:10.1111/jth.13811

15. Moher D, Liberati A, Tetzlaff J, Altman DG; Prisma Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med*. 2009;6(7):e1000097. doi:10. 1371/journal.pmed.1000097

16. Fischer K, van der Bom JG, Mauser-Bunschoten EP, et al. Endogenous clotting factor activity and long-term outcome in patients with moderate haemophilia. *Thromb Haemost*. 2000;84 (6):977–980. doi:10.1055/s-0037-1614159

17. den Uijl IEM, Biesma D, Grobbee D, Fischer K. Turning severe into moderate haemophilia by prophylaxis: are we reaching our goal? *Blood Transfus*. 2013;11(3):364–369. doi:10.2450/2012.0092-12

18. Gilbert L, Rollins L, Hilmes M, et al. Haemophilia A carriers demonstrate pathological and radiological evidence of structural joint changes. *Haemophilia*. 2014;20(6):e426–e429. doi:10.1111/hae.12535

19. Sidonio RF, Mili FD, Li T, et al. Females with FVIII and FIX deficiency have reduced joint range of motion. *Am J Hematol*. 2014;89(8):831–836. doi:10.1002/ajh.23754

20. Soucie JM, Monahan PE, Kulkarni R, Konkle BA, Mazepa MA. The frequency of joint hemorrhages and procedures in nonsevere hemophilia A vs B. *Blood Adv*. 2018;2(16):2136–2144. doi:10.1182/bloodadvances.2018020552

21. Osooli M, Donfield SM, Carlsson KS, et al. Joint comorbidities among Swedish carriers of haemophilia: a register-based cohort study over 22 years. *Haemophilia*. 2019;25(5):845–850. doi:10.1111/hae.13831

22. den Uijl IEM, Biesma D, Grobbee D, Fischer K. Outcome in moderate haemophilia. *Blood Transfus*. 2014;12(Suppl 1):s330–s336. doi:10.2450/2012.0091-12

23. Osooli M, Lovdahl S, Steen Carlsson K, et al. Comparative burden of arthropathy in mild haemophilia: a register-based study in Sweden. *Haemophilia*. 2017;23(2):e79–e86. doi:10.1111/hae.13166

24. Gomperts ED, Schwarz J, Donfield SM, et al. The importance of genetic factors for the development of arthropathy: a longitudinal study of children and adolescents with haemophilia A. *Thromb Haemost*. 2017;117(2):277–285. doi:10.1160/TH16-06-0440

25. Khanum F, Bowen DJ, Kerr BC, Collins PW. Joint health scores in a haemophilia A cohort from Pakistan with minimal or no access to factor VIII concentrate: correlation with thrombin generation and underlying mutation. *Haemophilia*. 2014;20(3):426–434. doi:10.11 11/hae.12326

26. Jayandharan GR, Nair SC, Poonnoose PM, et al. Polymorphism in factor VII gene modifies phenotype of severe haemophilia. *Haemophilia*. 2009;15(6):1228–1236. doi:10.1111/j.1365-2516.2009. 02080.x

27. Shankarkumar U, Ghosh K, Mohanty D. Novel HLA B*2714 and B*2708 allele associations in seronegative spondarthritis patients and haemophilia patients with chronic synovitis in India. *Tissue Antigens*. 2003;62(2):175–178. doi:10.1034/j.1399-0039.2003.00074.x

28. Ghosh K, Shankarkumar U, Shetty S, Mohanty D. Chronic synovitis and HLA B27 in patients with severe haemophilia. *Lancet*. 2003;361 (9361):933–934. doi:10.1016/S0140-6736(03)12763-8

29. Cruz E, Porto G, Morais S, Campos M, de Sousa M. HFE mutations in the pathobiology of hemophilic arthropathy. *Blood*. 2005;105 (8):3381–3382. doi:10.1182/blood-2004-11-4342

30. López-Jiménez JJ, Ortega-Cervantes R, Luna-Záizar H, et al. Genetic biomarkers related to hemarthrosis, inflammation, and cartilage structure in pediatric patients with hemophilic arthropathy. *Mol Genet Genomic Med*. 2019;7(11):e979. doi:10.1002/mgg3.979

31. Fischer K, van der Bom JG, Molho P, et al. Prophylactic versus on-demand treatment strategies for severe haemophilia: a comparison of costs and long-term outcome. *Haemophilia*. 2002;8 (6):745–752. doi:10.1046/j.1365-2516.2002.00695.x

32. Collins P, Faradji A, Morfini M, Enriquez MM, Schwartz L. Efficacy and safety of secondary prophylactic vs. on-demand sucrose-formulated recombinant factor VIII treatment in adults with severe hemophilia A: results from a 13-month crossover study. *J Thromb Haemost*. 2010;8 (1):83–89. doi:10.1111/j.1538-7836.2009.03650.x

33. Gringeri A, Lundin B, von Mackensen S, Mantovani L, Mannucci PM; Esprit Study Group. A randomized clinical trial of prophylaxis in children with hemophilia A (the ESPRIT study). *J Thromb Haemost*. 2011;9(4):700–710. doi:10.1111/j.1538-7836.2011.04214.x

34. Tagliaferri A, Feola G, Molinari AC, et al. Benefits of prophylaxis versus on-demand treatment in adolescents and adults with severe haemophilia A: the POTTER study. *Thromb Haemost*. 2015;114 (1):35–45. doi:10.1160/TH14-05-0407

35. Oldenburg J, Zimmermann R, Katsarou O, et al. Controlled, cross-sectional MRI evaluation of joint status in severe haemophilia A patients treated with prophylaxis vs. on demand. *Haemophilia*. 2015;21(2):171–179. doi:10.1111/hae.12539

36. Manco-Johnson MJ, Soucie JM, Gill JC. Prophylaxis usage, bleeding rates, and joint outcomes of hemophilia, 1999 to 2010: a surveillance project. *Blood*. 2017;129(17):2368–2374. doi:10.1182/blood-2016-02-683169

37. Oldenburg J, Kulkarni R, Srivastava A, et al. Improved joint health in subjects with severe haemophilia A treated prophylactically with recombinant factor VIII Fc fusion protein. *Haemophilia*. 2018;24 (1):77–84. doi:10.1111/hae.13353

38. Warren BB, Jacobson L, Kempton C, et al. Factor VIII prophylaxis effects outweigh other hemostasis contributors in predicting severe haemophilia A joint outcomes. *Haemophilia*. 2019;25(5):867–875. doi:10.1111/hae.13778

39. Fischer K, van der Bom JG, Mauser-Bunschoten EP, et al. The effects of postponing prophylactic treatment on long-term outcome in patients with severe hemophilia. *Blood*. 2002;99(7):2337–2341. doi:10.1182/blood.V99.7.2337

40. Bladen M, Main E, Hubert N, Koutoumanou E, Liesner R, Khair K. Factors affecting the haemophilia joint health score in children with severe haemophilia. *Haemophilia*. 2013;19(4):626–631. doi:10.1111/hae.12108

41. Nijdam A, Foppen W, van der Schouw YT, Mauser-Bunschoten EP, Schutgens REG, Fischer K. Long-term effects of joint bleeding before starting prophylaxis in severe haemophilia. *Haemophilia*. 2016;22(6):852–858. doi:10.1111/hae.12959

42. Warren BB, Thornhill D, Stein J, et al. Young adult outcomes of childhood prophylaxis for severe hemophilia A: results of the Joint Outcome Continuation Study. *Blood Adv*. 2020;4(11):2451–2459. doi:10.1182/bloodadvances.2019001311

43. Nijdam A, Foppen W, De Kleijn P, et al. Discontinuing early prophylaxis in severe haemophilia leads to deterioration of joint status despite low bleeding rates. *Thromb Haemost*. 2016;115(5):931–938. doi:10.1160/TH15-08-0637

44. Fischer K, Van Der Bom JG, Prejs R, et al. Discontinuation of prophylactic therapy in severe haemophilia: incidence and effects on outcome. *Haemophilia*. 2001;7(6):544–550. doi:10.1046/j.1365-2516.2001.00560.x

45. Wallny TA, Scholz DT, Oldenburg J, et al. Osteoporosis in haemophilia - an underestimated comorbidity? *Haemophilia*. 2007;13 (1):79–84. doi:10.1111/j.1365-2516.2006.01405.x

46. Nair AP, Jijina F, Ghosh K, Madkaikar M, Shrikhande M, Nema M. Osteoporosis in young haemophiliacs from western India. *Am J Hematol*. 2007;82(6):453–457. doi:10.1002/ajh.20877

47. Abdelrazik N, Reda M, El-Ziny M, Rabea H. Evaluation of bone mineral density in children with hemophilia: mansoura University children hospital (MUCH) experience, Mansoura, Egypt. *Hematology*. 2007;12(5):431–437. doi:10.1080/10245330701383700

48. Alioglu B, Selver B, Ozsoy H, Koca G, Ozdemir M, Dallar Y. Evaluation of bone mineral density in Turkish children with severe haemophilia A: Ankara hospital experience. *Haemophilia*. 2012;18 (1):69–74. doi:10.1111/j.1365-2516.2011.02587.x

49. Naderi A, Nikvarz M, Arasteh M, Shokoohi M. Osteoporosis/osteopenia and hemophilic arthropathy in severe hemophilic patients. *Arch Iran Med*. 2012;15(2):82–84. doi:1012152/AIM.006

50. Anagnostis P, Vakalopoulou S, Slavakis A, et al. Reduced bone mineral density in patients with haemophilia A and B in Northern Greece. *Thromb Haemost*. 2012;107(3):545–551. doi:10.1160/TH11-08-0556

51. Anagnostis P, Vakalopoulou S, Vyzantiadis TA, et al. The clinical utility of bone turnover markers in the evaluation of bone disease in patients with haemophilia A and B. *Haemophilia*. 2014;20(2):2 68–275. doi:10.1111/hae.12271

52. El-Mikkawy DME, Elbadawy MA, Abd El-Ghany SM, Samaha D. Serum sclerostin level and bone mineral density in pediatric hemophilic arthropathy. *Indian J Pediatr*. 2019;86(6):515–519. doi:10. 1007/s12098-019-02855-1

53. Wells AJ, McLaughlin P, Simmonds JV, et al. A case-control study assessing bone mineral density in severe haemophilia A in the UK. *Haemophilia*. 2015;21(1):109–115. doi:10.1111/hae.12565

54. Kempton CL, Antun A, Antoniucci DM, et al. Bone density in haemophilia: a single institutional cross-sectional study. *Haemophilia*. 2014;20(1):121–128. doi:10.1111/hae.12240

55. Kiper Unal HD, Comert Ozkan M, Atilla FD, et al. Evaluation of bone mineral density and related parameters in patients with haemophilia: a single center cross-sectional study. *Am J Blood Res*. 2017;7 (5):59–66.

56. Harris S, Boggio LN. Exercise may decrease further destruction in the adult haemophilic joint. *Haemophilia*. 2006;12(3):237–240. doi:10.1111/j.1365-2516.2006.01214.x

57. Groen WG, Takken T, van der Net J, Helders PJ, Fischer K. Habitual physical activity in Dutch children and adolescents with haemophilia. *Haemophilia*. 2011;17(5):e906–e912. doi:10.1111/j.1365-2516.2011. 02555.x

58. Cuesta-Barriuso R, Torres-Ortuno A, Pérez-Alenda S, Jose Carrasco J, Querol F, Nieto-Munuera J. Sporting activities and quality of life in children with hemophilia: an observational study. *Pediatr Phys Ther*. 2016;28(4):453–459. doi:10.1097/PEP.0000000 000000296

59. Timmer MA, Veenhof C, de Kleijn P, de Bie RA, Schutgens REG, Pisters MF. Movement behaviour patterns in adults with haemophilia. *Ther Adv Hematol*. 2020;11:2040620719896959. doi:10.1177/ 2040620719896959

60. Doria AS, Keshava SN, Mohanta A, et al. Diagnostic accuracy of ultrasound for assessment of hemophilic arthropathy: MRI correlation. *Am J Roentgenol*. 2015;204(3):W336–W347. doi:10.22 14/AJR.14.12501

61. De la Corte-rodriguez H, Rodriguez-Merchan EC, Alvarez-Roman MT, Martin-Salces M, Martinoli C, Jimenez-Yuste V. The value of HEAD-US system in detecting subclinical abnormalities in joints of patients with hemophilia. *Expert Rev Hematol*. 2018;11(3):253–261. doi:10.1080/17474086.2018.1435269

62. Foppen W, van der Schaaf IC, Beek FJA, Mali WPTM, Fischer K. MRI predicts 5-year joint bleeding and development of arthropathy on radiographs in hemophilia. *Blood Adv*. 2020;4(1):113–121. doi:10.1182/bloodadvances.2019001238

63. Foppen W, van der Schaaf IC, Beek FJA, Mali WPTM, Fischer K. Diagnostic accuracy of point-of-care ultrasound for evaluation of early blood-induced joint changes: comparison with MRI. *Haemophilia*. 2018;24(6):971–979. doi:10.1111/hae.13524

64. von Drygalski A, Moore RE, Nguyen S, et al. Advanced hemophilic arthropathy: sensitivity of soft tissue discrimination with musculoskeletal ultrasound. *J Ultrasound Med*. 2018;37(8):1945–1956. doi:10. 1002/jum.14541

65. Poonnoose PM, Hilliard P, Doria AS, et al. Correlating clinical and radiological assessment of joints in haemophilia: results of a cross sectional study. *Haemophilia*. 2016;22(6):925–933. doi:10.1111/hae.13023

66. Klukowska A, Czyrny Z, Laguna P, Brzewski M, Serafin-Krol MA, Rokicka-Milewska R. Correlation between clinical, radiological and ultrasonographical image of knee joints in children with haemophilia. *Haemophilia*. 2001;7(3):286–292. doi:10.1046/j.1365-2516.2001.00509.x

67. Fischer K, Nijdam A, Holmstrom M, et al. Evaluating outcome of prophylaxis in haemophilia: objective and self-reported instruments should be combined. *Haemophilia*. 2016;22(2):e80–e86. doi:10.1111/hae.12901

68. Pergantou H, Matsinos G, Papadopoulos A, Platokouki H, Aronis S. Comparative study of validity of clinical, X-ray and magnetic resonance imaging scores in evaluation and management of haemophilic arthropathy in children. *Haemophilia*. 2006;12(3):241–247. doi:10.1111/j.1365-2516.2006.01208.x

69. Feldman BM, Funk SM, Bergstrom BM, et al. Validation of a new pediatric joint scoring system from the International Hemophilia Prophylaxis Study Group: validity of the hemophilia joint health score. *Arthritis Care Res*. 2011;63(2):223–230. doi:10.1002/acr.20353

70. Fischer K, de Kleijn P. Using the Haemophilia Joint Health Score for assessment of teenagers and young adults: exploring reliability and validity. *Haemophilia*. 2013;19(6):944–950. doi:10.1111/hae.12197

71. Oymak Y, Yildirim AT, Yaman Y, et al. The effectiveness of tools for monitoring hemophilic arthropathy. *J Pediatr Hematol Oncol*. 2015;37(2):e80–e85. doi:10.1097/MPH.0000000000000210

72. Timmer MA, Foppen W, Schutgens RE, Pisters MF, Fischer K. Comparing findings of routine haemophilia joint health score and haemophlia early arthropathy detection with ultrasound assessments in adults with haemophilia. *Haemophilia*. 2017;23(2):e141–e143. doi:10.1111/hae.13147

73. Seuser A, Kurnik K, Mahlein AK. Infrared thermography as a non-invasive tool to explore differences in the musculoskeletal system of children with hemophilia compared to an age-matched healthy group. *Sensors*. 2018;18(2):518. doi:10.3390/s18020518

74. Seuser A, Navarrete-Duran M, Auerswald G, Mancuso ME. Muscle function deterioration in patients with haemophilia: prospective experience from Costa Rica. *Haemophilia*. 2018;24(4):e230–e241. doi:10.1111/hae.13455

75. García-Massó X, Ye-Lin Y, Garcia-Casado J, Querol F, Gonzalez LM. Assessment of haemophilic arthropathy through balance analysis: a promising tool. *Comput Methods Biomech Biomed Engin*. 2019;22(4):418–425. doi:10.1080/10255842.2018.1561877

76. Iem DU, De Schepper AMA, Camerlinck M, Grobbee DE, Fischer K. Magnetic resonance imaging in teenagers and young adults with limited haemophilic arthropathy: baseline results from a prospective study. *Haemophilia*. 2011;17(6):926–930. doi:10.1111/j.1365-2516.2011.02513.x

77. Lundin B, Ljung R, Pettersson H. MRI scores of ankle joints in children with haemophilia–comparison with clinical data. *Haemophilia*. 2005;11(2):116–122. doi:10.1111/j.1365-2516.2005.01061.x

78. Chowdary P. Extended half-life recombinant products in haemophilia clinical practice – expectations, opportunities and challenges. *Thromb Res*. 2020;196:609–617. doi:10.1016/j.thromres.2019.12.012

79. Zanon E, Tagliaferri A, Pasca S, et al. Physical activity improved by adherence to prophylaxis in an Italian population of children, adolescents and adults with severe haemophilia A: the SHAPE Study. *Blood Transfus*. 2020;18(2):152–158. doi:10.2450/2019.0040-19

80. Malec LM, Cheng D, Witmer CM, et al. The impact of extended half-life factor concentrates on prophylaxis for severe hemophilia in the United States. *Am J Hematol*. 2020;95(8):960–965. doi:10.1002/ajh.25844

81. Hua B, Olsen EHN, Sun S, et al. Serological biomarkers detect active joint destruction and inflammation in patients with haemophilic arthropathy. *Haemophilia*. 2017;23(4):e294–e300. doi:10.1111/hae.13196

82. Jansen NWD, Roosendaal G, Lundin B, et al. The combination of the biomarkers urinary C-terminal telopeptide of type II collagen, serum cartilage oligomeric matrix protein, and serum chondroitin sulfate 846 reflects cartilage damage in hemophilic arthropathy. *Arthritis Rheum*. 2009;60(1):290–298. doi:10.1002/art.24184

83. Melchiorre D, Milia AF, Linari S, et al. RANK-RANKL-OPG in hemophilic arthropathy: from clinical and imaging diagnosis to histopathology. *J Rheumatol*. 2012;39(8):1678–1686. doi:10.3899/jrheum.120370

84. Manetti M, Linari S, Romano E, et al. TNF-alpha/TNF-R system may represent a crucial mediator of proliferative synovitis in hemophilia A. *J Clin Med*. 2019;8(7):939. doi:10.3390/jcm8070939

85. Parameswaran N, Patial S. Tumor necrosis factor-alpha signaling in macrophages. *Crit Rev Eukaryot Gene Expr*. 2010;20(2):87–103. doi:10.1615/CritRevEukarGeneExpr.v20.i2.10

86. von Drygalski A, Barnes RFW, Jang H, et al. Advanced magnetic resonance imaging of cartilage components in haemophilic joints reveals that cartilage hemosiderin correlates with joint deterioration. *Haemophilia*. 2019;25(5):851–858. doi:10.1111/hae.13802

87. Acharya SS, Kaplan RN, Macdonald D, Fabiyi OT, DiMichele D, Lyden D. Neoangiogenesis contributes to the development of hemophilic synovitis. *Blood*. 2011;117(8):2484–2493. doi:10.1182/blood-2010-05-284653

88. Zetterberg E, Palmblad J, Wallensten R, Morfini M, Melchiorre D, Holmström M. Angiogenesis is increased in advanced haemophilic joint disease and characterised by normal pericyte coverage. *Eur J Haematol*. 2014;92(3):256–262. doi:10.1111/ejh.12227

89. Rodriguez-Merchan EC, Valentino LA. Increased bone resorption in hemophilia. *Blood Rev*. 2019;33:6–10. doi:10.1016/j.blre.2018.05.002

90. Eldash HH, Atwa ZT, Saad MA. Vitamin D deficiency and osteoporosis in hemophilic children: an intermingled comorbidity. *Blood Coagul Fibrinolysis*. 2017;28(1):14–18. doi:10.1097/MBC.0000000000000519

91. Gamal Andrawes N, Hashem Fayek M, Salah El-Din N, Atef Mostafa R. Effect of low-dose factor VIII prophylaxis therapy on bone mineral density and 25(OH) vitamin D level in children with severe haemophilia A. *Haemophilia*. 2020;26(2):325–332. doi:10.1111/hae.13917

92. Abbasnezhad A, Habibi M, Abdolkarimi B, Zare S, Fazeli Moghadam E, Choghakhori R. Serum concentrations of vitamin D, calcium, phosphorus and trace minerals in adults and children with haemophilia A: association with disease severity, quality of life, joint health and functional status. *Int J Hematol Oncol Stem Cell Res*. 2020;14(1):56–71.

93. Zhou JY, Barnes RFW, Foster G, Iorio A, Cramer TJ, von Drygalski A. Joint bleeding tendencies in adult patients with hemophilia: it's not all pharmacokinetics. *Clin Appl Thromb Hemost*. 2019;25:1076029619862052. doi:10.1177/1076029619862052

94. Lobet S, Meite N, Koninckx MI, et al. Implementation and assessment of a self- and community-based rehabilitation programme in patients with haemophilia from Cote d'Ivoire. *Haemophilia*. 2019;25(5):859–866. doi:10.1111/hae.13833

95. Timmer MA, Kloek CJJ, de Kleijn P, et al. A blended physiotherapy intervention for persons with hemophilic arthropathy: development study. *J Med Internet Res*. 2020;22(6):e16631. doi:10.2196/16631

96. Hashem F, Stephensen D, Drechsler WI, et al. Muscle strengthening intervention for boys with haemophilia: developing and evaluating a best-practice exercise programme with boys, families and health-care professionals. *Health Expectations*. 2020;23:1350–1361.

Severe, Refractory Immune Thrombocytopenia Occurring After SARS-CoV-2 Vaccine

Jackie M Helms, Kristin T Ansteatt, Jonathan C Roberts, Sravani Kamatam, Kap Sum Foong, Jo-mel S Labayog & Michael D Tarantino

CASE REPORT

Severe, Refractory Immune Thrombocytopenia Occurring After SARS-CoV-2 Vaccine

Jackie M Helms [1]
Kristin T Ansteatt [1]
Jonathan C Roberts [1,2]
Sravani Kamatam [3]
Kap Sum Foong [2,3]
Jo-mel S Labayog [4]
Michael D Tarantino [1,2]

[1]The Bleeding and Clotting Disorders Institute, Peoria, IL, USA; [2]Department of Medicine, University of Illinois College of Medicine-Peoria, Peoria, IL, USA; [3]Department of Medicine, Saint Francis Medical Center, Peoria, IL, USA; [4]Department of Medicine, OSF Sacred Heart Medical Center, Danville, IL, USA

Abstract: The rollout of the SARS-CoV-2 vaccine is underway, and millions have already been vaccinated. At least 25 reports of "immune thrombocytopenia" (ITP) or "thrombocytopenia" following the Moderna or Pfizer vaccine have been added to the Vaccine Adverse Event Reporting System (VAERS) in the US. ITP is a rare but known complication of several vaccinations. SARS-CoV-2 vaccine is new, with a novel mechanism of action, and understanding the epidemiology, clinical manifestations, treatment success and natural history of post-vaccination thrombocytopenia is evolving. We report a 74-year-old man who developed refractory thrombocytopenia within one day of receiving the Moderna SARS-CoV-2 vaccine. Several hours after vaccination, he developed significant epistaxis and cutaneous purpura. Severe thrombocytopenia was documented the following day, and he developed extremity weakness and encephalopathy with facial muscle weakness. Over a 14-day period, thrombocytopenia was treated first with high dose dexamethasone, intravenous immunoglobulin, platelet transfusions, rituximab, plasma exchange (for presumed acute inflammatory demyelinating polyneuropathy (AIDP)), and four daily doses of the thrombopoietin receptor agonist (TPO-RA) eltrombopag (Promacta™), without a platelet response. Three days later, he received the TPO-RA romiplostim (Nplate™). Five days later, his platelet count began to rise and by post-vaccination day 25, his platelet count was in the normal range. Thrombocytopenia was refractory to frontline and second-line treatment. The eventual rise in his platelet count suggests that one or both TPO-RAs may have impacted platelet recovery. Possibly, but less likely given the temporality, the drug-induced thrombocytopenia was subsiding. The aggressive use of immunosuppressive treatment may jeopardize the intended purpose of the SARS-CoV-2 vaccine, and earlier use of non-immunosuppressive second-line treatment for vaccine-related severe thrombocytopenia, such as with TPO-RAs, should be considered. While it is imperative to continue the global vaccination program, vigilance to the occurrence of post-vaccination severe thrombocytopenia is warranted.

Keywords: immune thrombocytopenic purpura, platelet, SARS-CoV-2, thrombocytopenia, thrombopoietin receptor agonist, vaccine

Introduction

Primary Immune Thrombocytopenic Purpura (ITP) is an autoimmune disorder characterized by increased platelet destruction and decreased platelet production.[1] The incidence of ITP is 6 per 100,000 adults/year.[1] ITP has been documented as a result of viral illnesses or vaccinations that are given for prevention of infectious illnesses.[2–11] The recent global pandemic of SARS-CoV-2, a novel coronavirus, has caused many deaths worldwide and a global vaccination program is imperative to achieving herd immunity.[12,13] Platelet aggregation and activation appear to be

Correspondence: Michael D Tarantino
Chief Medical Officer, The Bleeding and Clotting Disorders Institute, 9128 North Lindbergh Drive, Peoria, IL, 61615, USA
Tel +1 309 692-5337
Fax +1 309 693-3913
Email mtarantino@ilbcdi.org

deranged in hospitalized patients with COVID-19, however the effect on the platelet count is not significantly correlated, although severe (presumed immune-mediated) thrombocytopenia during COVID-19 has been reported in a small number of patients.[14–17] To date, the SARS-CoV-2 vaccine appears to rarely cause an immune-mediated platelet destruction, not unlike that seen with the rubella, pneumococcus, and influenza vaccines.[2,4,5] The mechanism of post-vaccination thrombocytopenia, in both live and inactivated vaccines, is presumed to be immune mediated and may be similarly related to hyperfunction of B-cells observed in ITP.[18,19] Tens of millions of people have been vaccinated with the new SARS-CoV-2 vaccine. A small but growing number of cases of "immune thrombocytopenia" or "thrombocytopenia" following the administration of the SARS-CoV-2 vaccine have been reported to the FDA's VAERS.[20] Review of available information regarding post SARS-CoV-2 vaccination-related thrombocytopenia suggests a heterogenous onset, severity, and duration. At least one reported patient had a good platelet response to frontline treatment with corticosteroids and IVIg.[21] Here we report a case of severe, multi-drug, refractory immune thrombocytopenia shortly after the initial dose of the Moderna SARS-CoV-2 vaccine. The thrombocytopenia eventually relented after treatment with the thrombopoietin receptor agonist (TPO-RA) romiplostim.

Case Report

A 74-year-old male with hypertension, gout, hyperlipidemia and nonischemic cardiomyopathy, presented with acute epistaxis and diffuse cutaneous purpura a few hours after receiving the first dose of the Moderna SARS-CoV2 vaccine. He received the influenza and pneumococcal vaccines three months and six weeks, respectively, prior to his presentation.

Two months prior to vaccination, the patient had a platelet count of 224 x 10^9/L. The SARS-CoV-2 vaccine was administered on 19 Jan 2021 and that evening he experienced severe epistaxis and diffuse purpura. The next day, he presented to a local emergency department with uncontrolled epistaxis, and was noted to have a platelet count of 10 x 10^9/L. He was hospitalized for five days and received high-dose dexamethasone (40 mg/day), five daily doses of intravenous immunoglobulin (400 mg/kg/day), three daily platelet transfusions, and two weekly doses of rituximab (375 mg/m²/dose). Immune suppression with dexamethasone (20 mg/day) was continued after hospital discharge. (See Figure 1) Due to persistent severe thrombocytopenia, platelet count 21×10^9/L, the TPO-RA eltrombopag (50 mg/day) was initiated on post-vaccination day ten.

On post-vaccination day 13, he again presented to the emergency department with severe thrombocytopenia, platelet count 12×10^9/L, but now with progressive, generalized weakness that first involved the left arm and the lower
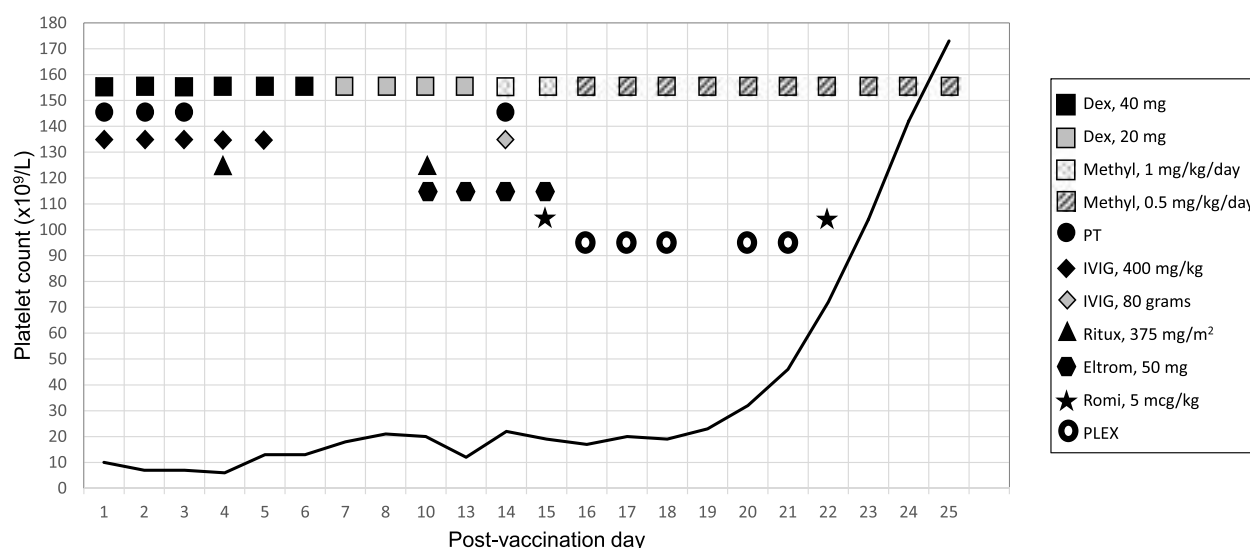
Figure 1 Post-vaccination platelet count.
Abbreviations: Dex, dexamethasone; Methyl, methylprednisolone; PT, platelet transfusion; IVIG, intravenous immunoglobulin; Ritux, rituximab; Eltrom, eltrombopag; Romi, romiplostim; PLEX, plasma exchange.

extremities bilaterally, back pain causing inability to ambulate, urinary retention, constipation and encephalopathy with dysarthria. These findings, although concurrent with the persistent, refractory severe thrombocytopenia, were thought to be unrelated. Due to suspicion of acute inflammatory demyelinating polyneuropathy (AIDP), plasma exchange was initiated on post-vaccination day 15. Magnetic resonance imaging on post-vaccination day 16 revealed severe L1-5 stenosis with multi-level disc herniation and fluid collections within the lumbar, posterior and paraspinal musculature.

On post-vaccination day 14, he received one pheresis unit of platelets for a platelet count of 22×10^9/L without improvement in his platelet count 1-hour post transfusion, and an additional dose of IVIg of 80 grams was given. On post-vaccination day 15, he received high dose methylprednisolone (1 mg/kg/day) and romiplostim (5 mcg/kg) with no increase in the platelet count for the next five days (See Figure 1).

Examination of the peripheral blood smear on post-vaccination day 14 revealed normal-to-large sized platelets. Blood and urine cultures grew methicillin-susceptible *Staphylococcus aureus* (MSSA) on post-vaccination day 13 that was treated with cefazolin. SARS-CoV-2 by a polymerase chain reaction assay was not detected on post-vaccination days 13 and 14. Additionally, tests for human immunodeficiency virus, Hepatitis B virus (HBV), Hepatitis C virus, Epstein-Barr virus, cytomegalovirus, and parvovirus B19 were negative.

On post-vaccination day 19, after the third episode of plasma exchange, his facial weakness improved. On post-vaccination day 22, his platelet count was 72×10^9/L, a second dose of romiplostim 5 mcg/kg was administered, and a corticosteroid taper was begun. He was transferred to a skilled nursing facility on post-vaccination day 25 with a platelet count of 173×10^9/L.

Discussion

Previous studies have shown that ITP is a rare complication following routine vaccinations, primarily rubella but also pneumococcus, *Haemophilus influenza* type B, HBV, human papilloma virus, varicella-zoster, diphtheria, tetanus, pertussis, and polio.[2–11]

Our patient presented with severe thrombocytopenia within one day of receiving the Moderna SARS-CoV-2 vaccine with virtually no response to standard ITP treatment, suggesting refractoriness or a different pathophysiology for the severe thrombocytopenia. The patient's

refractory thrombocytopenia ultimately responded after treatment with romiplostim (Nplate), much like many cases of ITP with an onset of action as soon as four days after the subcutaneous first dose.[22,23] This patient's poor response to frontline and second-line treatments suggests that TPO-RA agents may be useful in refractory ITP post SARS-CoV-2 vaccination.

Because of the concurrent, suspected AIDP and concern for jeopardizing the effect of the SARS-CoV-2 vaccine, thereafter, nonimmune suppressive treatment for severe thrombocytopenia was chosen. The deleterious effects of aggressive immunosuppression for thrombocytopenia should not be overlooked. In prior studies, immunosuppressive therapies post vaccination resulted in attenuation of the immune response to the administered vaccine.[24] TPO-RAs are not immunosuppressive and very effective for persistent or chronic ITP.[25] Knowing that TPO-RAs may increase the risk of venous or arterial thrombosis in a small proportion of patients, and that patients with SARS-CoV-2 infection are at higher risk for thrombotic events, patients selected for TPO-RA therapy for SARS-CoV-2 related severe thrombocytopenia should be chosen judiciously.[26,27]

Conclusion

Given the SARS-CoV-2 vaccine is new, with a novel mechanism of action, there exists the uncertainty of whether or not refractory ITP will be a rare adverse event. Notwithstanding that post-vaccination induced ITP is a rare adverse event, it should not limit the use of vaccines, including the SARS-CoV-2 vaccine. However, further investigation is imperative to explicate the pathological mechanism, epidemiology, clinical manifestations, and treatment outcomes.

Consent

Written informed consent for publication of their details was obtained from the patient. The governing institutional review board (IRB) for St. Francis Medical Center, the University of Illinois College of Medicine- Peoria IRB, gave approval for this case report.

Acknowledgments

Disclosure

Dr Michael D Tarantino reports personal fees for consulting and/or speaking from and past clinical trials investigator for Amgen and Dova, outside the submitted work. The authors report no other conflicts of interest in this work.

References

1. Weycker D, Hanau A, Hatfield M, et al. Immune thrombocytopenia in US clinical practice: incidence and healthcare burden in the first 12 months following diagnosis. *J Med Econ*. 2020;23(2):184–192. doi:10.1080/13696998.2019.1669329

2. Black C, Kaye JA, Jick H. MMR vaccine and idiopathic thrombocytopaenic purpura. *Br J Clin Pharmacol*. 2003;55(1):107–111. doi:10.1046/j.1365-2125.2003.01790.x

3. Garbe E, Andersohn F, Bronder E, et al. Drug induced immune thrombocytopaenia: results from the Berlin Case-Control Surveillance Study. *Eur J Clin Pharmacol*. 2012;68:821–832. doi:10.1007/s00228-011-1184-3

4. Tseng HF, Sy LS, Quan L. Pneumococcal conjugate vaccine safety in elderly adults. Open Forum Infect Dis. 2018;5(6):1–8.

5. Nagasaki J, Manabe M, Ido K, et al. Postinfluenza vaccination idiopathic thrombocytopenic purpura in three elderly patients. Case Reports in Hematol. Epub 2016 February 21.

6. Jin C, Dong H, Sun Z, et al. Acute immune thrombocytopenic purpura as adverse reaction to oral polio vaccine (OPV). *Hum Vaccin Immunother*. 2013;9(8):1739–1740. doi:10.4161/hv.24847

7. Genovese C, La Fauci V, Squeri A, Trimarchi G, Squeri R. HPV vaccine and autoimmune diseases: systematic review and metaanalysis of the literature. *J Prev Med Hyg*. 2018;59:E194–E199. doi:10.15167/2421-4248/jpmh2018.59.3.998

8. Miller E, Waight P, Farrington P, et al. Idiopathic thrombocytopenic purpura and MMR vaccine. *Arch Dis Child*. 2001;84:227–229. doi:10.1136/adc.84.3.227

9. Cecinati V, Principi N, Brescia L. Vaccine administration and the development of immune thrombocytopenic purpura in children. *Human Vacc Immunotherap*. 2013;9(5):1158–1162. doi:10.4161/hv.23601

10. France EK, Glanz J, Xu S, et al.; Vaccine Safety Datalink Team. Risk of immune thrombocytopenic purpura after measles-mumps-rubella immunization in children. *Pediatrics*. 2008;121(3):e687–e92. doi:10.1542/peds.2007-1578

11. Isai A, Durand J, Le Meur S, Hidalgo-Simon A, Kurz X. Autoimmune disorders after immunisation with Influenza A/H1N1 vaccines with and without adjuvant: eudraVigilance data and literature review. *Vaccine*. 2012;30(49):7123–7129. doi:10.1016/j.vaccine.2012.09.032

12. Centers for disease control and prevention.. Available from: https://www.cdc.gov/coronavirus/2019-ncov/index.html. Accessed February. 15, 2021

13. Omer SB, Yildirim I, Forman HP. Herd Immunity and Implications for SARS-CoV-2 Control. *JAMA*. 2020;324(20):2095–2096. doi:10.1001/jama.2020.20892

14. Leopold V, Pereverzeva L, Schuurman AR, et al. Platelets are hyperactivated but show reduced glycoprotein VI reactivity in COVID-19 patients. *Thromb Haemost*. 2021. doi:10.1055/a-1347-5555

15. Comer SP, Cullivan S, Sklanna PB, et al. COVID-19 induces a hyperactive phenotype in circulating platelets. *PLoS Biol*. 2021;19 (2):e3001109. doi:10.1371/journal.pbio.3001109

16. Slomka A, Kowalewski M, Zekanowska E. Coronavirus disease 2019 (COVID-19): a short review on hematological manifestations. *Pathogens*. 2020;9(6):493. doi:10.3390/pathogens9060493

17. Bhattacharjee S, Banerjee M. Immune thrombocytopenia secondary to COVID-19: a systematic review. *SN Comp Clin Med*. 2020;2:2048–2058. doi:10.1007/s42399-020-00521-8

18. Giordano P, Cascioli S, Lassandro G, et al. B-cell hyperfunction in children with immune thrombocytopenic purpura persists after splenectomy. *Ped Res*. 2016;79(2):262–270. doi:10.1038/pr.2015.211

19. Yokomichi H, Tanaka-Taya K, Koshida R, et al. Immune thrombocytopenic purpura risk by live, inactivated and simultaneous vaccinations among Japanese adults, children and infants: a matched case-control study. *Int J Hematol*. 2020;112(1):105–114. doi:10.1007/s12185-020-02866-1

20. Vaccine adverse event reporting system. Available from: https://vaers.hhs.gov. Accessed February 15, 2021.

21. Tarawneh OH, Tarawneh HS. Immune thrombocytopenia in a 22-year-old post Covid-19 vaccine. *Am J Hematol*. 2021. doi:10.1002/ajh.26106

22. Kuter DJ, Bussel JB, Lyons RM, et al. Randomized, controlled, 6-month evaluation of AMG-531 in patients with chronic immune thrombocytopenic Purpura. *Lancet*. 2008;371(9610):395–403. doi:10.1016/S0140-6736(08)60203-2

23. Wang B, Nichol JL, Sullivan JT. Pharmacodynamics and pharmacokinetics of AMG 531, a novel thrombopoietin receptor ligand. *Clin Pharmacol Ther*. 2004;76(6):628–638. doi:10.1016/j.clpt.2004.08.010

24. Papp KA, Haraoui B, Kumar D, et al. Vaccination guidelines for patients with immune-mediated disorders on immunosuppressive therapies. *J of Cut Med Surg*. 2019;21(1):50–74. doi:10.1177/1203475418811335

25. Tarantino MD, Chalmers S. Therapeutic Thrombopoietin Mimetics. In: Gresele P, Kleiman NS, Lopez JA, Page CP, editors. *Platelets in Thrombotic and Nonthrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics*, 2nd. Cambridge University Press; 2016: 1417–1429.

26. Kuter DJ, Bussel JB, Newland A, et al. Long-term treatment with romiplostim in patients with chronic immune thrombocytopenia: safety and efficacy. *Brit J of Haematol*. 2013;161:411–423. doi:10.1111/bjh.12260

27. Pavord S, Thachil J, Hunt BJ, et al. Practical guidance for the management of adults with immune thrombocytopenia during the COVID-19 pandemic. *Brit J of Haematol*. 2020;189(6):1038–1043. doi:10.1111/bjh.16775

Revisiting Autoimmunity in Chronic Lymphocytic Leukemia: Prognostic Value of Positive Direct Antiglobulin Test in a Retrospective Study and Literature Review

Shimaa A Ahmed, Ghada E M Abdallah, Mai M Aly, Eman M Nagiub Abdelsalam & Mostafa F Mohammed Saleh

ORIGINAL RESEARCH

Revisiting Autoimmunity in Chronic Lymphocytic Leukemia: Prognostic Value of Positive Direct Antiglobulin Test in a Retrospective Study and Literature Review

Shimaa A Ahmed[1]
Ghada EM Abdallah[2]
Mai M Aly[2]
Eman M Nagiub Abdelsalam[3]
Mostafa F Mohammed Saleh [ID][2]

[1]Department of Internal Medicine, Faculty of Medicine, South Valley University, Qena, Egypt; [2]Clinical Hematology Unit, Department of Internal Medicine, Faculty of Medicine, Assiut University, Assiut, Egypt; [3]Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt

Introduction: A positive direct antiglobulin test (DAT) with or without autoimmune hemolytic anemia is a frequent finding in chronic lymphocytic leukemia (CLL). The heterogenic clinical course of CLL mainly depends on different pathogenetic mechanisms which appears in a form of variable biological and clinical features. These features allow stratification of patients into subsets with different outcomes.

Patients and Methods: We evaluated the DAT as a prognostic marker in 120 CLL patients treated with chemoimmunotherapy. Clinical and laboratory features, treatment response, and survival outcomes of CLL patients were assessed in relation to their DAT test status. Additionally, the English literature was extensively reviewed regarding the prognostic impact of a positive DAT in CLL.

Results: DAT positivity was detected in 36 patients (30%) and was associated advanced disease staging ($P = 0.03$). No correlations were found with other clinical, laboratory, or biological factors such as ZAP-70 or CD38. Both a positive DAT and an Eastern Cooperative Oncology Group performance status >2 were predictors for non-response to first-line treatment in the multivariate analysis (OR = 0.3, 95% CI: 0.12–0.8 and OR = 0.2, 95% CI: 0.08–0.8, respectively). The five-year progression-free survival was significantly lower in the DAT-positive group ($P = 0.004$). No significant association was found with overall survival ($P = 0.2$). Sixteen reports analyzing more than 11,000 patients were identified in our review.

Conclusion: In conclusion, DAT positivity in CLL patients is associated with poor response to treatment and disease progression.

Keywords: autoimmunity, chronic lymphocytic leukemia, direct antiglobulin test, autoimmune hemolytic anemia

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia; it accounts for approximately 30% of all leukemias and primarily affects the elderly.[1] The clinical course is usually indolent, but some patients experience more aggressive disease. CLL is frequently associated with immune disturbances. A positive direct antiglobulin test (DAT) with or without frank autoimmune hemolytic anemia (AIHA) is strongly associated with CLL. Associations with immune thrombocytopenia (ITP) and pure red cell aplasia (PRCA) have been reported as well. The incidence of immune cytopenia has been reported to range from less than 5% to 38%.[2]

Correspondence: Mostafa F Mohammed Saleh
Tel +201013701057
Fax +2088-2332278-2080278
Email mostafafaisal2017@yahoo.com

The pathogenesis of autoimmunity in CLL is not well known. Different mechanisms have been proposed, including aberrant antigen presentation that induces IgG autoantibodies coating erythrocytes and platelets with consequent antibody-dependent cellular cytotoxicity and complement-mediated destruction and the development of an autoreactive T-cell repertoire that produces inhibitory cytokines.[3] The dysregulation of T regulatory cells in CLL with an imbalance of the T regulatory/T helper 17-cell ratio has also been noted.[4,5]

AIHA is primarily characterized by the presence of a positive DAT and the alteration of other hemolytic markers including the reticulocyte count, lactate dehydrogenase (LDH), and haptoglobin.[6] The DAT is positive at some point during the course of CLL in up to 35% of cases.[7] However, DAT positivity does not necessarily mean that AIHA is present, and only one-third of DAT-positive CLL cases develop clinically overt hemolysis.[8]

Regarding prognostic relevance, several reports have shown an association of AIHA in CLL patients with other clinical and biological prognostic factors.[7,9–11] Moreover, DAT positivity at diagnosis may provide insights about survival in CLL; however, conflicting conclusions have been reported.[12–14]

In this study, we sought to determine the prognostic role of the DAT in CLL patients treated with chemoimmunotherapy by evaluating the response rate and survival outcomes. An extensive literature review was performed on this subject to verify this potential and provide a platform for possible use in future prognostic models of CLL.

Patients and Methods

Patients with newly diagnosed CLL between January 2011 and December 2019 who attended the Clinical Hematology Unit, Internal Medicine Department, Assiut University Hospital were recruited for this study. Patients were eligible if they had a confirmed diagnosis of CLL and the treatment was as per the International Workshop on Chronic Lymphocytic Leukemia (IWCLL).[15] For diagnosis, peripheral blood smears and bone marrow morphology examinations with immunophenotyping by flowcytometry (strong expressions of CD5 and CD23, low or absent expressions of CD79b, sIgM, and FMC7) were performed. All patients were classified according to Rai staging.[16] Any patient with a history of prior systemic autoimmune diseases and prior cancer or who had received previous chemotherapy and radiotherapy had been ruled out. Baseline data of different demographic and clinical

parameters were collected, including age, sex, performance status, presence of B-symptoms, Rai stage, hepatitis status, and DAT test at diagnosis. The expressions of surface CD38 and cytoplasmic ZAP-70 were illustrated as the percentage of gated cells and were considered positive when ZAP-70 $\geq$ 20% and CD38 $\geq$ 30%. A flowcytometric analysis was performed by fluorescence-activated cell sorter Calibur flow cytometry, and data were obtained and analyzed using Cell Quest software (Becton Dickinson Biosciences).

The peripheral blood samples were obtained using K3EDTA vacutainers for the complete count (CBC), and DAT analyses. Another peripheral blood sample was collected without anticoagulants, and plain tubes were used for the assessments of serum LDH, urea, creatinine, and uric acid. Hematological parameters were determined by the automated CBC analyzer "Cell Dyne Ruby" (Abbott, Diagnostic ®). Biochemical markers were assessed using "Hitachi 912" (Japan) by photometric assay. The DAT was performed in all cases using microtube column agglutination Gel technique systems for antibody screening (Diamed). AIHA was diagnosed based on the presence of unexplained anemia; a positive DAT for IgG, C3d, or both; and/or an increased reticulocyte count and an increase in indirect bilirubin with no other cause for anemia identified prior to treatment or any blood product transfusion.

Treatment Course and Outcomes

Indications for treatment in the CLL patients followed the IWCLL guidelines.[15] The following treatment regimens were administered:

- Thirty-five patients received an alkylating agent, chlorambucil, at a dose of 6–10 mg/d (0.1–0.2 mg/kg/d) PO for 7–14 days in 28-day cycles until the disease stabilized (usually 6–12 cycles).
- Twenty-one patients received a purine analog therapy, FCR (fludarabine (25 mg/m^2/d intravenously (IV) × 5 days q28), cyclophosphamide (250 mg/m^2 IV × 5 days), and rituximab (375 mg/m^2 day 1 prior to each chemotherapy cycle by slow intravenous infusion)).
- Fifty-seven patients received CVP plus rituximab treatment on day 1 of a 21-day cycle with cyclophosphamide, 750 mg/m^2 IV; vincristine, 1.4 mg/m^2 (maximum of 2.0 mg); and prednisone, 100 mg by mouth daily for 5 days. Rituximab was administered

as 375 mg/m^2 one day prior to each chemotherapy cycle by slow intravenous infusion.

The response to treatment was classified as complete remission,[16] which was defined by the disappearance of the disease in the clinical and imaging studies; partial response, which was defined by a >50% decrease of the involved site; progressive disease, which was defined by a >50% increase in the size of the involved lesions or the appearance of new lesions; or as relapse, which was defined by recurrence of the disease after CR. Stable disease was defined as a change of −45% to +45% in size of the involved lesions.[17] The overall response included both CR and PR.

Overall survival (OS) was defined as the duration between the date of diagnosis and the last follow-up or date of death. Progression-free survival (PFS) is the period from the date of first-line therapy to the date of relapse, progression, the last follow-up, or death from any cause. The study was approved by our Institutional Review Board in accordance with the Declaration of Helsinki, and written informed consent was obtained from all recruited patients.

A literature search for relevant studies was performed using PubMed, Scopus, and Google Scholar. The following key words were used: autoimmunity, DAT test, Coombs test, AHIA, and CLL. Relevant references in the cited studies were also included in the review process. Case reports or studies of other autoimmune cytopenias such as ITP or PRCA in CLL were excluded.

Statistical Analysis

Comparisons between DAT-positive and -negative groups were performed regarding CBC, LDH levels, staging, treatment outcomes, and survival analysis. Numeric variables were expressed as the mean ± standard deviation or the median (interquartile range) depending on tests of normality, and categorical variables were described as relative frequencies. The *t*-test, Wilcoxon Sum-Rank test, and the chi-square test were used for comparisons between groups. The differences were considered statistically significant at $P < 0.05$. The survival curves were determined by the Kaplan–Meier method and analyzed by a cox regression hazard ratio. The difference between groups was calculated by the Log Rank test. Data were analyzed using the Statistical Package for Social Science version 24 (IBM Corp., Armonk, NY).

Results

The baseline data of 120 patients were collected, and two patients had completed their treatment and follow-up in another center and thus only their baseline data were analyzed. The mean age of the cohort population was 60 ± 12.5 years, and 56 patients (46.7%) were females. DAT positivity was recorded at study entry in 36 patients (30%). The baseline clinical and laboratory parameters of the cohort according to the DAT test at diagnosis are illustrated in Table 1.

CD38 and ZAP-70 were analyzed for 90 patients at diagnosis, and the results indicated that 32 (35.6%) patients were positive for ZAP-70 and 25 (27.8%) were positive for CD38. The frequencies of the positive CD38 and ZAP-70 cases within positive and negative DATs cases are shown in Table 2.

A total of 113 patients in the cohort required treatment with either fludarabine-based chemotherapy or non-fludarabine-based chemotherapy (chlorambucil ± R or CVP ± R), five patients remained under a wait and see protocol throughout the entire recorded follow-up period, and there no data were available regarding the therapy response in two patients. The percentages of the patients treated by the different therapy lines in DAT-positive and DAT-negative cases are illustrated in Table 3. The details of the response are illustrated in Table 4. The univariate logistic regression analysis of the overall response of the patients who received first-line treatment was associated with a positive DAT at diagnosis (OR = 0.3, 95% CI: 0.12–0.65), an Eastern Cooperative Oncology Group (ECOG) performance score >2 (OR = 0.2, 95% CI: 0.08–0.5), and older age (OR = 0.96, CI: 0.93–0.99); conversely, there was no association with being positive for CD38 at diagnosis (OR = 0.5, 95% CI: 0.21–1.4), being positive for ZAP-70 (OR = 0.7, 95% CI: 0.3–1.9), or advanced Rai stage (OR = 1.2, 95% CI: 0.5–2.6). The multivariate model including performance status, age, and positive DAT is illustrated in Table 5.

The median follow-up was 35.5 months (range, 2–84 months). The median survival time of the patients with a negative DAT was 84 months, which did not differ significantly from the group with a positive Coombs test (60 months, $P = 0.2$) (Figure 1). The five-year PFS of patients with positive DATs was significantly lower than that of patients with negative Coombs tests (22% vs 60%, $P = 0.004$) (Figure 2).

We included parameters that were reported as predictors of PFS in the univariate cox regression hazards analysis (Table 6) and used them in the multivariate cox

Table 1 Clinical and Laboratory Characteristics of Patients with CLL Based on DAT Status

Patient Characteristics		DAT-Positive (N = 36)	DAT-Negative (N = 84)	P-value
Age (years) mean ± SD		60.9 ± 12.2	59.8 ± 12.7	0.7
Male		15 (41.7%)	49 (58.3%)	0.1
Performance stage ECOG (0–1)		23 (63.9%)	67 (79.8%)	0.07
B-symptoms		19 (54.3%)	44 (68.8%)	0.2
Rai stage	Stage 0–2	9 (25%)	39 (46.4%)	0.03*
	Stage 3–4	27 (75%)	45 (54.8%)	
HCV (Positive cases)		8 (22.2%)	13 (15.5%)	0.4
White blood cells × 10^9/L (median, IQR)		141 (191)	65 (99.5)	0.1
Hemoglobin gm/dl (mean ± SD)		8.4 ± 2.5	9.7 ± 2.4	0.006*
Platelets × 10^9/L (median, IQR)		137.5 (116.3)	141 (99)	0.8
Lactate dehydrogenase U/L (median, IQR)		529 (402)	391 (233.5)	0.4
Albumin mg/dl (mean ± SD)		36.8 ± 4.7	37.5 ± 5.7	0.3

Notes: *Statistically significant at <0.05; chi-square test was used.
Abbreviations: SD, standard deviation; ECOG, Eastern cooperative oncology group; HCV, hepatitis C virus; IQR, interquartile range; LDH, lactate dehydrogenase; DAT, direct antiglobulin test.

Table 2 Associations of CD38 and ZAP-70 with DAT

	DAT-Positive (N = 26)	DAT-Negative (N = 64)	p-value
CD38 positive cases (n, %)	8 (30.8%)	17 (26.6%)	0.7
ZAP-70 positive cases (n, %)	9 (34.9%)	23 (35.9%)	0.9

Note: Chi-square test was used.
Abbreviation: DAT, direct antiglobulin test.

Table 3 Proportions of the Therapy Lines in DAT-Positive and DAT-Negative Cases

	DAT-Negative (n = 79)	DAT-Positive (n = 34)	p-value
Chlorambucil	24 (30.4%)	11 (32.4%)	0.6
Fludarabine-based	13 (16.5%)	8 (23.5%)	
CVP-R	42 (53.2%)	15 (44.1%)	

Abbreviation: CVP-R, cyclophosphamide, vincristine, prednisone, and rituximab.

Table 4 Response Assessment-Based DAT

	DAT-Positive (N = 34)	DAT-Negative (N = 79)	P-value
CR	3 (8.8%)	13 (16.5%)	0.03
PR	13 (38.2%)	47 (59.5%)	
Progression	18 (53%)	19 (24%)	
Death	5 (14.7%)	8 (10.1%)	0.5

Abbreviations: CR, complete response; PR, partial response.

Table 5 Multivariate Analysis of Overall Response to the First-Line Therapy

	Regression Coefficient	p-value	OR
Positive DAT	−1.1	0.013	0.3
ECOG >2	−1.4	0.02	0.2
Age in years	−0.006	0.9	0.9

Abbreviations: OR, odds ratio; DAT, direct antiglobulin test; ECOG, Eastern cooperative oncology group.

regression model. This model indicated that a positive DAT (HR = 2.3, 95% CI: 1.1–4.8) and being positive for CD38 (HR = 2.5, CI: 1.1–5.1) could serve as predictors.

Only 12 patients with a positive DAT had confirmed AHIA, and 24 patients had no obvious anemia. As shown in Figure 3, there was no difference in PFS between patients who developed AIHA and those who did not (median PFS of 36 and 42 months, respectively, $P = 0.9$).

We thoroughly searched for previous studies performed on CLL patients with AIHA or a positive DAT and identified 16 reports with more 11,000 total patients. Table 7 summarizes the impact of a positive DAT on CLL patient outcomes reported by those studies.

Discussion

A positive DAT with or without AIHA is a common finding in CLL. Although there are convincing data that the
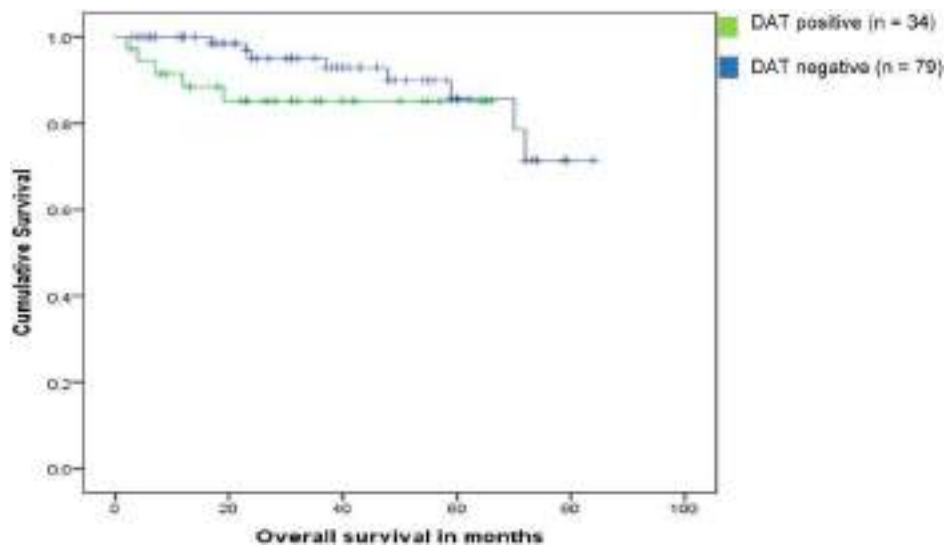
Figure 1 Kaplan–Meier estimates of overall survival in CLL patients according to DAT status (P = 0.2).
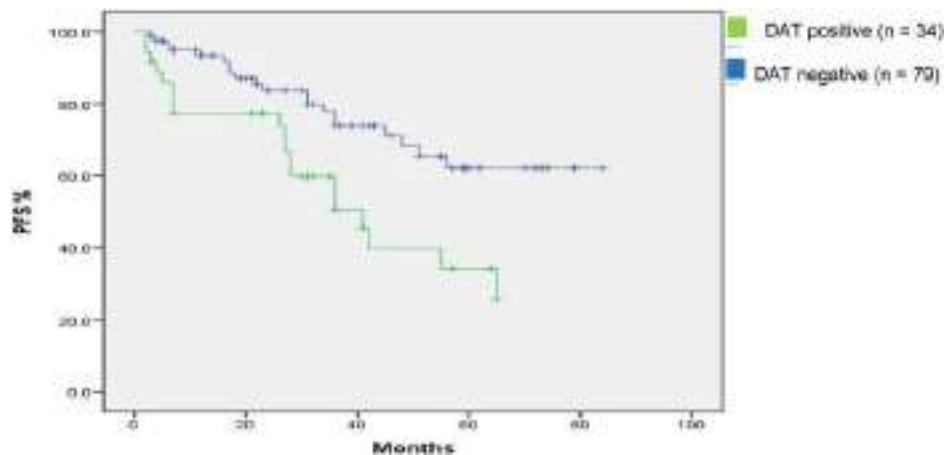
Figure 2 Kaplan–Meier estimates of PFS in CLL patients according to DAT status (P = 0.004). Median PFS of positive and negative cases: 40.9 and 84 months, respectively.

clinical outcome of CLL patients who have a positive DAT differs from that of patients with a negative result, the conclusions remain inconsistent.

In the present study, the frequency of a positive DAT was 30%. This is very similar to previous studies from Asia that were conducted in Chinese and Pakistani populations.[13,18] Reports from western countries had lower prevalence.[10,19–21] The higher incidence might be explained by ethnic background or late patient medical consultation resulting in a higher percentage (60%) of advanced disease stages at diagnosis, as noted in our cohort.

Among the different baseline clinical and biological factors of CLL, DAT positivity was associated with advanced stage CLL. This association between either a positive DAT[10]

or AIHA and advanced disease has been consistently reported in several studies.[12,19] Other studies have reported that autoimmune diseases or positive DATs in CLL were

Table 6 Univariate Cox Regression Hazard Analysis of Possible Predictors of PFS

Variables	HR	95% CI	P-value
DAT-positive	2.5	1.3–4.6	0.005
Advanced Rai stage	1	0.5–1.9	0.5
Zap-70 positive	1.9	1–4.3	0.07
CD38 positive	2.5	1.2–5.3	0.02
Age	0.97	0.95–0.99	0.05
Fludarabine-based chemotherapy as first-line treatment	1.2	0.5–3.1	0.7

Abbreviations: HR, hazard ratio; CI, confidence interval; DAT, direct antiglobulin.
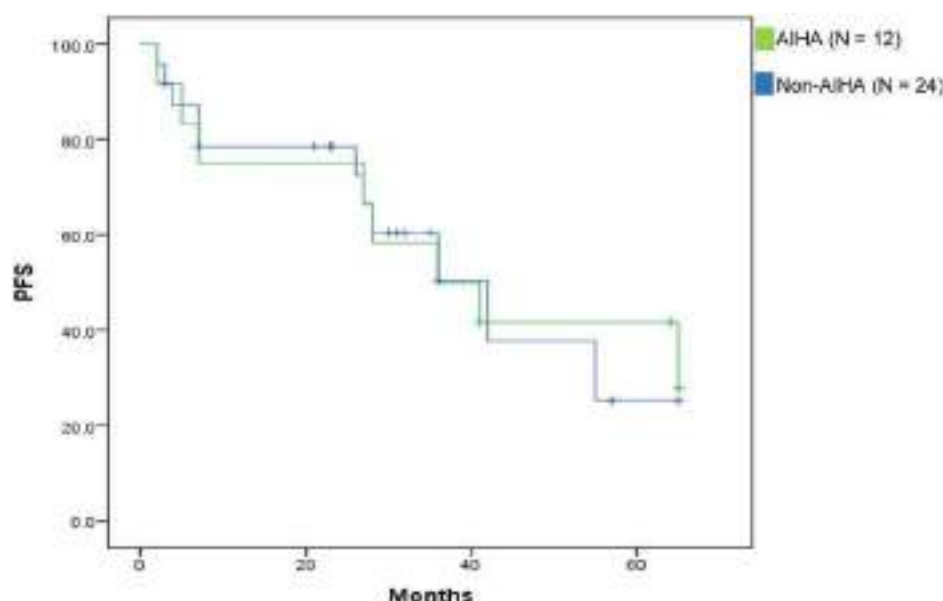
Figure 3 Kaplan–Meier estimates of PFS in DAT-positive CLL patients according to AIHA development (P = 0.9).

associated with older age,[13,20] male gender,[20] higher lymphocytic count at presentation,[10,20] high serum LDH,[18,22] and high β2-microglobulin levels.[10,14,23]

AIHA occurred in 33.3% of the patients with a positive DAT, and this accounted for approximately 10% of the entire cohort, which is in agreement with other studies.[8,12] This could indicate that a positive DAT without anemia in CLL might serve as an early unique prognostication of the disease.

In the present study, the subgroup comparison based on DAT positivity revealed no differences regarding the presence of other biologic prognostic parameters such as ZAP-70 or CD38, and this was partially agreed by Xu et al who found no correlation with CD38 despite the presence of a correlation with ZAP-70.[18] Others reported that a positive DAT was associated with ZAP-70 and CD38.[10] Nevertheless, because such assessments did not account for all patients in this study, this finding should be interpreted with caution.

Unfortunately, baseline cytogenetic and molecular data of our cohort were not recorded and were not included in our analysis to verify our assumption; however, several reports have shown that DAT positivity was associated with poor risk cytogenetics[22] and unmutated IgVH status.[8,18,22]

In the response assessment, a positive DAT and worse ECOG performance status seemed to be the only independent predictors for an unfavorable response to the different chemo-immunotherapies. Dearden et al reported lower overall response rates for positive DATs that reflected poor PFS,[12] which is quite similar to our findings. The outcomes of the DAT-positive patients treated with a fludarabine-based regimen were not any better, which is consistent with the results of Barcellini et al.[9] However, the combination of cyclophosphamide with fludarabine may have a protective effect against the development of AIHA with a better response and PFS compared with fludarabine alone. Interestingly, positive DATs have been reported to have shorter treatment-free survival in untreated CLL.[8]

Previous reports have shown that anemia caused by autoimmune mechanisms results in better survival compared with anemia secondary to bone marrow infiltration with advanced disease stages.[24] Herein, the PFS did not differ between the patients who developed AIHA and those who did not among the DAT-positive cohort. An interesting finding by Dearden et al was that PFS and OS were better for DAT-negative patients, even after excluding those who developed AHA.[12] This strongly indicates that a positive DAT represents a risk factor independently from developing anemia for the survival of CLL patients.

A positive DAT was not associated with OS in our cohort. This finding is in accordance with previous reports;[1,10,13,25] however, Quinquenel et al reported that a positive DAT was an independent adverse prognostic factor for OS.[8] In our study, PFS may not be a surrogate

Table 7 Studies About the Prognostic Impact of a Positive DAT on CLL Outcomes

Study	CLL	DAT-Positive N (%)	AIHA N (%)	CLL Patients with DAT Positive Characteristics
Atef et al; 2019[25]	101	28 (27.7%)	20 (19.8)	Higher WBC, lower HB, lower PLT Positive CD38 Positive ZAP-70 Less BM infiltration
Demir and Ekinci; 2017[28]	192	8 (4%) +DAT (n = 4)	3 (2.5)	Older age ($P = 0.036$), Advanced disease stage ($P = 0.004$) Received more first-line treatments ($P = 0.003$)
Visentin et al; 2013[29]	795	27 (3.4%)	27 (3.8)	Autoimmune disease more in females, advanced disease stage and 11q deletion by FISH Autoimmune diseases have better survival compared to other CLL complications as major infections or secondary cancers
Quinquenel et al; 2015[8]	378	56 (14.8%)		In Binet stage A correlated only with IgHV unmutated status ($p < 0.001$) In Binet stage A, positive DAT had a significantly shorter OS, regardless of their IGHV mutational status By multivariate analysis, a positive DAT was found to be an independent adverse prognostic factor for OS.
Abbas et al; 2015[13]	60	14 (23.3%)	14 (23.3)	Advanced Rai stage ($P<0.01$) Anemia ($P<0.001$)
Ricci et al; 2013[14]	146*	20 (14%)	8 (5.5) #	Higher β2-microglobulin Shorter treatment free survival (TFS)
Shvidel et al; 2013[23]	1477	93 (6.3%)	80 (5.4)#	66 patients with DAT positive have no clinical or laboratory signs of hemolysis at time of CLL diagnosis Shorter OS compared to DAT negative CLL ($P =0.001$)
Moreno et al; 2010[10]	960	49 (5%)	50 (5.2)#	Higher WBC, shorter lymphocyte doubling time Higher β2 microglobulin Higher CD38 Advanced (Binet stage C) disease 2nd to autoimmune mechanism had better survival vs Advanced stage2nd to a massive bone marrow infiltration ($P = 0.02$).
Xu et al; 2009[18]	123	34 (27.6%)	12 (9.8)#	Advanced Binet stage ($p < 0.001$), Higher level of serum lactate dehydrogenase (LDH) ($p = 0.003$) Higher β2 microglobulin ($p = 0.011$), Higher IgVH un mutated status ($p < 0.001$), Positive ZAP-70 ($p = 0.012$), and Trisomy 12 cytogenetic aberration ($p = 0.004$)
Ricci et al; 2009[22]**	158	42 (26.6%)	8 (5.5)#	Higher β2 microglobulin ($P=0.011$) Higher IgVH un mutated status ($P=0.0014$). Higher LDH ($P=0.051$). Positive ZAP-70 ($P=0.083$) and More frequency of deletion in 17p13, 11q22 or 6q21 ($P=0.064$),
Zent et al; 2008[24]	1750	41 (2.3%)	41 (2.3)	Survival from onset of cytopenia was significantly better for patients with autoimmune mechanism (median 9.1 years) compared to patients with BM failure (median 4.4 years, $p < 0.001$)

(*Continued*)

Table 7 (Continued).

Study	CLL	DAT-Positive N (%)	AIHA N (%)	CLL Patients with DAT Positive Characteristics
Dearden et al; 2008[12]	637	89 (14%)	89 (14)	Less Overall response (66% vs 81%, *P* = 0.004) Less likely to have had a good (complete or nodular partial) response (18% vs 41%, *P* <0.001) Lower 5-year PFS (9% vs 18%; *P*=0.001)
Duek et al; 2006[30]	964	62 (6.4%)	55 (5.7)	Higher atypical prolymphocytes Higher CD38 Higher β2-microglobulin
Barcellini et al; 2006[9]	3150 (18 center) 194 had AIC	126/3150 (4%) 126/194 (66%)	129 (4)	Advanced stage (*P*<0.001). Older age (OR 3.43, CI 1.22–9.63) First line therapy (OR 15.62, CI 5.00–48.82) Second line therapy (OR 48.64, CI 10.00–239.19) Multivariate analysis showed that Fludarabine as a second line did not show difference in RR
Kyasa et al; 2003[21]	132	16 (18%)	6 (4.5)[#]	No CLL/SLL patients had DAT-negative hemolytic anemia 10 DAT positive CLL/SLL patients, did not have AIHA, 2 had ITP.
Mauro et al; 2000[20]	1203	52 (4.3%)	45 (3.7)[#]	More LC > 60 x 10(9)/L (*P* <0.00001) Age above 65 years (*P* <0.01) Male gender (*P* <0.01)

Notes: *Untreated CLL; **Abstract ASH 2009; #studies that not all DAT positive patients suffer from AIHA.
Abbreviations: WBC, white blood cells; Hb, hemoglobin; PLT, platelet; LC, lymphocytic count; LDH, lactate dehydrogenase; ZAP, zeta-associated protein; IgVH, immunoglobulin variable heavy chain; PFS, progression-free survival; OS, overall survival; DAT, direct antiglobulin.

for OS because this association became weaker in diseases with longer survival post progression, and it may require a longer follow-up duration to detect it.[26]

This study has some limitations. It is a retrospective cohort study with a relatively short follow-up duration. The unavailability of data about cytogenetic parameters and other molecular variables such as IgVH status or del 17 prevented us from conducting further analyses to prove our hypothesis. Another limitation was that patients receiving novel agents such as Bruton tyrosine kinase inhibitors like ibrutinib or BCL2 inhibitors like Venetoclax were not included in our study; thus, our conclusions might not extend to this population.

Conclusion

When assessing CLL, anemia is a cornerstone of Rai and Binet staging systems;[16,27] however, whether it is immune-related has not yet been incorporated in those models, which might limit better disease evaluation and prognostication. DAT positivity can be considered a surrogate marker for advanced clinical stage and progression of the disease course in patients with CLL, and we suggest incorporating it with other developing molecular stratifying parameters currently being investigated to develop a novel risk stratification system for CLL.

Abbreviations

CLL, Chronic lymphocytic leukemia; CR, Complete remission; DAT, Direct antiglobulin test; ECOG, Eastern Cooperative Oncology Group; IWCLL, International Workshop on Chronic Lymphocytic Leukemia; OR, Or advanced Rai; OS, Overall survival; PFS, Progression-free survival; PR, Partial response; PRCA, Pure red cell aplasia.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Rebecca S, Siegel M, Kimberly D, Miller M, Ahmedin Jemal D. Cancer statistics. *CA Cancer J Clin*. 2017;67(27):7–30.
2. Hodgson K, Ferrer G, Montserrat E, Moreno C. Chronic lymphocytic leukemia and autoimmunity: a systematic review. *Haematologica*. 2011;96(5):752–761. doi:10.3324/haematol.2010.036152
3. Fattizzo B, Barcellini W. Autoimmune cytopenias in chronic lymphocytic leukemia: focus on molecular aspects. *Front Oncol*. 2020;9:1435. doi:10.3389/fonc.2019.01435
4. D'Arena G, Simeon V, D'Auria F, et al. Regulatory T-cells in chronic lymphocytic leukemia: actor or innocent bystander? *Am J Blood Res*. 2013;3(1):52–57.
5. Lad DP, Varma S, Varma N, Sachdeva MUS, Bose P, Malhotra P. Regulatory T-cell and T-helper 17 balance in chronic lymphocytic leukemia progression and autoimmune cytopenias. *Leuk Lymphoma*. 2015;56(8):2424–2428. doi:10.3109/10428194.2014.986479
6. Zent CS, Kay NE. Autoimmune complications in chronic lymphocytic leukaemia (CLL). *Best Pract Res Clin Haematol*. 2010;23(1):47–59. doi:10.1016/j.beha.2010.01.004
7. Borthakur G, O'Brien S, Wierda WG, et al. Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab–incidence and predictors. *Br J Haematol*. 2007;136(6):800–805. doi:10.1111/j.1365-2141.2007.06513.x
8. Quinquenel A, Al Nawakil C, Baran-Marszak F, et al. Old DAT and new data: positive direct antiglobulin test identifies a subgroup with poor outcome among chronic lymphocytic leukemia stage A patients. *Am J Hematol*. 2015;90(1):E5–E8. doi:10.1002/ajh.23861
9. Barcellini W, Capalbo S, Agostinelli RM, et al. Relationship between autoimmune phenomena and disease stage and therapy in B-cell chronic lymphocytic leukemia. *Haematologica*. 2006;91(12):1689–1692.
10. Moreno C, Hodgson K, Ferrer G, et al. Autoimmune cytopenia in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance. *Blood*. 2010;116(23):4771–4776.
11. Zanotti R, Frattini F, Ghia P, et al. ZAP-70 expression is associated with increased risk of autoimmune cytopenias in CLL patients. *Am J Hematol*. 2010;85(7):494–498. doi:10.1002/ajh.21737
12. Dearden C, Wade R, Else M, et al. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. *Blood*. 2008;111(4):1820–1826.
13. Abbas SA, Zeeshan R, Sultan S, Irfan S. Direct Coombs test positivity in B-chronic lymphoid leukemia: a marker of advanced clinical disease. *Asian Pac J Cancer Prev*. 2015;16(14):6007–6010. doi:10.7314/apjcp.2015.16.14.6007
14. Ricci F, Tedeschi A, Vismara E, et al. Should a positive direct antiglobulin test be considered a prognostic predictor in chronic lymphocytic leukemia? *Clin Lymphoma Myeloma Leuk*. 2013;13(4):441–446. doi:10.1016/j.clml.2013.02.024
15. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008;111(12):5446–5456. doi:10.1182/blood-2007-06-093906
16. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood*. 1975;46(2):219–234. doi:10.1182/blood.V46.2.219.219
17. Hallek M, Cheson BD, Catovsky D, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood*. 2018;131(25):2745–2760. doi:10.1182/blood-2017-09-806398
18. Xu W, Li J-Y, Miao K-R, et al. The negative prognostic significance of positive direct antiglobulin test in Chinese patients with chronic lymphocytic leukemia. *Leuk Lymphoma*. 2009;50(9):1482–1487. doi:10.1080/10428190903111930
19. Hamblin TJ, Oscier DG, Young BJ. Autoimmunity in chronic lymphocytic leukaemia. *J Clin Pathol*. 1986;39(7):713–716. doi:10.1136/jcp.39.7.713
20. Mauro FR, Foa R, Cerretti R, et al. Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. *Blood*. 2000;95(9):2786–2792. doi:10.1182/blood.V95.9.2786.009k30_2786_2792
21. Kyasa MJ, Parrish RS, Schichman SA, Zent CS. Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma. *Am J Hematol*. 2003;74(1):1–8. doi:10.1002/ajh.10369
22. Ricci F, Tedeschi A, Vismara E, et al. *Positive Direct Antiglobulin Test (DAT) in Chronic Lymphocytic Leukemia (CLL) Patients: Correlation with Prognostic Factors*. American Society of Hematology; 2009.
23. Shvidel L, Tadmor T, Braester A, et al. Pathogenesis, prevalence, and prognostic significance of cytopenias in chronic lymphocytic leukemia (CLL): a retrospective comparative study of 213 patients from a national CLL database of 1518 cases. *Ann Hematol*. 2013;92(5):661–667. doi:10.1007/s00277-012-1663-3
24. Zent CS, Ding W, Schwager SM, et al. The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma. *Br J Haematol*. 2008;141(5):615–621. doi:10.1111/j.1365-2141.2008.07086.x
25. Atef B, Azmy E, Aladle D, Mabed M. The prevalence and prognostic significance of autoimmune cytopenias in a cohort of Egyptian patients with chronic lymphocytic leukemia. *Hematol Oncol Stem Cell Ther*. 2019;12(2):97–104. doi:10.1016/j.hemonc.2019.01.004
26. Amir E, Seruga B, Kwong R, Tannock IF, Ocaña A. Poor correlation between progression-free and overall survival in modern clinical trials: are composite endpoints the answer? *Eur J Cancer*. 2012;48(3):385–388. doi:10.1016/j.ejca.2011.10.028
27. Binet JL, Leporrier M, Dighiero G, et al. A clinical staging system for chronic lymphocytic leukemia: prognostic significance. *Cancer*. 1977;40(2):855–864. doi:10.1002/1097-0142(197708)40:2<855::AID-CNCR2820400239>3.0.CO;2-1
28. Demir C, Ekinci Ö. Clinical and serological autoimmune complications in chronic lymphocytic leukemia. *Wien Klin Wochenschr*. 2017;129(15–16):552–557. doi:10.1007/s00508-017-1208-9
29. Visentin A, Imbergamo S, Gurrieri C, et al. Major infections, secondary cancers and autoimmune diseases occur in different clinical subsets of chronic lymphocytic leukaemia patients. *Eur J Cancer*. 2017;72:103–111. doi:10.1016/j.ejca.2016.11.020
30. Duek A, Shvidel L, Braester A, Berrebi A. Clinical and immunologic aspects of B chronic lymphocytic leukemia associated with autoimmune disorders. *Isr Med Assoc J*. 2006;8(12):828–831.

Journal of Blood Medicine

Dovepress

Publish your work in this journal

The Journal of Blood Medicine is an international, peer-reviewed, open access, online journal publishing laboratory, experimental and clinical aspects of all aspect pertaining to blood based medicine including but not limited to: Transfusion Medicine; Blood collection, Donor issues, Transmittable diseases, and Blood banking logistics; Immunohematology; Artificial and alternative blood based therapeutics; Hematology; Biotechnology/nanotechnology of blood related medicine; Legal aspects of blood medicine; Historical perspectives. The manuscript management system is completely online and includes a very quick and fair peer-review system. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.

Submit your manuscript here: http://www.dovepress.com/journal-of-blood-medicine-journal

Correlation of Serum Ferritin and Liver Iron Concentration with Transient Liver Elastography in Adult Thalassemia Intermedia Patients with Blood Transfusion

Tubagus Djumhana Atmakusuma & Anna Mira Lubis

ORIGINAL RESEARCH

Correlation of Serum Ferritin and Liver Iron Concentration with Transient Liver Elastography in Adult Thalassemia Intermedia Patients with Blood Transfusion

Tubagus Djumhana Atmakusuma
Anna Mira Lubis

Division of Hematology-Medical Oncology, Department of Internal Medicine, Dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

Introduction: Iron overload is a common feature of thalassemia intermedia due to regular blood transfusion and increased gastrointestinal iron absorption. Early detection and adequate iron chelator can decrease morbidity and mortality from iron overload. Liver iron concentration (LIC) by MRI T2* is the best non-invasive way to measure body iron stores. However, this method is expensive and not available nationwide in Indonesia. The aim of this study was to identify liver iron overload and correlation of transferrin saturation, serum ferritin, liver MRI T2* and LIC with transient liver elastography in adult thalassemia intermedia patients.

Methods: This is a cross-sectional study of 45 patients with thalassemia intermedia with blood transfusion and with and without iron chelator therapy. The study was conducted at Cipto Mangunkusumo Hospital from August through October 2016. We performed measurements of transferrin saturation, serum ferritin level, transient liver elastography and liver MRI T2*. Pearson and Spearman correlation tests were used to evaluate the correlation between transient liver elastography with transferrin saturation, serum ferritin, liver MRI T2*and LIC.

Results and Discussion: This study showed that 64.4% of study subjects are β-Hb E thalassemia intermedia. Furthermore, 84.4% of study subjects have regular transfusion. Based on liver MRI T2*all subjects suffered from liver iron overload, 48.9% had severe degree. Median value of liver MRI T2* was 1.6 ms. Mean serum ferritin was 2831 ng/mL, with median transferrin saturation of 66%. Mean of LIC corresponding to liver MRI T2* and mean liver stiffness measurement was 15.36±7.37 mg Fe/gr dry weight and 7.7±3.8 kPa, respectively. Liver stiffness correlated with serum ferritin (r=0.651; p=0.000), liver MRI T2* (r=−0.357; p=0.016), and LIC (r=0.433; p=0.003). No correlation was found between liver elastography and transferrin saturation (r=0.204; p=0.178).

Conclusion: Serum ferritin, liver MRI T2*and LIC correlated with liver elastography. No correlation was found between transferrin saturation and liver elastography.

Keywords: liver MRI T2*, LIC, serum ferritin, thalassemia intermedia, transient liver elastography

Correspondence: Tubagus Djumhana Atmakusuma
Division of Hematology-Medical Oncology, Department of Internal Medicine, Dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine Universitas Indonesia, Jalan Diponegoro No. 71, Jakarta Pusat, DKI, Jakarta, Indonesia
Tel +622 13162497
Email hom_fkui@yahoo.com

Introduction

Thalassemia is a red blood cells genetic disorder characterized by the abnormality formation of globin chain alpha, beta, or both. Thalassemia major (TM) is generally characterized by severe anemia and clinically appears in infancy (<2 years), requiring red blood cell transfusions on a regular basis for growth and live, while thalassemia trait is generally asymptomatic and do not require red blood cell

transfusion.[1,2] Thalassemia intermedia (TI) is a group of thalassemia phenotype spectrum which is between the major and trait thalassemia.

Indonesia is one of the countries with the highest prevalence of thalassemia genetic, called "thalassemia belt".[3] Based on data from Thalassemia Center in RSCM, there are 9031 TM patients in Indonesia,[4] and this is expected to rise in line with the high number of thalassemia gene carriers who are asymptomatic, and generally they have not done premarital screening.

Unlike the TM, iron overload is still an important issue in TI, and associated with the increase of morbidity and mortality.[5] Even if thalassemia intermedia patients do not get a blood transfusion, they are still at risk for iron overload, due to increased iron absorption in the gastrointestinal tract.[6] Iron overload can be monitored by serum ferritin, which is a very quick, inexpensive, non-invasive, and generally widely available examination. But the value of this serum ferritin can be elevated in other conditions such as infection, inflammation, malignancies, etc. Liver iron concentration (LIC) is the gold standard in estimating the value of iron body load and can predict accurately total body iron.[7–9]

Liver biopsy is the gold standard in assessing the LIC directly. However, it is associated with several side effects and disadvantages such as pain, bleeding, infection, extensive sampling variability, and inter-observer variability. Therefore, a non-invasive examination has been developed in assessing LIC, such as magnetic resonance imaging (MRI). T2* MRI examination of liver is now a validated examination in assessing LIC.[10,11] This examination is non-invasive, fast, accurate and reproducible.[2] But it has not been equally distributed across thalassemia service center in Indonesia. In thalassemia patients, liver toxicity due to iron overload can lead to fibrosis, cirrhosis, and even liver cancer.

An accurate assessment of iron load in patients with thalassemia during long-term follow-up are essential, not only for the prevention of complications due to iron but also for monitoring the adequacy of iron chelation treatment. Until now, iron chelation adequacy treatment was assessed by measurement of serum ferritin and LIC.[12] It is a serial measurement that makes it a very expensive examination. So far, we have not found yet a publication about the correlation between liver elastography and liver MRIT2* on IT, transfusion-dependent thalassemia (TDT) and non-transfusion-dependent thalassemia (NTDT) patients. Liver elastography is used to measure liver

fibrosis since it is a non-invasive method and correlates well with the degree of fibrosis.[13–15] This method is a vital tool that can be added to the armamentarium of assessing chronic liver injury due to iron overload. The examination can be performed bedside, using a portable machine, and provide instantaneous results. The limitation of this method is the inability to assess fibrosis status when there is ascites or tumor present. This study is expected to assess the correlation of liver elastography and liver MRI T2* as standard tests to assess the LIC.

Methods

This cross-sectional study aimed to identify liver and blood iron overload in adult patients with TI and the correlation between transferrin saturation, serum ferritin, MRI T2* liver, and LIC assessed by MRI T2* liver examination with liver elastography value in Hematology–Oncology Division of the Internal Medicine Department Policlinic General Hospital National Center Cipto Mangunkusumo (RSUPNCM) in August 2016 to October 2016.

Patients diagnosed with thalassemia (thalassemia α, thalassemia β and thalassemia β-Hb E) using the method of Hb microcapillary electrophoresis, high-performance liquid chromatography (HPLC) or by DNA analysis, aged at least 18 years and do not have Hepatitis B or hepatitis C, human Immunodeficiency Virus (HIV), severe infection, massive ascites, hepatic failure, congestive heart failure, BMI>30 kg/m^2, total bilirubin>3g/dl, claustrophobia (phobia of narrow or closed place) and willing to participate in this research were included in this study.

We conducted demographic data such as age, gender, ethnicity, age of first thalassemia diagnosis and age of first transfusion. Clinical data such as splenectomy status, iron chelation therapy, and drug inducers of hemoglobin F were collected through interviews and medical records. Complete physical examination, and venous blood sampling for peripheral blood complete test, liver function test (AST, ALT, total bilirubin, direct, indirect), the levels of serum ferritin and transferrin saturation were conducted in all subjects. On the same day, we conducted liver Elastography examination using FibroScan® by Echosens™ with M probe in Hepatology Procedure Room RSUPNCM, performed by two operators (consultants of gastro-entero-hepatology and one trainee) who do not have prior knowledge about the patient iron status. The elasticity was obtained though multiple measurements, averaged by the machine. Normal measurement ranges from 2 to 7 kPa. T2* MRI examination of

the liver using 1.5 T MRI Siemens™ Avanto Magnetom® completed by CMR Tools software, carried out in the Department of Radiology RSUPNCM, with the funding coming from National Health Insurance (JKN) as routine checks, which were analyzed by two consultant radiologists who did not know the status of the iron and liver FibroScan results, with a maximum distance of 1 month.

This study has obtained proper ethical clearance, established by Ethics Committee of The Faculty of Medicine, University of Indonesia; Ethical Approval Number: 600/UN2.F1/ETIK/2016. Appropriate informed consent to participate in this study has been obtained from the subjects prior to study commencement. This study was conducted in accordance with the Declaration of Helsinki. Data were analyzed with SPSS 20 for Windows. Pearson's correlation coefficient (r) was performed to analyze the distribution of normal data or correlation coefficient.

Results

There were 45 subjects of the study, 28 (62.2%) were women, the median age of subjects was 33 (18–84) years (Table 1). A total of 42.4% of the subjects were in the range of 18–30 years age group. β-Thalassemia Hb E showed to be the highest proportion of thalassemia (64.4%). A total of 48.9% of the subjects diagnosed with thalassemia at the age of 20 years and the majority of subjects (71.1%) is TI with regular blood transfusions, with the median age of first receiving blood transfusion was 19 years old. Almost all subjects (95.6%) received iron chelation therapy, iron chelation with deferiprone is the most used.

Based on the assessment of iron load by checking transferrin saturation and serum ferritin, majority of the subjects had complications of iron overload. A total of 66.7% of the subjects had a transferrin saturation levels ≥55%, with a median value of 66%, with a range of 31–100%. A total of 93.3% of the research subjects had a mean serum ferritin >800 ng/mL, with the largest group of >2000 ng/mL, which is about 60% of the study subjects. T2* MRI examination of the liver obtained all the study subjects had hemosiderosis complications (median 1.6 ms) with 48.9% of subjects experienced severe hemosiderosis. LIC mean values obtained through T2* MRI examination was 15.36±7.37 mg iron/g dry weight liver. Elastography mean value and AST liver was 7.7±3.8 kPa and 33.3 U/L (Table 2).

Table 1 Characteristic Data Based on Demographic Research Subjects

Characteristics	N=45
Gender, n(%)	
Female	28(62.2)
Male	17(37.8)
Age, median (IQR)	33(22)
Age group, n(%)	
18–30 years	19(42.2)
31–40 years	11(24.4)
41–50 years	10(22.2)
51–60 years	3(6.7)
>60 years	2(4.4)
Thalassemia type, n(%)	
Thalassemia α	2(4.4)
Thalassemia β	14(31.1)
Thalassemia βHbE	29(64.4)
Age of thalassemia diagnosis, median (IQR)	20(33)
2–6 years, n(%)	11(24,4)
7–18 years, n(%)	10(22.2)
> 18 years, n(%)	24(53.3)
First transfusion age (years), median (IQR)	19(27)
2–6 years, n(%)	12(26.7)
7–18 years, n(%)	10(22.2)
> 18 years, n(%)	23(52.1)

Liver Iron Concentration Based on the Liver MRI T2*

LIC values obtained from the calculation of MRI T2* liver showed as much as 49.9% of the study subjects study experienced severe complications of hemosiderosis (>15 mg iron/g of liver dry weight), and only 11.3% of research subjects with a light hemosiderosis (Table 3).

Correlation Between Serum Ferritin and Liver Elastography Values

Based on Pearson correlation test, there was a positive correlation between serum ferritin and liver elastography values ($r = 0.651$; $p < 0.001$; Figure 1). Pearson correlation of test results obtained a weak correlation between LIC and liver elastography value ($r=0.433$; $p=0.003$; Figure 2), with a positive direction.

Table 2 Characteristic Thalassemia-Related Data of Study Subjects

Characteristics	N=45
Transfusion requirement of the year, n (%)	
1–3 times a year	7 (15.6)
4–6 times a year	6 (13.3)
> 6 times a year	32 (71.1)
A history of splenectomy, n (%)	
Yes	44 (97.8)
No	1 (2.2)
Iron chelation, n (%)	
Not getting iron chelating agent	2 (4.4)
Deferiprone	31 (68.9)
Deferasirox	11 (24.4)
Combination deferiprone + deferasirox	1 (2.2)
Pre-transfusion hemoglobin (current research), mean (SD)	7.7 (1.5)
<7 g/dl, n (%)	9 (20.0)
7–9 g/dl, n (%)	23 (51.1)
> 9 g/dl, n (%)	13 (28.9)
Mean Hb pre-transfusions per year, median (IQR)	8 (1.1)
<7 g/dl, n (%)	9 (20.0)
7–9 g/dl, n (%)	32 (71.1)
> 9 g/dl, n (%)	4 (8.9)
AST U/L mean (SD)	33.3 (19.4)
ALT U/L mean (SD)	28.8 (21.1)
MRI T2* liver ms, median (IQR)	1.6 (1.64)
LIC mg iron/g dry weight liver, mean (SD)	15.36 (7.37)
Liver elastography, kPa mean (SD)	7.7 (3.8)

Abbreviations: AST, aspartate transaminase; ALT, alanine aminotransferase; hsCRP, high-sensitive C-reactive protein; MRI, magnetic resonance imaging; LIC, liver iron concentration; IQR, inter quartile range; SD, standard deviation.

Correlation Between Liver Elastography with Liver MRI T2*

Spearman correlation test results obtained weak correlation between the value of liver elastography and MRI T2* of liver ($r=-0.357$; $p=0.016$), with a negative direction (Figure 3).

Discussion

Gastrointestinal iron absorption is increased due to chronic anemia and erythropoiesis, and also repeated blood transfusions which render thalassemia intermedia patients at

Table 3 Liver MRI T2* and LIC Result

Variables	N (%)
Liver MRI T2*	**45**
Light hemosiderosis (3,8–11 ms)	5 (11.1)
Moderate hemosiderosis (1,8–3,8 ms)	18 (40.0)
Severe hemosiderosis (<1,8 ms)	22 (48.9)
LIC values based on liver MRI T2*	**45**
Mild iron overload (2–7 mg/g)	6 (11.3)
Moderate iron overload (7–15 mg/g)	17 (37.8)
Severe iron overload (>15 mg/g)	22 (49.9)

Abbreviations: MRI, magnetic resonance imaging; LIC, liver iron concentration.

high risk for the occurrence of iron overload.[16] In the study, iron overload was assessed by serum ferritin, transferrin saturation and LIC by MRI T2*. Iron load varies greatly with time, depending on the intensity of the transfusion, administration of chelating iron and iron absorption in the gastrointestinal tract, which is heavily influenced by the severity of ineffective erythropoiesis and chronic anemia.[17–24] Serum ferritin level also changes and varies in line with changes in body iron load.[25–27] Because of that, in this experiment, we used the mean serum ferritin in the past year to avoid the presence of other factors that affect serum ferritin values such as infection, inflammation, etc.[28–30] The mean serum ferritin showed a chronic condition compared with serum ferritin levels only. The mean ferritin per year was 2831±1828 ng/mL. A total of 60% of the subjects experienced a severe iron overload in ferritin values >2000 ng/mL.

In this study, most of the subjects received blood transfusions on a regular basis; hence, the mean serum ferritin levels were high. Furthermore, the iron which was distributed to the reticuloendothelial system (RES), increased ferritin synthesis and was released into the circulation, leading to a high serum ferritin.[31–35] One out of three subjects with serum ferritin <800 ng/mL (229 ng/mL) had LIC 7.81 mg iron/gram dry weight of the liver and did not receive iron chelation. This showed the importance for monitoring the serum ferritin level as well as charge of excess iron.[36] Monitoring of LIC should be done regularly if possible, to achieve optimal iron chelation.[37–39]

Based on MRI T2* examination of the liver, we found all subjects experienced liver toxicity; 48.9% of the subjects experienced severe liver hemosiderosis (MRI T2*<1.8 ms). The proportion of subjects experienced severe liver hemosiderosis (LIC >15 mg iron/g of liver dry weight) was not
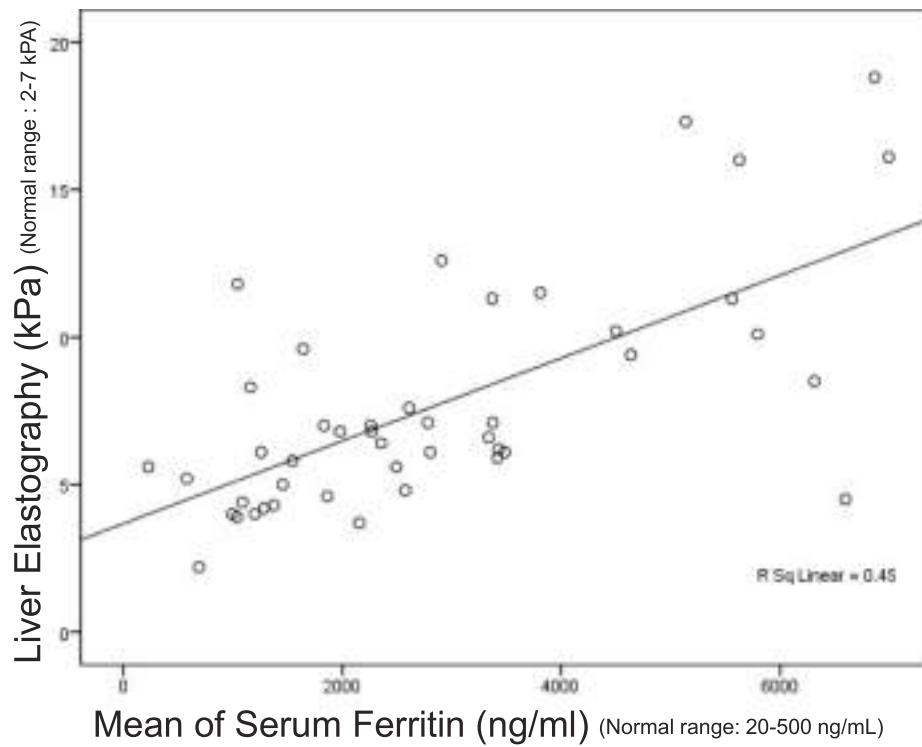
Figure 1 Correlation between serum ferritin and liver elastography.
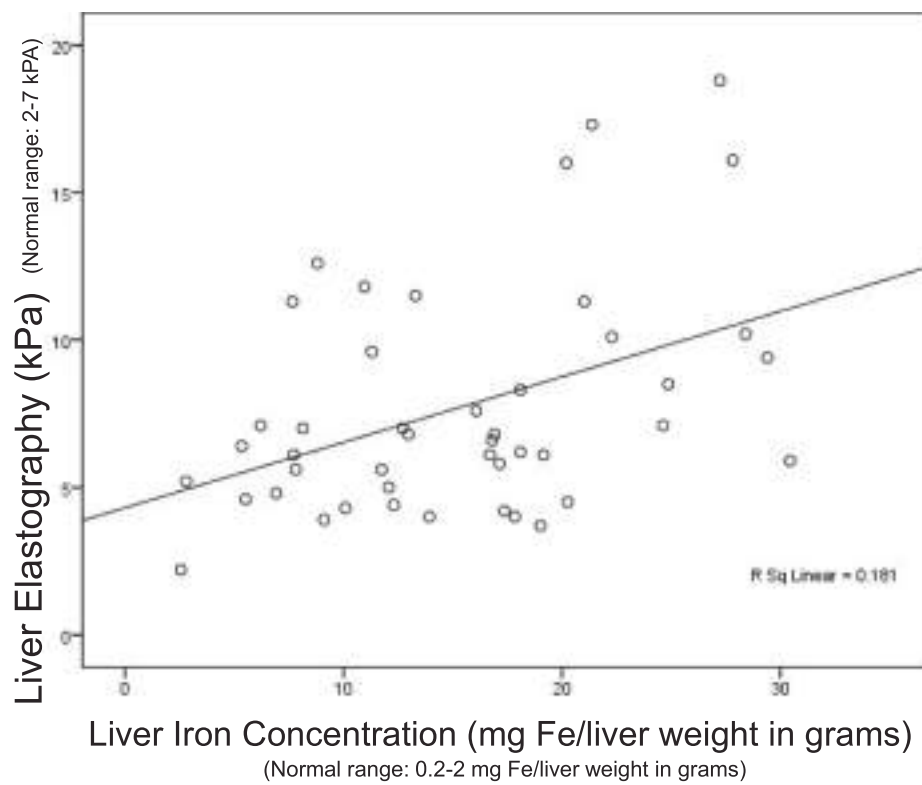
Figure 2 Correlation between liver iron concentration (LIC) and liver elastography.
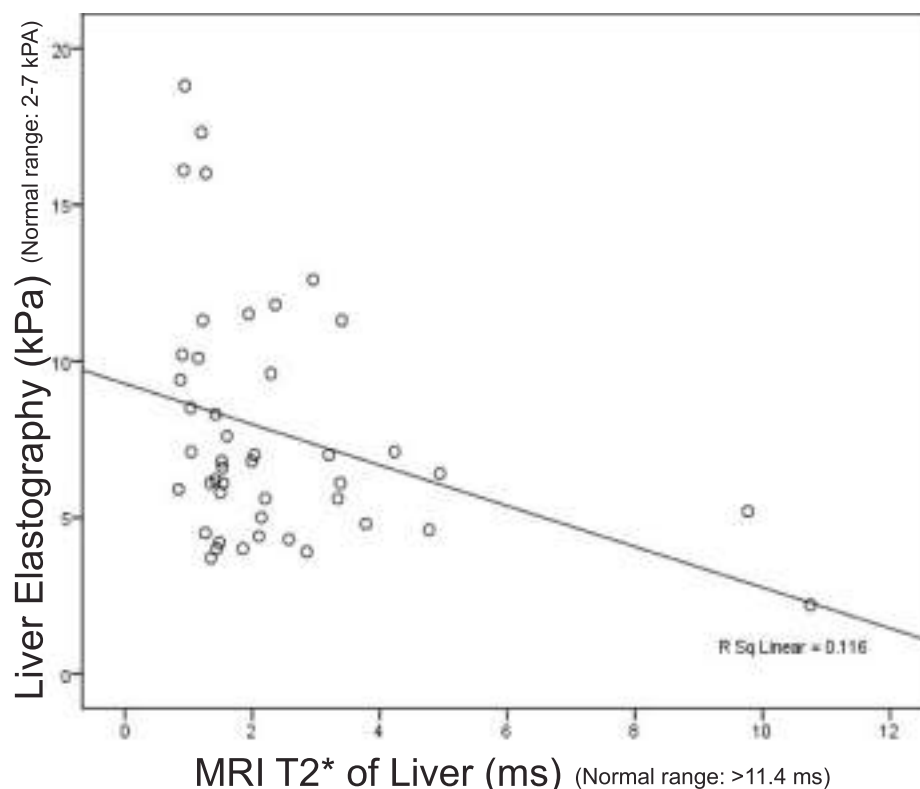
Figure 3 Correlation between MRI T2* and liver elastography.

much different (49.9%). Clinical spectrum of TI patients was very wide and varied, from mild and did not need blood transfusions, to severe with regular transfusions such as TM. However, non-transfusion-dependent thalassemia (NTDT) patients were still at risk of having excess iron load. The ineffective erythropoiesis and chronic anemia caused reduced hepcidin levels that lead to increase iron absorption in the gastrointestinal tract and increase iron release from macrophages of the RES.[40–47]

It must always be remembered that, in contrast to TM, serum ferritin can not be used to assess the severity of iron overload in thalassemia intermedia, especially IT NTDT.[8,9] A significant association between serum ferritin and LIC has been clear in the TM patients who receive regular transfusions. This is similar to the study conducted by Taher, et al[11] in Lebanon. In this study, where the majority of study subjects were IT with regular transfusion, with a mean serum ferritin 2831 (1828) ng/mL, we found a high iron overload in the liver, with an average LIC 15.36 (7.37) gr iron/gr liver dry weight. The high LIC value was predicted because of the high requirement for blood transfusions.[48–58]

In this study, a serial transferrin saturation examination was not obtained from medical records, so we could not see the trend of transferrin saturation in the past year. The other was the use of iron chelation in the study subjects, deferiprone was the most used iron-chelating agent in this study. It showed a declining optimal transferrin saturation within 2 hours after deferiprone ingestion and increased within 6 hours.[59–63] Deferiprone time lapse between consumption and time of blood sampling for the examination, which was not uniform in each research subject, could affect transferrin saturation values, related to the concentration of iron chelation and a half-life in the blood. Severe hepatic impairment (liver damage/necrosis of hepatocytes) can also lead to an increase in ferritin and transferrin decline in production.[64–68]

In this research, study subjects were IT patients with the majority of blood transfusions on a regular basis. In this study, serum ferritin used is the mean serum ferritin for 1 year, which was considered to represent the trend and chronicity of the charge of iron overload. Then, compared with the measurements of serum ferritin during the course,

this was different from the previous study using serum ferritin at the time of the study. In this study, moderate correlations were shown, between the mean serum ferritin and elastography. Iron overload and liver iron exposure in the long term might cause iron toxicity in liver which proceed into fibrosis. Liver elastography was used to assess liver fibrosis.[69]

In this study, the factors that can affect the liver fibrosis had been excluded, which were an infection of hepatitis B or C, as the long-term complications resulting from transfusions in patients with thalassemia. Thalassemia patients with excess iron load and hepatitis infection, liver fibrosis was not only due to iron toxicity but also infection such as hepatitis provided a major contribution.[55–57] In contrast to the study by Pipaliya et -al,[13] a weak correlation in this study was predicted because of difference in MRI methods used. In the study by Pipaliya,[13] MRI T2* was used. Besides, variation of patient populations especially in subjects with TM might influence the results.[69]

Conclusion

The proportion of liver iron overload in adult patients with thalassemia intermedia was 100% in General Hospital National Center Cipto Mangunkusumo (RSUPNCM) assessed by MRI T2* examination 100%. There was no correlation between transferrin saturation with a liver elastography level. There was a moderate correlation between serum ferritin by liver elastography value. Furthermore, it showed a weak correlation between LIC based on liver T2* MRI examination and liver elastography value. Adding to that, there was a weak negative correlation between the liver elastography and MRI T2* liver.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

References

1. Wood B, Molecular Higgs D. Basis of thalassemia syndromes. In: Beaumont C, Beris P, Beauzard Y, Brugnara C, editors. *Disorders of Erythropoiesis, Erythrocytes, and Iron Metabolism*. Italy: European School of Hematology; 2009:251–262.
2. Taher AT, Vichinsky E, Musallam K, Cappellini MD, Viprakasit V. Guidelines for the management of non-transfusion dependent thalassemia (NTDT). In: *Thalassemia International Federation*. Cyprus: Publisher Thalassemia International Federation; 2013:1–8.
3. Musallam KM, Rivella S, Vichinsky E, Rachmilewtz EA. Review articles. Non-transfusion dependent thalassemias. *Hematol J*. 2013;98 (6):833–844. doi:10.3324/haematol.2012.066845
4. Hematology Oncology Indonesia Working Group. *Data Registri Thalassemia Indonesia*. Children Thalassemia Center RSUPNCM Jakarta; 2016.
5. Viprakasit V, Porter J. Iron overload and chelation. In: Capellini M, Cohen A, Porter J, Taher A, Viprakasit V, editors. *Guidelines for the Management of Transfusion-Dependent Thalassemia (TDT)*. 3rd ed. Thalassemia International Federation: Thalassemia International Federation Publishers; 2014:42–96. TIF Publication No. 20.
6. Taher AT, Musallam KM, Beshlawy A, et al. Efficacy and safety of long-term (>7 year) alemtuzumab therapy for refractory T-cell large granular lymphocytic leukaemia. *Br J Haematol*. 2010;150 (4):480–497. doi:10.1111/j.1365-2141.2010.08218.x
7. Musallam KM, Cappellini MD, Wood JC, et al. Elevated liver iron concentration is a marker of increased morbidity in patients with β-thalassemia intermedia. *Hematol J*. 2011;96(11):1605–1612. doi:10.3324/haematol.2011.047852
8. Musallam KM, Cappellini MD, Taher AT. Evaluation of the 5 mg/g liver iron concentration threshold and its association with morbidity in patients with β- thalassemia intermedia. *Blood Cells Mol Dis*. 2013;51(1):35–38. doi:10.1016/j.bcmd.2013.01.015
9. Pantalone GR, Lace D, Valenza F, et al. Hepatocellular carcinoma in patients with thalassemia syndrome: clinical characteristics and outcome in a long-term single center experience. *Br J Haematol*. 2010;150(2):226–248. doi:10.1111/j.1365-2141.2010.08179.x
10. Taher AT, Viprakasit V, Musallam KM, Cappellini MD. Critical review: treating iron overload in patients with non-transfusion-dependent thalassemia. *Am J Hematol*. 2013;88(5):409–415. doi:10.1002/ajh.23405
11. Taher AT, Musallam KM, Wood JC, Cappellini MD. Magnetic resonance evaluation of hepatic and myocardial iron deposition in thalassemia intermedia independent transfusion – compared to regularly transfused thalassemia major patients. *Am J Hematol*. 2010;85 (4):288–290. doi:10.1002/ajh.21626
12. Puliyel M, Sposto R, Bardoukas V, et al. Ferritin trends do not predict changes in total body iron in patients with transfusional iron overload. *Am J Hematol*. 2014;89(4):391–394. doi:10.1002/ajh.23650
13. Pipaliya N, Solanke D, Parikh P, et al. Comparison of tissue elastography with magnetic resonance imaging T2 * and serum ferritin quantification in detecting liver iron overload in the patient with thalassemia major. *Clin Gastroenterol Hepatol*. 2016.
14. Musallam KM, Motta I, Salvatori M, et al. Longitudinal changes in serum ferritin levels correlate with measures of hepatic stiffness transfusion-independent in patients with β-thalassemia intermedia. *Blood Cells Mol Dis*. 2012;49(3–4):136–139. doi:10.1016/j.bcmd.2012.06.001
15. Fraquelli M, Cassinerio E, Roghi A, et al. Transient elastography in the assessment of liver fibrosis in adult thalassemia patients. *Am J Hematol*. 2010;85(8):564–568. doi:10.1002/ajh.21752
16. Weatherall DJ. The definition and epidemiology of non-transfusion-dependent thalassemia. *Blood Rev*. 2012;26S:S3–6. doi:10.1016/S0268-960X(12)70003-6

17. Sengsuk C, Tangvarasittichai O, Chantanaskulwong P, et al. Association of iron overload with oxidative stress, hepatic damage, and dyslipidemia in transfusion-dependent β-thalassemia/HbE patients. *Ind J Clin Biochem*. 2014;29(3):298. doi:10.1007/s12291-013-0376-2

18. Viprakasit V, Tyan P, Rodmai S, Taher AT. Review. Identification and key management of non-transfusion-dependent thalassemia patients: not a rare but under-recognized potentially condition. *Orphanet J Rare Dis*. 2014;9(131):1–11. doi:10.1186/s13023-014-0131-7

19. Cappellini MD, Musallam KM, Cesarwtti C, Taher A. Thalassemia intermedia. In: Beaumont C, Beris P, Beauzard Y, Brugnara C, editors. *Disorders of Erythropoiesis, Erythrocytes, and Iron Metabolism*. Italy: European School of Hematology; 2009:287.

20. Maharani EA, Soedarmono Y, Nainggolan IM. Frequency of thalassemia carrier and Hb variant and the quality of stored donor blood. *Med J Indones*. 2014;23(4):209–212. doi:10.13181/mji.v23i4.766

21. Musallam KM, Cappellini MD, Wood JC, Taher AT. Iron overload in non-transfusion-dependent thalassemia: a clinical perspective. *Blood Rev*. 2012;26S:S16–9. doi:10.1016/S0268-960X(12)70006-1

22. Musallam KM, Taher AT, Rachmilewitz EA. B-thalassemia intermedia: a clinical perspective. *Cold Spring Harb Perspect Med*. 2012;2(7):1–15. doi:10.1101/cshperspect.a013482

23. Taher AT, Musallam KM, Cappellini MD, Weatherall DJ. Optimal management of β thalassemia intermedia. *Br J Haematol*. 2011;152(5):512–523. doi:10.1111/j.1365-2141.2010.08486.x

24. Taher A, Isma'eel H, Cappellini MD. Thalassemia intermedia: revisited. *Blood Cells Mol Dis*. 2006;37(1):12–20. doi:10.1016/j.bcmd.2006.04.005

25. Kohgo Y, Ikuta K, Ohtake T, Torimoto Y, Kato J. Body of iron metabolism and the pathophysiology of iron overload. *Int J Haematol*. 2008;88(1):7–15. doi:10.1007/s12185-008-0120-5

26. Rivella S. The role of ineffective erythropoiesis in a non-transfusion-dependent thalassemia. *Blood Rev*. 2012;26S:S12–5. doi:10.1016/S0268-960X(12)70005-X

27. Batts KP. Iron overload syndromes and the liver. *Mod Pathol*. 2007;20(S1):S31–9. doi:10.1038/modpathol.3800715

28. Taher A, Hershko C, Cappellini MD. Iron overload in thalassemia intermedia: reassessment of iron chelation strategies. *Br J Haematol*. 2009;147(5):634–640. doi:10.1111/j.1365-2141.2009.07848.x

29. Musallam KM, Cappellini MD, Daar S, et al. Serum ferritin levels and morbidity risk in transfusion-independent in patients with β-thalassemia intermedia: the ORIENT Study. *Hematol J*. 2014;99(11):e218–21. doi:10.3324/haematol.2013.097220

30. Musallam KM, Cappellini MD, Daar S, Karimi M, El-Beshlawy A, Taher AT. Serum ferritin levels and morbidity in β-thalassemia intermedia: a 10-year cohort study. [Abstract]. *Am Soc Hematol*. 2012.

31. Hershko C. Pathogenesis and management of iron toxicity in thalassemia. *Ann NY Acad Sci*. 2010;1202(1):1–9. doi:10.1111/j.1749-6632.2010.05544.x

32. Pietrangelo A. Mechanism of iron hepatotoxicity. *Hepatol*. 2016;65(1):226–227. doi:10.1016/j.jhep.2016.01.037

33. Britton RS, Leicester KL, Bacon BR. Iron toxicity and chelation therapy. *Int J Haematol*. 2002;76(3):219–228. doi:10.1007/BF02982791

34. Taher A, Rassi F, Isma'eel H, Koussa S, Inati A, Cappellini MS. Correlation of liver iron concentration determined by R2 magnetic resonance imaging with serum ferritin in patients with thalassemia intermedia. *Hematol J*. 2008;93(10):1584–1586. doi:10.3324/haematol.13098

35. Pakbaz Z, Fischer R, Fung E, Nielsen P, Harmatz P, Vichinsky E. Serum ferritin underestimates liver iron concentration in patients with thalassemia transfusion independent as compared to regularly transfused thalassemia and sickle cell patients. *Pediatr Blood Cancer*. 2007;49(3):329–332. doi:10.1002/pbc.21275

36. Shah R, Treban A, Das R, Marwaha R. Serum ferritin in thalassemia intermedia. *Indian J Hematol Blood Transfus*. 2014;30(4):281–285. doi:10.1007/s12288-013-0267-y

37. Taher AT, Porter JB, Kattamis A, et al. Defining serum ferritin thresholds to predict clinically relevant liver iron concentrations for guiding deferasirox therapy when MRI is unavailable in patients with non-transfusion-dependent thalassaemia. *Br J Haematol*. 2015;168(2):284–290. doi:10.1111/bjh.13119

38. Bacon BR, Adams PC, Kowdley KV, Powell LW, Tavill US. Diagnosis and management of hemochromatosis: 2011 practice guideline by the American association for the study of liver disease. *Hepatology*. 2011;54(1):328–343. doi:10.1002/hep.24330

39. Garbowski M, Carpenter J, Smith G, et al. Biopsy-based calibration of T2 * magnetic resonance for estimation of liver iron concentration and comparison with R2 Ferris can. *J Cardiovasc Magn Reson*. 2014;16(40):1–11. doi:10.1186/1532-429X-16-40

40. Szurowska E, Sikorska K, Swieszewska E, et al. The role of MR imaging in the detection of hepatic iron overload in patients with cirrhosis of different origins. *BMC Gastroenterol*. 2010;10(13):1–14.

41. Timothy G, Pierre CP, Chua-anusom W, et al. Noninvasive measurement and imaging of liver iron concentrations using proton magnetic resonance. *Blood*. 2005;105(2):855–856. doi:10.1182/blood-2004-01-0177

42. Echeverria J, Castiella A, Emparanza J. Quantification of iron concentration in the liver by MRI. *Insights Imaging*. 2012;3(2):173–180. doi:10.1007/s13244-011-0132-1

43. Henninger B, Kremser C, Rauch S, et al. Evaluation of MR imaging with T1 and T2 * mapping for the determination of hepatic iron overload. *Eur Radiol*. 2012;22(11):2478–2486. doi:10.1007/s00330-012-2506-2

44. Gandon Y, Oiivie D, Guyader D, Aube C, Oberti F, Sebille V. Non-invasive assessment of hepatic iron stores by MRI. *Lancet North Am*. 2004;363(9406):357–362. doi:10.1016/S0140-6736(04)15436-6

45. Wood JC, Enriquez C, Ghugre N, et al. MRI R2 and R2 * mapping accurately estimates hepatic iron concentration in transfusion-dependent thalassemia and sickle cell disease Patients. *Blood*. 2005;106(4):1460–1465. doi:10.1182/blood-2004-10-3982

46. Sarigianni M, Liakos A, Vlachaki E, et al. Accuracy of magnetic resonance imaging in the diagnosis of liver iron overload: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol*. 2015;13(1):55–63. doi:10.1016/j.cgh.2014.05.027

47. Angelucci E, Giovagnoni A, Valeri G, et al. Limitations of magnetic resonance imaging in measurement of hepatic iron. *Blood*. 1997;90(12):4736–4742. doi:10.1182/blood.V90.12.4736

48. Verissimo MP, Loggetto SR, Fabron A Jr., et al. Special articles Brazilian protocol for thalassemia association of iron chelation therapy in patient under regular transfusion. *Reu Braz Hematol*. 2013;35(6):428–434.

49. Castiella A, Zapata E. Non-invasive prediction methods for liver fibrosis in hemochromatosis: one step beyond. *World J Hepatol*. 2010;2(7):251–255. doi:10.4254/wjh.v2.i7.251

50. Papastergiou V, Tsochatzis E, Burroughts AK. Non-invasive assessment of liver fibrosis. *Ann Gastroenterol*. 2012;25(3):218–231.

51. Adhoute X, Foucher J, Laharie D, et al. Diagnosis of liver fibrosis and other FibroScan using noninvasive methods in patients with hemochromatosis: a prospective study. *Gastroenterol Clin Biol*. 2008;32(2):180–187. doi:10.1016/j.gcb.2007.12.021

52. Talwalkar J, Kurtz D, Schoenleber S, West C, Montori V. Ultrasound-based transient elastography for the detection of hepatic fibrosis: systematic review and meta-analysis. *Clin Gastroenterol Hepatol*. 2007;5(10):1214–1220. doi:10.1016/j.cgh.2007.07.020

53. Paparo F, Cevasco L, Zefiro D, et al. Diagnostic value of real-time elastography in the assessment of hepatic fibrosis in patients with liver iron overload. *Eur J Radiol*. 2013;82(12):e755–61. doi:10.1016/j.ejrad.2013.08.038

54. Marco V, Bronte F, Cabibi D, et al. Noninvasive assessment of liver fibrosis in thalassemia major patients by transient elastography (TE) – lack of interference by iron deposition. *Br J Haematol*. 2009;148(3):476–479. doi:10.1111/j.1365-2141.2009.07996.x

55. Taher AT, Cappellini MD, Musallam KM. Recent advances and treatment challenges in patients with non-transfusion-dependent thalassemia. *Blood Rev*. 2012;26S:S1–2. doi:10.1016/S0268-960X (12)00028-8

56. Telfer PT, Prestcott E, Holden S, Walker M, Hoffbrand AV, Wonke B. Hepatic iron concentration combined with long term monitoring of serum ferritin to predict complication of iron overload in thalassemia major. *Br J Haematol*. 2000;110(4):971–977. doi:10.1046/j.1365-2141.2000.02298.x

57. Children Thalassemia Center RSUPNCM. Data MRI T2 * liver and heart in Dept. pediatrics 2014 Jakarta. Anak: Thalassemia Center; 2014

58. Olivieri NF, Gary MB, Doreen M, Matitiahu B, Laurence MB. Iron-chelation therapy with oral deferiprone in patients with major thalassemia. *N Engl J Med*. 1995;332(14):918–922. doi:10.1056/NEJM199504063321404

59. Galanello R, Piga A, Forni GL, et al. Phase II clinical evaluation of deferasirox, a once daily oral chelating agent, in pediatric patients with B-thalassemia major. *Hematol J*. 2006;91:1343–1351.

60. Adams P, Barton J. A diagnostic approach to hyperferritinemia with a non-elevated transferrin saturation. *J Hepatol*. 2011;55(2):453–458. doi:10.1016/j.jhep.2011.02.010

61. Al-Refaie FN, Silva CE, Wonke B. Hoffbrand AV changes in transferrin saturation after treatment with the oral iron chelator deferiprone in patients with iron overload. *J Clin Pathol*. 1996;48 (2):110–114. doi:10.1136/jcp.48.2.110

62. Sinakos E, Vasilios P, Efthimia V, Ioanna T, Maria RG. Is liver stiffness really unrelated to liver iron concentration? *Br J Haematol*. 2010;150(2):247–248. doi:10.1111/j.1365-2141.2010.08183.x

63. Ezzat H. Utility of transient elastography (FibroScan) in estimating hepatic iron concentration in comparison to MRI in patients with transfusion dependent hemoglobinopathies. [Abstract]. *Blood*. 2014;124(21):4888. doi:10.1182/blood.V124.21.4888.4888

64. Majd Z, Haghpanah S, Ajami G, et al. Serum ferritin levels correlation with heart and liver MRI and LIC in patients with transfusion-dependent thalassemia. *Iran Red Crescent Med J*. 2015;17(4):e24959. doi:10.5812/ircmj.17(4)2015.24959

65. Origa R, Barella S, Argiolas GM, Bina P, Agus A, Galanello R. No evidence of cardiac iron in 20 never or minimally transfused patients with thalassemia intermedia. *Hematol J*. 2008;93(7):1095–1096. doi:10.3324/haematol.12484

66. Aessopos A, Kati M, Farmakis D. Heart disease in thalassemia intermedia: a review of the underlying pathophysiology. *J Hematol*. 2007;92(5):658–665. doi:10.3324/haematol.10915

67. Aessopos A, Farmakis D, Deftereos S, et al. Thalassemia heart disease: a comparative evaluation of thalassemia major and thalassemia intermedia. *Chest*. 2005;127(5):1523–1530. doi:10.1378/chest.127.5.1523

68. Xiao G, Yang J, Yan L. Comparison of diagnostic accuracy of aspartate aminotransferase to platelet ratio index and fibrosis-4 for detecting liver fibrosis in adult patients with chronic hepatitis B virus infection: a systematic review and meta-analysis. *Hepatology*. 2015;61(1):29. doi:10.1002/hep.27382

69. Lubis AM, Atmakusuma TD, Kurnawan J, Harimurti K. *Korelasi antara muatan besi berlebih darah dan hati dengan nilai elastografi hati pada pasien thalasemia intermedia dewasa yang mendapatkan transfusi darah*. [Correlation between blood and liver excess iron load and liver elastographic values in adult intermedia thalassemia patients receiving blood transfusions] [Thesis]; 2016. Indonesian.

What Does the Economic Burden of Acute Myeloid Leukemia Treatment Look Like for the Next Decade? An Analysis of Key Findings, Challenges and Recommendations

Anna Forsythe & Karen Sandman

REVIEW

What Does the Economic Burden of Acute Myeloid Leukemia Treatment Look Like for the Next Decade? An Analysis of Key Findings, Challenges and Recommendations

Anna Forsythe
Karen Sandman

Purple Squirrel Economics, New York, NY, USA

Abstract: Acute myeloid leukemia (AML) is conventionally treated with chemotherapy in eligible patients. Potentially curative regimens are associated with significant toxicity, and the major cost drivers in AML historically have been hospitalization and hematopoietic stem cell transplantation. The past several years have seen a dramatic increase in the number of treatment options, including oral therapies and drugs targeted to biological pathways implicated in AML. Major current and future drivers of cost in AML include hospitalization and medical costs, stem cell transplantation for eligible patients, and medication costs. It is likely that hospitalization and medical costs will decline as more AML treatment moves to the outpatient setting. Stem cell transplantation costs may increase, if more patients are eligible for improved procedures, although the overall cost of transplantation could decrease if new procedures reduce the need for hospitalization. Medication costs are likely to increase, with various branded drugs available and in development. From a broader perspective, another driver of cost is the proportion of patients with AML who can undergo treatment. Patients who may previously have been unable to tolerate chemotherapy are more likely to be treated with the range of less intensive, more tolerable options now available. The effectiveness of newer AML treatment options also suggests that, overall, there may be more patients staying alive and on treatment longer than in the past. While certain advances, such as increased use of oral and outpatient therapies, could potentially reduce costs, the overall economic impact of AML is likely to increase as more patients are eligible for novel therapies across several phases from induction to maintenance to relapsed/refractory disease. While these novel therapies have the potential to deliver value in the form of improved efficacy, safety, and convenience, payers will need to determine how to cover a longer, more complex AML treatment pathway.

Keywords: AML, leukemia, cost of illness, economic burden

AML: A Rare and Costly Cancer

Acute myeloid leukemia (AML) arises in the bone marrow from the abnormal clonal expansion of myeloid blood cell precursors. Leukemic blast cells are found in the circulating blood as well as in the bone marrow, where they disrupt normal blood cell production, leading to myelosuppression.[1] Consequences of AML include anemia, with weakness and pallor, thrombocytopenia, resulting in bleeding, and leukopenia, leading to fever and infection.

Correspondence: Anna Forsythe
Managing Partner and CEO, Purple Squirrel Economics, 4 Lexington Ave, Suite 15K, New York, NY, 10010, USA
Tel +1-646-477-0936
Email Annaforsythe@pshta.com

AML, which accounts for about 30% of all leukemia cases, is a relatively rare cancer, accounting for about 1.1% of all cancers in the US, with about 20,000 new cases and 11,000 deaths per year.[2] Global incidence of AML in 2018 was estimated to be around 130,000.[3] AML tends to affect older individuals, with a median age at diagnosis of 68 years.[2]

Without treatment, acute leukemias can be rapidly fatal, but with prompt initiation of intensive treatment, survival of several years or more is achievable.[1] Conventional treatment for AML involves aggressive, cytotoxic induction chemotherapy, with the goal of allogeneic hematopoietic stem cell transplant (HSCT) in eligible patients. This intensive approach, while successful in some patients, has not been feasible in many elderly patients due to poor performance status and comorbidities.[4] Thus, while long-term survival approaches 50% in patients under age 65, it drops to about 10% in patients over 65.[5] The 5-year overall survival rate in the US from 2010 to 2016 was 28.7%.[2]

Based on the low overall survival rates in AML, as well as the disparities in treatment outcomes for younger and older patients, there has been considerable research in recent years to develop treatment pathways for different subgroups of patients. At the same time, an evolving understanding of AML biology has sparked the development of targeted therapies that can be tailored to patients based on genetic features of their cancer cells.[4] The current standard approach is to evaluate patients' fitness for intensive chemotherapy (IC) and HSCT. If a patient is an IC candidate, they typically receive an induction regimen, such as cytarabine plus daunorubicin, followed by HSCT if possible. Patients may also undergo consolidation therapy with high-dose cytarabine. If a patient is a candidate for non-intensive chemotherapy (NIC), they may receive a low-dose hypomethylating agent (HMA) such as azacitidine or decitabine, or they may receive low-dose cytarabine (LDAC). While these NIC options have relatively modest efficacy, in recent years there have been a series of approvals of new, targeted drugs that can be used with or without NIC, providing patients with a range of options beyond conventional chemotherapy. Patients who achieve complete remission may receive maintenance therapy with recently approved oral options (oral azacitidine approved by the US Food and Drug Administration [FDA] and midostaurin approved by the European Medicines Agency [EMA]). Relapsed or refractory (R/R) patients may receive an IC or NIC reinduction regimen, with or without a targeted agent, or single-agent targeted therapy.[1,4,6]

Conventional therapies for AML incur considerable costs and healthcare resource utilization (HCRU), with hospital-based chemotherapy infusions, the need for frequent monitoring, and the inevitable need to treat serious adverse effects of treatment.[7] HSCT, while potentially curative, is a costly inpatient procedure. Newer treatments, with more manageable safety profiles, may help to limit medical and hospitalization costs, but in general these novel drugs cost more than conventional chemotherapy. Moreover, the higher tolerability of newer treatments increases the number of patients who can initiate and stay on treatment, potentially expanding the overall budget impact of AML on healthcare systems.

In conducting this review, we sought to first characterize the current landscape of cost and value in AML, identifying the major cost drivers and how they have evolved in recent years. We then aimed to consider how upcoming advances in treatment and care delivery may impact the economic impact of AML, so that we could identify opportunities for manufacturers, treatment centers, and others to deliver value in AML in the coming years.

AML: A Therapeutic Landscape in Transition
Historical Cost Drivers in AML

We conducted a systematic review of studies reporting economic outcomes in AML. The systematic review was performed in accordance with the methodological principles of conduct for systematic reviews as detailed in the University of York CRD's "Guidance for Undertaking Reviews in Health Care" and in accordance with methodology established in the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement.[8,9] SLR searches (from database inception to December 2020) were conducted in the MEDLINE, Embase, EconLit and Cochrane databases. In addition to the database searches, keyword searches of the annual proceedings of scientific meetings (American Society for Clinical Oncology [ASCO], European Haematology Association [EHA], European Society for Medical Oncology [ESMO] and American Society for Hematology [ASH]). A total of 54 records were selected from 48 original studies reporting on healthcare resource use or costs in AML. Among selected studies, 31 included US data, and 14 included EU data.

One of the large retrospective database studies on the economic burden of AML in the US before the approval of

targeted agents (2008 to 2016) examined HCRU and direct costs in AML in a commercial payer database.[10] The most expensive episodes of care were R/R AML ($439,104), HSCT ($329,621), induction IC ($198,657), consolidation IC ($73,428), and NIC ($53,081). Across all these groups, the main driver of cost was inpatient hospitalization, which accounted for about 70% of costs. AML symptoms and treatment toxicity were associated with higher costs, suggesting that less toxic alternatives to chemotherapy may help to control healthcare costs in AML. Several other retrospective studies confirmed these findings, noting very high costs associated with relapse/disease progression; the largest cost driver was inpatient utilization in both private and public healthcare settings.[11–13]

Outside of the US, the economic picture of AML is similar, as demonstrated in a claims database study (1997 to 2015) covering nearly 40,000 patients with AML in Spain.[14] With mean annual direct costs of €30,775 per patient that increased by 3.7-fold from 1999 to 2011, the primary drivers were hospitalization and HSCT. A retrospective study in the Netherlands aimed at calculating the cost of initial treatment in AML also concluded that hospitalization was the major cost driver.[15] A large study based on a Swedish registry (N=2954, 2007 to 2015) noted that among all AML treatment phases, the total cost from date of HSCT to death is the largest, amounting to over US$160,000, with inpatient costs accounting for 60% of the total.[16]

These and other database studies exploring AML costs prior to the advent of novel therapeutics depict a scenario likely to change as less toxic and more effective therapies increase in uptake. The remainder of this review explores the likely transition of key cost drivers in AML in the coming years.

Evolving Clinical and Economic Picture of AML

Following the development of the cytarabine + daunorubicin regimen and HSCT for AML in the 1970s, there were several decades without significant innovation in AML treatment.[4,17] This situation changed in 2017, and the past several years have seen nine new products approved in AML. A growing understanding of the genetic features of AML cells has allowed many of these new therapies to target to specific biological pathways implicated in AML development and progression.[18] Relevant to

the HCRU associated with these treatments, seven of the nine newly approved drugs are orally administered.

Two are for patients with fms-like tyrosine kinase 3 positive (FLT3+) cancer: oral midostaurin may be added to IC in newly diagnosed patients, and patients with R/R AML may receive oral gilteritinib.

Patients with isocitrate dehydrogenase (IDH) mutations may receive oral enasidenib for IDH2+ RR AML, while elderly patients with IDH1+ AML may receive oral ivosidenib in the newly diagnosed or relapsed/refractory setting.

Patients with newly diagnosed AML eligible for IC who have CD33 expression may receive intravenous (IV) gemtuzumab ozogamicin in combination with IC.

A liposomal, IV administered combination of cytarabine and daunorubicin, CPX-351, is available for newly diagnosed therapy-related AML or AML with myelodysplasia-related changes (AML-MRC).

Two targeted therapy options that can be added to NIC for newly diagnosed patients are oral venetoclax and oral glasdegib.

Lastly, oral azacitidine has been approved as a maintenance therapy in patients who achieved first complete remission (CR) or complete remission with incomplete blood count recovery (CRi) following intensive induction chemotherapy and who are not able to complete intensive curative therapy.

In addition to the rapid growth in treatment options for patients with AML, including those eligible for IC or NIC, there have been progressive improvements in supportive care for patients undergoing AML treatment. While IC remains an intensive treatment that can result in serious complications or even death, there have been substantial advances such as the introduction of broad-spectrum oral antifungals and improvements in transfusion medicine. These changes, combined with increased emphasis on patient and caregiver quality of life and management of costs, have allowed many patients to receive a greater proportion of AML treatment in the outpatient setting. Most recently, the COVID-19 pandemic has accelerated the push towards using telehealth and outpatient care when appropriate, and it is likely that these new approaches to care will influence treatment practices in the long term.[7]

Major drivers of cost in AML include hospitalization and medical costs, stem cell transplantation for eligible patients, and medication costs (conventional chemotherapy and novel agents). From a broader health plan or societal perspective,

another driver of cost is the proportion of patients with AML who undergo treatment. Patients who may previously have been unable to tolerate chemotherapy are more likely to be treated with the range of NIC options now available. The effectiveness of newer AML treatment options also suggests that, overall, there may be more patients staying alive and on treatment longer than in the past.

Cost Drivers in Transition: Hospitalization and Medical Costs

Inpatient hospital care, and medical costs more broadly, have historically been major cost drivers in AML. Several recent factors are likely to reduce these costs, which in principle could reduce the overall economic burden of AML. These include the advent of oral AML therapies and chemotherapy regimens that can be administered in the outpatient setting, as well as improved supportive care that may reduce the need for emergency care and prolonged hospitalizations.[7]

AML treatments that can be administered in the outpatient setting include the seven recently approved oral options. Some of the oral therapies are administered as single agents and others as add-on therapy; choice of agent and regimen depends on biological features of the patient's AML and the stage of treatment. In addition to the oral options, which avoid administration costs, the new liposomal formulation of daunorubicin and cytarabine, CPX-351, has a simplified dosing schedule[19] that allows it to be administered to suitable patients in the outpatient setting, potentially reducing inpatient costs. In a pilot study, 14 patients were able to receive induction chemotherapy as outpatients, reducing their mean overall hospitalization by over 2 days compared with those who received the same regimen as inpatients.[20] Another pilot study found that patients were able to safely receive IC for AML and high-risk myelodysplastic syndromes (MDS) as outpatients.[21] Fourteen of 17 patients completed IC without needing hospital admission, although most eventually required admission for supportive care typical of patients following induction. Outpatient administration of induction regimens is not expected to prevent hospitalization altogether, but it may reduce costs by decreasing the total number of inpatient days.

Cost Drivers in Transition: HSCT and Other Cellular Therapies

Beyond hospitalization and medical costs, the other major historical cost driver in AML has been HSCT. This intensive procedure requires specialized care, with patients often hospitalized for prolonged periods and requiring extensive follow-up care. While many patients with AML have historically not been considered candidates for HSCT, improved methods for donor selection and relaxed requirements for patient fitness have expanded the eligible patient population, potentially increasing overall HSCT costs in AML.

In recent years, the ability to identify patients who are likely to benefit from allogeneic HSCT has been improved by advances in the cytogenetic and molecular risk stratification of AML as well as early assessments of measurable residual disease (MRD).[22,23] Among patients who are considered suitable HSCT candidates, there are increasing options even if they do not have a matched sibling donor. Over 30 million adults are registered worldwide as potential volunteer donors, cryopreserved umbilical cord blood is growing in availability, and there is growing evidence to support the safe transplantation of haploidentical (half-matched) stem cells.[24] These factors have led to a sharp increase in the number of allogeneic HSCT procedures performed in AML in the last decade.

There are several new transplantation technologies that potentially expand the donor pool. Despite the promise of umbilical cord blood stem cells in increasing the pool of potential matched donors, one challenge in using this approach in adults has been the relatively low dose of stem cells available, which can lead to graft failure and delayed bone marrow recovery.[25,26] One strategy being investigated to address this challenge is UM171, a hematopoietic stem cell self-renewal agonist, which expands umbilical cord blood stem cells, thus allowing for a higher stem cell dose. Initial results in hematologic malignancies demonstrate the feasibility of this approach, with a potential for low risk of chronic graft-versus-host disease and relapse.[27] An ongoing study (NCT03913026) is assessing the use of UM171-expanded cord blood cells in patients with high-risk acute leukemia/myelodysplasia. In a pilot UM171 trial (NCT02668315), among 22 patients who received a single UM171 cell bank transplant, the rate of GVHD (10%) was low, with no moderate-to-severe chronic GVHD.[27] If methods such as UM171 enter clinical practice, there may be an increase in the number of patients who undergo transplants, and thus in the associated costs—both for the new technology as well as for the transplant procedure and post-transplant care.

Another possible cellular therapy on the horizon for AML is chimeric antigen receptor T-cell (CAR-T) therapy. To date, the CAR-T products that have been approved are

used in B-cell malignancies, which lend themselves to this approach because they express antigens that are unique to the B-cell lineage. Myeloid cancers, by contrast, tend to express tumor antigens that are also found on various healthy cells, including hematopoietic stem or progenitor cells, making it challenging to design CAR-T therapy for AML.[28] The depletion of stem cells by CAR-T cell therapy would cause prolonged myeloablation, with consequences such as infection and transfusion dependence. Despite these significant challenges, there are ongoing efforts to develop CAR-T treatments for RR AML, with ten ongoing trials identified in a search of clinicaltrials.gov on January 31, 2021 (Table 1).

If investigational cellular therapies such as those outlined in Table 1 show efficacy with acceptable safety profiles in AML, they would likely carry a very high cost. The currently available CAR-T therapies cost in the range of $375,000 to $475,000 for a single infusion, in addition to the medical costs and management of complications.[29] If a large number of patients with AML were considered candidates for cellular therapy, there could be a significant impact on the overall cost burden in AML. There are, however, substantial payer restrictions on coverage for the currently available CAR-T therapies. The actual cost impact therefore may be less, if only a small fraction of patients with AML are able to access the treatment.

The high cost of CAR-T therapies may be offset by their benefits if these approaches are found to be cost-effective relative to other options. Our systematic literature review did not identify any cost-effectiveness analyses for CAR-T treatments in AML. CAR-T therapy was found to be cost-effective, with an incremental cost-effectiveness

ratio (ICER) of $64,600/quality-adjusted life year (QALY), in a microsimulation model of pediatric acute lymphoblastic leukemia (ALL).[30] The authors of the pediatric ALL analysis note that longer-term efficacy data for CAR-T may change their findings, and it is not clear whether comparable findings would apply in adults with AML.

Cost Drivers in Transition: Drug Costs

Conventional chemotherapy for AML utilizes largely generic drugs, so that the primary costs are in administration and toxicity management rather than in direct drug costs. The large number of new AML therapies approved in the past several years largely stems from the explosion in research into abnormal genetic pathways in cancer cells and how to disrupt these pathways.[18] The tailoring of therapies to specific biological pathways helps to get the right treatment to the right patient, but it also requires genetic profiling of a patient's cancer cells to inform treatment decisions. The higher demand for genetic testing will likely contribute to the economic burden of AML.

The impact of new AML drugs on costs would best be assessed by analysing treatment patterns. While currently published treatment pattern studies are largely based on data from before new drugs became available in 2017, the novel therapies are by now fully integrated into prominent treatment guidelines, such as the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines. The NCCN guidelines include IC regimens that utilize novel branded therapies such as gemtuzumab ozogamicin, midostaurin, venetoclax, and CPX-351, alongside conventional chemotherapy options.[6] For NIC induction therapy, the NCCN guidelines include a variety of novel branded therapies, such as venetoclax, glasdegib, gemtuzumab ozogamicin, ivosidenib, and enasidenib. The guidelines also include most of the novel agents as options for post-remission or maintenance therapy, as well as for R/R AML, with specific recommendations based on patient fitness, biological factors, and prior therapies.

These new targeted therapies, with more on the horizon, offer many potential benefits in terms of efficacy, safety, ease of administration, and reduced hospital time, but as branded drugs and combinations, they are likely to substantially increase spending on medications in AML, both for patients and payers. In the US, patients with private insurance may face substantial out-of-pocket copayments for the self-administered drugs, and those on Medicare may have access challenges as they may be required to justify the branded therapies over established generic options.

Table 1 Ongoing Trials of CAR-T Therapies in AML

Interventions	Study Phase	Estimated N	NCT Number
CD19 CAR-T	2/3	10	NCT04257175
CD123/CLL1 CAR- T	2/3	20	NCT03631576
CD123 CAR-T	1/2	45	NCT04265963
CD123 CAR-T	1/2	40	NCT04272125
CD123 CAR-T	1	12	NCT04678336
CD123 CAR-T	1	12	NCT03766126
CD123 UCAR-T	1	59	NCT03190278
CD38 CAR-T	1/2	20	NCT04351022
CD33 CAR-T	1/2	34	NCT03971799
CLL-1 CAR T	1	18	NCT04219163

Notes: Source: ClinicalTrials.gov, accessed 31 January 2021.
Abbreviations: AML, acute myeloid leukemia; CAR-T, chimeric antigen receptor T-cell; NCT, National Clinical Trial; UCAR-T, universal chimeric antigen receptor T-cell.

In the coming years, the costs of genetic testing and novel therapies are likely to grow as more targeted therapies become available. As of January 2021, there were 621 ongoing interventional Phase 1 to Phase 3 studies in AML registered on ClinicalTrials.gov, over 450 of which are Phase 2 or phase 3. Among the ongoing studies, 99 evaluate biological treatments, 25 are transplantation studies, and the remaining 496 are evaluating drug treatments and combinations. While many of these studies involve new combinations of existing drugs, the innovative pipeline is strong. Table 2 presents ongoing phase 2 or phase 3 studies of treatments targeting specific biologically or genetically defined patient subgroups or molecular pathways.

Cost Drivers in Transition: Utilization of AML Treatment

The previous three major cost drivers discussed in this review—hospitalization/medical costs, HSCT/cellular therapies, and drug costs—relate to how advances in AML treatment may impact the costs of treating a patient with AML. The final category relates to how the evolution of AML treatment may impact the overall economic burden of AML by making high-cost treatment a possibility for a larger proportion of patients with AML.

Despite the availability of NIC options, a recent literature review found that up to one-third of patients in the US and Europe receive only best supportive care for AML,

Table 2 Ongoing Phase 2 and Phase 3 Trials of Therapies Targeting Biologically or Genetically Defined Patient Subgroups or Molecular Pathways in AML

Target Sub-Population	Treatment	AML Treatment Phase	NCT Number
FLT-3	Crenolanib	Maintenance	NCT02400255; NCT03258931
		RR AML	NCT03250338
	Crenolanib, Midostaurin	ND AML	NCT03258931
	Gilteritinib, Midostaurin	ND AML	NCT03836209; NCT03836209
	Gilteritinib	ND AML	NCT02752035; NCT04293562; NCT02997202
		Maintenance	NCT02927262
	Midostaurin	ND AML	NCT03379727; NCT03280030
	Quizartinib	ND and maintenance	NCT02668653
		ND AML	NCT04676243; NCT04209725
	Ibrutinib	RR AML	NCT03642236
	ASP2215	RR AML	NCT03182244; NCT02421939
	SKLB1028	RR AML	NCT04716114; NCT04015024
IDH-1/IDH-2	Ivosidenib/enasidenib	ND IC AML	NCT03839771; NCT04493164
	Enasidenib	RR AML	NCT04203316
	Ivosidenib	ND NIC AML	NCT03173248
	AG-221 (CC-90007)	RR AML	NCT02577406
	Olaparib	RR AML	NCT03953898
NPM1	Gemtuzumab ozogamicin	ND IC AML	NCT00893399
	Pembrolizumab	RR AML	NCT03769532
	Oral arsenic trioxide	AML	NCT04689815
	SNDX-5613	RR AML	NCT04065399

(*Continued*)

Table 2 (Continued).

Target Sub-Population	Treatment	AML Treatment Phase	NCT Number
CEBPA	HAD	ND IC AML	NCT04415008
HOX overexpression	Nintedanib	ND IC AML	NCT02665143
TP53	Decitabine	RR AML	NCT03063203
	Magrolimab	ND AML	NCT04778397
	APR-246 (eprenetapopt)	Maintenance	NCT03931291
CD33	Gemtuzumab ozogamicin	RR AML	NCT03839446
CD123+ or BPDCN-IPh-like+	Tagraxofusp	RR AML	NCT04342962
PD-1	Nivolumab	ND and RR AML AML in remission Maintenance	NCT02397720 NCT02275533 NCT02532231
		NIC RR AML	NCT03825367
	Visilizumab, azacitidine	RR AML	NCT04722952
	Tislelizumab + HMA	ND NIC AML	NCT04541277
	Pembrolizumab + decitabine Pembrolizumab + azacitidine Pembrolizumab + chemo Pembrolizumab + azacitidine +venetoclax	ND NIC and RR RR AML ND AML ND NIC AML	NCT03969446 NCT02845297 NCT04214249 NCT04284787
	Pembrolizumab	AML in remission	NCT02771197
	Camrelizumab (SHR-1210)	RR AML	NCT04353479
	Visilizumab	RR AML	NCT04722952
RARA pathway associated biomarker	SY-1425	AML	NCT02807558
TET2 mutations	Azacitidine	AML	NCT03397173

Note: Source: ClinicalTrials.gov, accessed 31 January 2021.
Abbreviations: AML, acute myeloid leukemia; BPDCN-IPh, blastic plasmacytoid dendritic cell neoplasm-like phenotype; CEBPA, CCAAT enhancer-binding protein alpha; FLT-3, fms-like tyrosine kinase 3; HAD, homoharringtonine + cytarabine + daunorubicin; HMA, hypomethylating agent; HOX, homeobox; IC, intensive chemotherapy; IDH, isocitrate dehydrogenase; NCT, National Clinical Trial; ND, newly diagnosed; NIC, non-intensive chemotherapy; NPM1, nucleophosmin-1; PD-1, programmed cell death-1; RARA, retinoic acid receptor alpha; RR, relapsed refractory; TET2, Tet methylcytosine dioxygenase 2; TP53, tumor protein 53.

with advanced age, comorbidities, and poor performance status as major factors in the decision not to administer active treatment.[31] There is, however, a growing trend to use broader criteria to assess fitness for treatment: the NCCN guidelines refer to "physiologic age" rather than chronological age, to avoid declining treatment to elderly patients who are likely to tolerate and benefit from treatment.[32]

New treatment options may increase the likelihood of physicians to offer anticancer treatment to older patients.

Glasdegib and venetoclax, for example, are approved specifically for use in elderly or unfit patients, based on pivotal trials in these populations.[33,34] CPX-351 is approved without age restriction following a pivotal trial demonstrating superior efficacy and comparable safety to conventional cytarabine + daunorubicin in patients age 60 to 75.[19] With increasing awareness of physiologic over chronological age, expanded options for targeted therapy with manageable safety profiles, and improved strategies for managing adverse effects of treatment, it is likely that

the proportion of treated patients will increase, leading to higher overall costs in AML.

As noted above, the NCCN guidelines include a variety of options for maintenance therapy in AML. The growing role of maintenance therapy in AML is likely to increase the overall number of patients receiving treatment, as patients would continue to be treated rather than waiting for relapse before starting treatment again. Regimens of IV cytarabine and daunorubicin with or without gemtuzumab ozogamicin are recommended by the NCCN for patients under 60 years of age who are eligible for intensive chemotherapy.[32] The EMA, but not the FDA, approved oral midostaurin as maintenance therapy on the basis of its phase 3 study (RATIFY; NCT00651261), which included the use of midostaurin in the induction, consolidation and maintenance settings (with progressively fewer patients completing each phase of treatment).[35] Maintenance with the hypomethylating agents, IV or oral azacitidine and IV decitabine, has also shown efficacy and is recommended by the NCCN guidelines.[6,36,37] With increasingly effective treatments, maintenance therapy may be of long duration: in the phase 3 trial of oral azacitidine, 71% of patients stayed on therapy for at least 6 months, while 49% were exposed for over 1 year.[37]

Likewise, there has been an expansion in the number of options for R/R AML, with the NCCN guidelines for R/R AML including oral therapies such as gilteritinib, enasidenib, ivosidenib, and venetoclax, and other treatments such as gemtuzumab ozogamicin.[6] These novel therapies have generally manageable toxicity profiles, making treatment for R/R AML a feasible option for a broader set of patients.

There are likely to be even more options for maintenance and R/R therapy in AML in upcoming years. As of January 2021, there were 14 ongoing studies registered with ClinicalTrials.gov investigating novel targeted therapies as maintenance. Furthermore, among the 621 ongoing interventional studies in AML, 171 were specifically in the R/R population; of these, 46 were phase 2 or phase 3 studies with primary completion dates ranging from 2020 to 2029. Table 3 presents a selection of studies in R/R AML that are expected to have primary results by 2023. With the number of existing and upcoming treatment options for R/R disease and maintenance therapy, patients plausibly could stay on therapy for several years, effectively converting AML to a disease that can be managed chronically. Such a scenario would have major implications in terms of the typical patient journey and associated costs.

Lastly, as life expectancy increases, there has been a modest uptick in AML incidence, from 3.4 per 100,000 persons in the US in 1975 to 4.3 per 100,000 in 2017.[2] Thus, the overall population of patients with AML has grown, contributing to the cumulative economic impact.

Table 3 Notable Ongoing Phase 2 and Phase 3 Studies in RR AML with Primary Results Expected by 2023

Intervention	Study Phase	Estimated N	Primary Completion Date	NCT Number
Uproleselan (GMI-1271) + IC	3	380	2022	NCT03616470
Crenolanib + IC	3	322	2021	NCT03250338
^{131I}apamistamab (Iomab-B)	3	150	2021	NCT02665065
Gilteritinib	3	318	2021	NCT03182244
Ibrutinib + IC ± sorafenib	2/3	122	2022	NCT03642236
Alvocidib	2	134	2022	NCT03969420
Cladribine + idarubicin + cytarabine + quizartinib	2	86	2022	NCT04047641
Isatuximab + IC	2	96	2021	NCT03860844
Liposomal daunorubicin-cytarabine + venetoclax	2	52	2021	NCT03629171
Liposomal daunorubicin-cytarabine + gemtuzumab ozogamicin	2	50	2022	NCT03672539
Nivolumab + azacitidine ± ipilimumab	2	182	2022	NCT02397720
Olaparib	2	94	2022	NCT03953898
Pembrolizumab + azacitidine	2	67	2021	NCT02845297
Venetoclax + decitabine	2	400	2023	NCT03404193

Note: Source: ClinicalTrials.gov, accessed 31 January 2021.
Abbreviations: AML, acute myeloid leukemia; IC, intensive chemotherapy; NCT, National Clinical Trial.

Opportunities to Deliver Value in AML

The rapid evolution of treatment options for AML leaves patients and physicians with notably more decisions than in the past, while presenting payers with more costs and offsets to consider. Where previously the AML treatment pathway hinged on age and fitness, it now must begin with genetic profiling followed by consideration of a range of conventional and novel regimens based on patient factors and preferences. Patients may choose to undergo induction therapy followed by HSCT, or perhaps their initial therapy may induce a sufficient response to allow a direct transition to maintenance therapy. Those who undergo HSCT may require less time in the hospital for the procedure as methods have improved, or they may, in the near future, undergo alternative forms of stem cell transplant or cellular therapies. Patients who eventually develop R/R disease can consider a variety of targeted options, many of which can be self-administered at home. As the number of drugs with different mechanisms increases, there may be an opportunity to sequence treatments in AML as patients experience longer-term survival even in the absence of cure. These potential survival gains and clinical benefits will require investment on the part of payers, and it will be incumbent on those developing novel treatments to demonstrate the economic value with compelling evidence.

Historically, hospitalization has been a major cost driver in AML. While some evolving treatments, such as stem cell transplant and cellular therapies, include substantial hospital or medical costs, the major driver of costs in AML in the next decade is likely to be the rapid uptake of a range of novel targeted therapies. The use of these therapies in multiple phases of treatment—newly diagnosed, maintenance, and R/R—will prolong the amount of time that patients are able to stay on treatment. With survival gains will come increases in the overall cost burden of AML. The AML landscape may develop in a manner similar to what has been observed in recent years in multiple myeloma, where a treatment "desert" transformed over two decades into an opportunity to sequence patients through multiple lines of therapy while maintaining quality of life.

One opportunity to deliver value in AML treatment is the use of oral and other self-administered therapies and keeping patients in outpatient settings when feasible. While these approaches are likely to reduce costs for payers, they may shift a greater cost burden to patients, particularly in the US where novel oral drugs incur substantial copayments. The advanced age of the typical patient with AML means that they are unlikely to be employed and may be unable to cover these costs.

Patients receiving outpatient anticancer therapy still require substantial healthcare resources due to the toxicity profiles of most available treatments. Outpatient regimens such as glasdegib + LDAC or venetoclax + azacitidine are myelosuppressive, and patients may require transfusions and other supportive measures.[33,34] Therefore, another way to deliver value in AML treatment is to develop treatments and regimens with improved safety profiles, with the goal of reduced spending on monitoring and treatment of adverse events. The targeting of treatments based on biological factors, while requiring investment in molecular testing, can help to focus spending on treatments most likely to be effective.

Despite the opportunities for cost offsets with innovative therapies, it would be naïve to imply that novel therapies for AML will ultimately reduce healthcare costs. The value of most cancer treatments lies in the opportunity to prolong survival while maintaining quality of life. In undertaking a holistic cost-benefit assessment of AML treatments, one must consider whether the costs translate into measurable outcomes such as increased survival, diminished symptom burden, reduced need for emergency and inpatient treatment, and decreased strain on caregivers. Such a value assessment can be used to determine the appropriate costs of innovative therapies.

Conclusion

AML is a relatively rare but costly cancer, currently characterized by high-cost intensive treatments that often require hospitalization, alongside a substantial fraction of patients who receive little or no anticancer treatment due to age or performance status. The past several years have seen a dramatic increase in the number of treatment options, and research is ongoing to further expand the therapeutic landscape in AML. While certain advances, such as increased use of oral and outpatient therapies, could potentially reduce costs, the overall economic impact of AML is likely to increase as more patients are eligible for novel therapies across several phases from induction to maintenance to R/R disease. These novel therapies have the potential to deliver value in the form of improved efficacy, safety, and convenience. In the coming years, value assessments will form the basis for price

negotiations as payers determine how to cover a longer and more complex treatment pathway in AML.

Disclosure

KS and AF are both employees of Purple Squirrel Economics, a Cytel company, which acts as a consultant for various pharmaceutical clients. The authors report no other conflicts of interest in this work.

References

1. PDQ Adult Treatment Editorial Board. PDQ Cancer Information Summaries. In: *Adult Acute Myeloid Leukemia Treatment (PDQ®): Health Professional Version*. National Cancer Institute (US); 2002.
2. National Cancer Institute. *Acute Myeloid Leukemia - Cancer Stat Facts*. SEER; 2013.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394–424. doi:10.3322/caac.21492
4. Carter JL, Hege K, Yang J, et al. Targeting multiple signaling pathways: the new approach to acute myeloid leukemia therapy. *Signal Transduct Target Ther*. 2020;5.
5. Shallis RM, Wang R, Davidoff A, Ma X, Zeidan AM. Epidemiology of acute myeloid leukemia: recent progress and enduring challenges. *Blood Rev*. 2019;36:70–87. doi:10.1016/j.blre.2019.04.005
6. Pollyea DA, Bixby D, Perl A, et al. NCCN Guidelines Insights: acute Myeloid Leukemia, Version 2.2021: featured Updates to the NCCN Guidelines. *J Nat Comprehen Cancer Network*. 2021;19(1):16–27. doi:10.6004/jnccn.2021.0002
7. Halpern AB, Walter RB. Practice patterns and outcomes for adults with acute myeloid leukemia receiving care in community vs academic settings. *Hematology Am Soc Hematol Educ Program*. 2020;2020(1):129–134. doi:10.1182/hematology.2020000097
8. Moher D, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ*. 2009;339:b2535–b2535. doi:10.1136/bmj.b2535
9. Centre for Reviews and Dissemination. *Systematic Reviews: CRD's Guidance for Undertaking Reviews in Health Care*; 2009.
10. Pandya BJ, Chen -C-C, Medeiros BC, et al. Economic and Clinical Burden of Acute Myeloid Leukemia Episodes of Care in the United States: a Retrospective Analysis of a Commercial Payer Database. *J Manag Care Spec Pharm*. 2020;26(7):849–859. doi:10.18553/jmcp.2020.19220
11. Irish W, Ryan M, Gache L, Gunnarsson C, Bell T, Shapiro M. Acute myeloid leukemia: a retrospective claims analysis of resource utilization and expenditures for newly diagnosed patients from first-line induction to remission and relapse. *Curr Med Res Opin*. 2017;33(3):519–527. doi:10.1080/03007995.2016.1267615
12. Meyers J, Yu Y, Kaye JA, Davis KL. Medicare fee-for-service enrollees with primary acute myeloid leukemia: an analysis of treatment patterns, survival, and healthcare resource utilization and costs. *Appl Health Econ Health Policy*. 2013;11(3):275–286. doi:10.1007/s40258-013-0032-2
13. Reyes C, Engel-Nitz NM, DaCosta Byfield S, et al. Cost of Disease Progression in Patients with Chronic Lymphocytic Leukemia, Acute Myeloid Leukemia, and Non-Hodgkin's Lymphoma. *Oncologist*. 2019;24(9):1219–1228. doi:10.1634/theoncologist.2018-0019
14. Marsà A, Ascanio M, Diaz-García J, Darbà J. Epidemiology, management, and economic impact of acute myeloid leukemia and myelodysplastic syndrome in Spain at the hospital level: a claims database analysis. *J Med Econ*. 2020;1–8. doi:10.1080/13696998.2019.1678170
15. Leunis A, Blommestein HM, Huijgens PC, Blijlevens NMA, Jongen-Lavrencic M, Uyl-de Groot CA. The costs of initial treatment for patients with acute myeloid leukemia in the Netherlands. *Leuk Res*. 2013;37(3):245–250. doi:10.1016/j.leukres.2012.09.018
16. Hernlund E, Redig J, Rangert Derolf A, et al. Costs per Treatment Phase for AML Patients Receiving High-Dose Chemotherapy in Sweden. *Blood*. 2019;134(Supplement_1):2154. doi:10.1182/blood-2019-127957
17. Patel SA, Gerber JM, User's A. Guide to Novel Therapies for Acute Myeloid Leukemia. *Clin Lymphoma Myeloma Leuk*. 2020;20(5):277–288. doi:10.1016/j.clml.2020.01.011
18. Yu J, Li Y, Zhang D, Wan D, Jiang Z. Clinical implications of recurrent gene mutations in acute myeloid leukemia. *Exp Hematol Oncol*. 2020;9(1):4. doi:10.1186/s40164-020-00161-7
19. Lancet JE, Uy GL, Cortes JE, et al. CPX-351 (cytarabine and daunorubicin) Liposome for Injection Versus Conventional Cytarabine Plus Daunorubicin in Older Patients With Newly Diagnosed Secondary Acute Myeloid Leukemia. *J Clin Oncol*. 2018;36(26):2684–2692. doi:10.1200/JCO.2017.77.6112
20. Kubal TE, Salamanca C, Komrokji RS, et al. Safety and feasibility of outpatient induction chemotherapy with CPX-351 in selected older adult patients with newly diagnosed AML. *JCO*. 2018;36(15_suppl):e19013. doi:10.1200/JCO.2018.36.15_suppl.e19013
21. Mabrey FL, Gardner KM, Shannon Dorcy K, et al. Outpatient intensive induction chemotherapy for acute myeloid leukemia and high-risk myelodysplastic syndrome. *Blood Adv*. 2020;4(4):611. doi:10.1182/bloodadvances.2019000707
22. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447.
23. Schuurhuis GJ, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. 2018;131(12):1275–1291. doi:10.1182/blood-2017-09-801498
24. Loke J, Malladi R, Moss P, Craddock C. The role of allogeneic stem cell transplantation in the management of acute myeloid leukaemia: a triumph of hope and experience. *Br J Haematol*. 2020;188(1):129–146. doi:10.1111/bjh.16355
25. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood*. 2013;122(4):491–498. doi:10.1182/blood-2013-02-453175
26. Maung KK, Horwitz ME. Current and future perspectives on allogeneic transplantation using ex vivo expansion or manipulation of umbilical cord blood cells. *Int J Hematol*. 2019;110(1):50–58. doi:10.1007/s12185-019-02670-6
27. Cohen S, Roy J, Lachance S, et al. Hematopoietic stem cell transplantation using single UM171-expanded cord blood: a single-arm, phase 1–2 safety and feasibility study. *Lancet Haematol*. 2020;7(2):e134–e145. doi:10.1016/S2352-3026(19)30202-9
28. Mardiana S, Gill S. CAR T Cells for Acute Myeloid Leukemia: state of the Art and Future Directions. *Front Oncol*. 2020;10. doi:10.3389/fonc.2020.00697
29. Kansagra A, Farnia S, Majhail N, Expanding access to chimeric antigen receptor T-cell therapies: challenges and opportunities. *Am Soc Clin Oncol Educ Book*. 2020;40:e27–e34. doi:10.1200/EDBK_279151
30. Sarkar RR, Gloude NJ, Schiff D, Murphy JD. Cost-Effectiveness of Chimeric Antigen Receptor T-Cell Therapy in Pediatric Relapsed/Refractory B-Cell Acute Lymphoblastic Leukemia. *J Natl Cancer Inst*. 2019;111(7):719–726. doi:10.1093/jnci/djy193
31. Sikirica S. *Patterns of Undertreatment Among Patients with Acute Myeloid Leukemia (AML) Not Receiving Standard Intensive Induction Chemotherapy*. ASH; 2020.
32. National Comprehensive Cancer Network, Inc. *NCCN® Clinical Practice Guidelines in Oncology (NCCN Guidelines®) for Acute Myeloid Leukemia*; 2021.

33. DiNardo CD, Jonas BA, Pullarkat V, et al. Azacitidine and Venetoclax in Previously Untreated Acute Myeloid Leukemia. *N Engl J Med.* 2020;383(7):617–629. doi:10.1056/NEJMoa2012971

34. Cortes JE, Heidel FH, Hellmann A, et al. Randomized comparison of low dose cytarabine with or without glasdegib in patients with newly diagnosed acute myeloid leukemia or high-risk myelodysplastic syndrome. *Leukemia.* 2019;33(2):379–389. doi:10.1038/s41375-018-0312-9

35. Stone RM, Mazzola E, Neuberg D, et al. Phase III open-label randomized study of cytarabine in combination with amonafide L-malate or daunorubicin as induction therapy for patients with secondary acute myeloid leukemia. *J Clin Oncol.* 2015;33(11):1252–1257. doi:10.1200/JCO.2014.57.0952

36. Foran JM, Sun Z, Claxton DF, et al. Maintenance Decitabine (DAC) Improves Disease-Free (DFS) and Overall Survival (OS) after Intensive Therapy for Acute Myeloid Leukemia (AML) in Older Adults, Particularly in FLT3-ITD-Negative Patients: ECOG-ACRIN (E-A) E2906 Randomized Study. *Blood.* 2019;134 (Supplement_1):115. doi:10.1182/blood-2019-129876

37. Wei AH, Döhner H, Pocock C, et al. Oral Azacitidine Maintenance Therapy for Acute Myeloid Leukemia in First Remission. *N Engl J Med.* 2020;383(26):2526–2537. doi:10.1056/NEJMoa2004444

Primary Pancreatic Lymphoma: Recommendations for Diagnosis and Management

Davide Facchinelli, Enrico Boninsegna, Carlo Visco & Cristina Tecchio

REVIEW

Primary Pancreatic Lymphoma: Recommendations for Diagnosis and Management

Davide Facchinelli[1]
Enrico Boninsegna[2]
Carlo Visco[3]
Cristina Tecchio[3]

[1]Hematology Unit, Ospedale San Bortolo, Vicenza, Italy; [2]Department of Radiology, Azienda Socio Sanitaria Territoriale, Mantova, Italy; [3]Hematology and Bone Marrow Transplant Unit, Department of Medicine, University of Verona, Verona, Italy

Video abstract

Point your SmartPhone at the code above. If you have a QR code reader the video abstract will appear. Or use:
https://youtu.be/xyNm7FAu4p8

Correspondence: Davide Facchinelli
Hematology Unit, Ospedale San Bortolo, Vicenza, Italy
Email davide.facchinelli@aulss8.veneto.it

Background: Primary pancreatic lymphoma (PPL) is a rare disease representing 0.1% of all malignant lymphomas, which lacks well-defined diagnostic and therapeutic protocols. We conducted a systematic review to analyze demographic, diagnostic and therapeutic features of PPL.

Methods: This review identified small series and single case reports. Sources were MEDLINE, PubMed, and the Cochrane library from January 2001 to December 2020. Data were screened, extracted and the risk of bias analyzed by three independent reviewers.

Results: A total of 107 eligible papers (17 small series, 90 single case reports) describing 266 patients were identified. Patients had a median age of 53.1 (range 3–86) years and were males in 64.6% of cases. Abdominal pain and jaundice were the most common presenting symptoms, affecting 75.3% and 41.8% of patients, respectively. PPL had a median size of 60.6 mm (range 16–200) and it was localized in the pancreatic head in 63.7% of cases. At diagnosis most patients underwent ultrasonography followed by computed tomography. PPL typically showed low echogenicity, and lower contrast enhancement than solid tumors. Histopathological specimens were obtained by percutaneous or endoscopic biopsies in 47.7% of patients; abdominal surgery was performed in 33.5% of cases. Overall, diffuse large B-cell lymphoma was the most frequent histological diagnosis (53.6%). However, patients aged <18 years were affected by Burkitt lymphoma in 52.4% of cases. Most patients (53.6%) received immunochemotherapy (IC) or IC plus radiotherapy (14%). Demolitive surgery appeared to be associated with impaired survival. Central nervous system (CNS) relapse or progression was observed in 20% of patients.

Conclusion: PPL is a rare entity, with some peculiar features at modern imaging. For diagnostic purposes percutaneous or endoscopic biopsies might be preferable, as opposed to surgery. No definite data is available about the optimal treatment, which should be tailored on the histological type and associated with CNS prophylaxis.

Keywords: primary pancreatic lymphoma, diffuse large B cell lymphoma, Burkitt lymphoma

Introduction

Primary Pancreatic Lymphoma (PPL) is a rare disease representing only 0.1% of malignant lymphomas, 0.6% of extranodal lymphomas, and 0.2% of all pancreatic tumors.[1,2] On the contrary, secondary pancreatic involvement occurs quite commonly in lymphomas, especially in the presence of widespread nodal or extranodal disease, and may be observed in up to 30% of cases.[3]

Over the years, different definitions of PPL have been suggested.[3,4] More recently, the World Health Organization (WHO) has provided the following diagnostic criteria: i) the bulk of disease has to be located in the pancreas, ii) although

adjacent lymph nodes involvement and distant spread may exist, the primary clinical presentation has to involve the pancreatic gland.[5]

PPL can develop at any age, but usually affects elderly patients, with a male prevalence. Immunosuppression, related to HIV infection or solid organ transplantation, may favor its development.[6] From a clinical standpoint, the main presenting symptom of PPL is the abdominal pain. However, other common clinical findings include systemic symptoms (fever, night sweats and weight loss), jaundice, pancreatitis and/or gastric or duodenal obstruction.[2,7,8] Overall, these symptoms resemble those of other pancreatic diseases, often resulting in diagnostic problems.[9,10] PPL can be located in any portion of the gland, but it mainly involves the pancreatic head, which contains the greatest amount of lymphoid tissue.[9] Histopathological analysis is usually consistent with diffuse large B cell lymphoma (DLBCL) not otherwise specified (NOS); nevertheless, other subtypes of lymphomas, including marginal zone lymphoma (MZL) or follicular lymphoma (FL), may be detected.[5,9,10] Anecdotal cases of Hodgkin (HL) and T cell non Hodgkin lymphoma (T-NHL) have been also described.[11,12]

A diagnosis of PPL can be obtained through percutaneous/endoscopic biopsy, exploratory laparotomy or demolition surgery.[7] With regard to treatment, no definite guidelines can be drawn from literature. In fact, most reports on PPL are retrospective and describe undersized and heterogeneously treated groups of patients.

The aim of this review was to retrieve, analyze and summarize data obtained from case collections and single case reports published in the last two decades.

The final goal was to identify specific characteristics of this rare lymphoma entity in order to provide evidence to establish future diagnostic and therapeutic guidelines.

Methods

This systematic review is reported in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analysis guidelines.[13] We conducted an extensive systematic literature search since 01 January 2000 through 31 December 2020. Sources were PubMed, Medline, and Cochrane library. We deemed eligible all types of reports (case collections and single cases). This search was limited to studies published in English.

The eligibility criteria were (a) adult and pediatric population (b) pancreatic lymphomas fulfilling diagnostic criteria for PPL according to WHO.[5]

All titles were downloaded into an Endnote library and duplicated removed automatically. Three reviewers (DF, EB, CV) screened the titles and abstracts for eligibility. Then full text was screened again. A senior reviewer (CT) resolved any disagreement. For each article, at least one reviewer extracted the following information: demographics, presenting symptoms, diagnostic methods, and histological classification. Whenever available data about treatment and outcome were also retrieved.

A total of 107 papers reporting on 266 patients were deemed eligible.[10–12,14–113] Figure 1 provides the PRISMA flow diagram.

Percentages regarding each item analyzed were calculated based on the number of available data as specified in each table.

Results
Demographics and Clinical Characteristics

Among the 266 patients retrieved from literature, 172 (64.6%) were males, 94 (35.4%) females. The mean age was 53.1 (range 3–86) years. Twenty-one (7.9%) patients were aged less than 18 years.

As reported in Table 1, abdominal pain (75.4%) was the most common presenting symptom, followed by jaundice (41.8%), and B symptoms such as fever, night sweats and weight loss (31.9%). Acute pancreatitis and gastric or duodenal obstruction were the first clinical presentation in 25.9% and 10.7% of patients, respectively. Two patients were immunosuppressed after a simultaneous pancreas-kidney transplant.[61,107]

Laboratory Tests

Pretreatment laboratory tests were available for a minority of patients. Serum levels of lactate dehydrogenase (LDH) were elevated in 50.4% of patients, carbohydrate antigen 19–9 (CA 19–9) in 26.9%. The blood count showed anemia in 20.9% of patients, leukocytosis (>10.000/mmc) in 18.3%, and thrombocytopenia in 4.8%. Laboratory tests are reported in Table 1.

Imaging

Imaging features were available in 256 patients (Table 2). PPL had a mean size of 60.6 mm (range 16–200) and it was located in the pancreatic head in 163 (63.7%) cases. Early-stage Ann-Arbor disease (I–II) was diagnosed in 76% of patients, advanced (III–IV) in the remaining 24%.
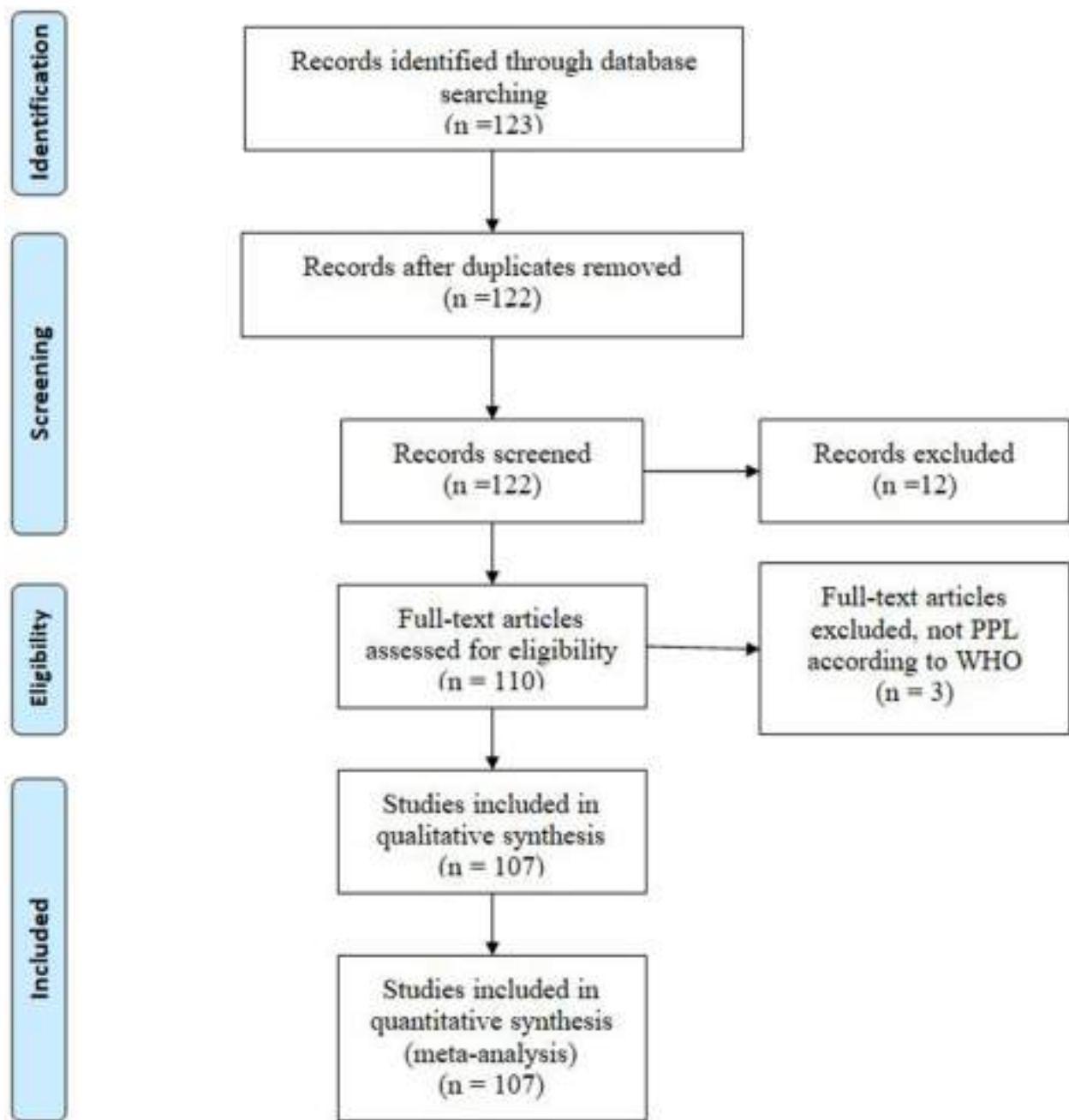
Figure 1 PRISMA flow diagram.

Ultrasonography (US) was performed in 214/256 patients (83.6%) at disease onset. Computed tomography (CT) was performed in 249/256 cases (97.3%), often following US; multidetector technology and contrast agent enhanced protocols were always used.

Magnetic Resonance Imaging (MRI) was performed in 36/256 cases (14.0%), nuclear medicine, in particular positron emission tomography-CT (PET-CT) with evaluation

of 18fluoro-2-deoxy-d-glucose (^{18}FDG) intake, in 51/256 patients (19.9%).

US is extensively used since it enables a rapid evaluation of pancreas size, borders, echostructure and vessels.[54,92] The most common finding is enlargement of the parenchyma, focal or diffuse; the affected pancreas presents also lower echogenicity, appearing darker than normal. At color-Doppler evaluation major vessels can be surrounded by

Table 1 Patient Characteristics

Characteristics	n (%)
Presenting symptoms	
Abdominal pain	175/232 (75.4)
Jaundice	100/239 (41.8)
B symptoms	77/241 (31.9)
GI obstruction	24/225 (10.7)
Acute pancreatitis	56/218 (25.9)
Laboratory values	
LDH > upper limits	65/129 (50.4)
CA 19–9 > upper limits	32/119 (26.9)
Anemia	19/91 (20.9)
Thrombocytopenia	4/84 (4.8)
WBC > 10.000/mmc	17/93 (18.3)

Abbreviations: GI, gastrointestinal; LDH, lactate dehydrogenase; CA, carbohydrate antigen; WBC, white blood cells.

Table 2 Imaging Characteristics

Imaging	n (%)
Imaging method	
US	214/256 (83.6)
CT	249 (97.3)
MRI	36 (14.1)
PET-CT	51 (19.9)
Pancreatic localization	
Head	163/256 (63.6)
Body-tail	76 (29.6)
All gland	17 (6.6)
Tumor diameter, mean (range)	60.6 mm (range 16–200 mm)

Abbreviations: US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; PET-CT, positron emission tomography-CT.

pathological tissue, but always patent (contrary to other solid neoplasms like adenocarcinoma).[110,114] When a pancreatic disease is identified by US a second line radiological examination, usually CT, is mandatory.

At CT, PPL presents as a large solid lesion, potentially involving the whole gland; contrast enhancement is lower than healthy pancreas. Fat stranding in the peri-pancreatic region is typical; dilation of the bile and pancreatic duct is not common, contrary to pancreatic adenocarcinoma. Vascular encasement can be observed, without irregularities in vessels wall.[51,88,114]

MRI can be used if CT is inconclusive or to avoid radiation exposure in younger patients. Involved parenchyma shows lower signal intensity on T1-weighted images and higher signal on T2-weighetd images compared to healthy pancreas; diffusion-weighted sequences present

very high sensitivity to depict lymphomatous tissue and lymph nodes.[114] PET-CT is indicated to assess the metabolic activity of the primary neoplasm and to depict involved lymph nodes in the whole body.[98]

Diagnostic Procedures and Histological Assessment

Information about diagnostic procedures was available in 224 patients (Table 3). Histopathological specimens were obtained mostly by percutaneous or endoscopic biopsies in 68 (30.3%) and 39 (17.4%) cases, respectively. Fine needle aspiration (FNA) was performed in 33 (14.8%) cases, abdominal surgery in 75 (33.5%) (26 surgical biopsy, 49 demolition surgery). Demolition surgery consisted mainly of Whipple procedure and spleno-pancreasectomy. In the remaining 9 (4.0%) patients the diagnosis was autoptic. The main histological diagnosis was DLBCL in 143 (53.6%) patients, followed by FL in 26 (9.8%) and BL in 20 (7.5%). In 31 (11.6%) cases the histology was not specified. Overall, this data is in agreement

Table 3 Diagnostic Methods, Histology and Clinical Stage

Diagnosis	n (%)
Diagnostic method	
Percutaneous biopsy	68/224 (30.3)
Endoscopic biopsy	39 (17.4)
FNA	33 (14.8)
Surgical biopsy	26 (11.6)
Demolition surgery	49 (21.9)
Autopsy	9 (4.0)
Histology	
DLBCL	143/266 (53.6)
FL	26 (9.8)
BL	20 (7.5)
High grade B cell lymphoma	14 (5.2)
T-NHL	18 (6.7)
HL	4 (1.5)
LPL	4 (1.5)
MZL	2 (0.7)
SLL	2 (0.7)
PTLD	2 (0.7)
Lymphoblastic lymphoma	1 (0.4)
Unknown	31 (11.6)
Clinical stage (Ann Arbor)	
I–II	152/200 (76)
III–IV	48/200 (26)

Abbreviations: FNA, fine needle aspiration; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; NHL, non Hodgkin lymphoma; HL, Hodgkin lymphoma; LPL, lymphoplasmacytic lymphoma; MZL, marginal zone lymphoma; SLL, small lymphocytic lymphoma; PTLD, post-transplant lymphoproliferative disorder.

with that reported by a recent paper by Mukhija et al[115] evaluating 835 patients affected by pancreatic lymphomas, though the analysis was not restricted to the PPLs only.

According to data published in literature, percutaneous or endoscopic biopsies are reliable and scarcely invasive. On the other hand, despite providing a significant diagnostic accuracy for adenocarcinoma (sensitivity of 86.8% [95% CI 85.5–87.9] and specificity of 95.8% [95% CI 94-6-96.7]),[116] endoscopic ultrasound guided fine needle aspiration (EUS-FNA) is a poor diagnostic tool for lymphoma.[117] Immunophenotypic analysis may be useful to integrate cytology.[118,119] In a study involving 11 patients with PPL, cytology alone revealed a correct diagnosis in 28% compared to 100% by integrated immunophenotype.[118] However, tissue architecture in addition to cytomorphology is crucial in the diagnosis of lymphoma. Overall, a non-invasive diagnostic approach is preferable to demolition surgery, which was associated with a higher mortality rate.[10,34,51,120] Figure 2 shows the temporal variation in biopsy techniques.

Treatment and Pattern of Relapse

Information about treatment was available in 207 of the 266 patients retrieved from literature (Table 4). The initial treatment consisted of immunochemotherapy (IC) alone in 111 patients (53.6%), IC plus radiotherapy (RT) in 29 (14.0%), RT alone in 1 case (0.5%), and demolition surgery in 59 (28.5%). Surgery alone was reserved to 15 patients, it was followed by IC in 39, RT in 3 and both IC and RT in 2 cases. Seven patients (3.4%) were not treated at all.

There is no consensus on the ideal treatment approach for PPL patients; studies from the pre-rituximab era suggested an aggressive local management of disease with demolition surgery.[3,34] In contrast, more recent papers suggested a less invasive approach with IC as the cornerstone of treatment.[121,122] There is no consensus on the role of RT in patients affected by PPL.[39]

Among 169 patients for whom a follow up was provided, 30 (17.7%) presented a disease relapse after a median time of 15 months (range 2–108 months).[10,19,28,33,49,51,53,64,73,101] Interestingly, 6 relapses (20%) (2 DLBCL, 2 BL, 1 T-NHL, and 1 high grade B cell lymphoma) involved the CNS.

PPL in the Pediatric Population

Our analysis identified 21 cases of PPL in patients under 18 years of age.[15,24,30,35,37,51,55,67,70,81,82,84,87,92,96,99–102] The mean age was 10.3 (range 3–16) years, with most of patients (19, 90.5%) being males (Table 5).

As in adults, the main presenting symptom was abdominal pain. The percentage of jaundice was higher than in adults (61.9% vs 39.9%), while the number of patients aged <18 years with B symptoms was very low (9.5% vs 34.1%). Interestingly 10 patients (47.6%) had an acute pancreatitis at disease onset.

Laboratory tests, compared to adults, showed elevated LDH values in the large majority of patients (80% vs 47.9%), while CA 19–9 never increased (0% vs 27.6%).
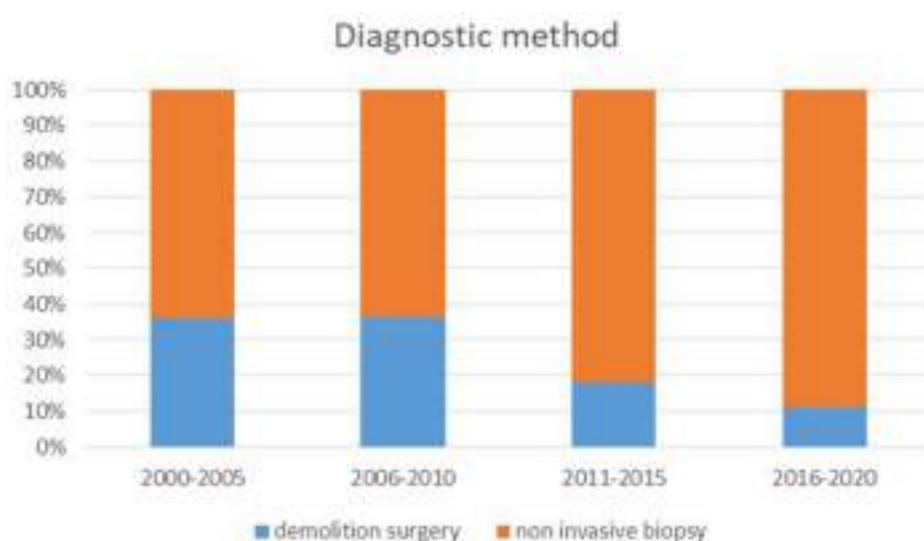
Figure 2 Temporal variation in biopsy techniques.

Table 4 Patient Survival According to Treatment and Histology

	Alive		Deceased	
	n (%)	OS, Months (Range)	n (%)	TTD, Months (Range)
Treatment*				
DS	4 (50)	11 (3–19)	4 (50)	2.5 (1–6)
DS-IC	13 (76.47)	37.8 (4–160)	4 (23.53)	13.5 (8–27)
DS-RT	2 (100)	34 (6–62)	/	/
DS-IC-RT	2 (100)	44.5 (24–65)	/	/
IC	26 (74.28)	34.7 (5–192)	9 (25.72)	13.4 (1–88)
IC-RT	15 (78.94)	45.4 (2–128)	4 (21.06)	36.5 (9–67)
Histology****				
DLBCL	48 (68.57)	39.3 (2–132)	22 (31.43)	18.9 (1–88)
FL	11 (84.61)	31.4 (6–62)	2 (15.39)	67 (63–72)
T-NHL	3 (23.07)	10.3 (4–15)	10 (76.93)	4.3 (0–8)
BL	8 (66.66)	63.25 (8–192)	4 (33.34)	2.9 (1–7)
HGBCL	3 (50)	34 (3–94)	3 (50)	10.3 (8–12)

Notes: *Data available in 83 patients; **data available in 113 patients.
Abbreviations: OS, overall survival; TTD, time to death; DS, demolition surgery; IC, Immuno-chemotherapy; RT, radiotherapy; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; NHL, non Hodgkin lymphoma; BL, Burkitt lymphoma; HGBCL, high grade B cell lymphoma.

Table 5 PPL Characteristics in Pediatric vs Adult Patients

Characteristics	Patients <18 Year n (%)	Patients >18 Years n (%)
Presenting symptoms		
Abdominal pain	16/21 (76.2)	159/211 (75.4)
Jaundice	13 (61.9)	87/218 (39.9)
B symptoms	2 (9.52)	75/220 (34.1)
GI obstruction	1 (4.76)	23/204 (11.3)
Acute pancreatitis	10 (47.6)	55/197 (27.9)
Laboratory values		
LDH > upper limits	8/10 (80)	57/119 (47.9)
CA 19–9 > upper limits	0/3 (0)	32/116 (27.6)
Pancreatic localization		
Head	13/21 (61.9)	150/235 (63.8)
Body-tail	2 (9.5)	74/235 (31.5)
All gland	6 (28.6)	11 (4.7)
Histology		
DLBCL	5/21 (23.8)	138/245 (56.3)
BL	11 (52.4)	9 (3.7)
High grade B cell lymphoma	1 (4.8)	13 (5.3)
T-NHL	2 (9.5)	17 (6.9)
Unknown	2 (9.5)	29 (11.8)
Clinical stage (Ann Arbor)		
I–II	5/13 (38.5)	147/187 (78.6)
III–IV	8 (61.5)	40/187 (21.4)

Abbreviations: GI, gastrointestinal; LDH, lactate dehydrogenase; CA 19–9, carbohydrate antigen; WBC, white blood cells; DLBCL, diffuse large B cell lymphoma; BL, Burkitt lymphoma; NHL, non Hodgkin lymphoma.

The mean size was similar (56.6 mm vs 61.1 mm) and also the pancreatic location, with a predilection for the pancreatic head (61.9% vs 63.8%).

Interestingly, in the pediatric cohort the main histological diagnosis was BL in 11 (52.4%), DLBCL in 5 (23.8%) patients.

Ng et al[123] reviewed the imaging findings of 80 children affected by NHL, with pancreatic involvement being present in 3 cases (3.75%). Similarly, Vade and Blane[124] reviewed the diagnostic imaging of 19 pediatric patients with BL and found 2 children with pancreatic involvement (10%).

The initial treatment consisted of IC alone in 16 patients (88.9%), and chemotherapy after demolition surgery in 2 (11.2%). Information about follow-up was available in 15 patients only. Fourteen/15 (93.3) reached a complete remission (CR) with first line therapy and one patient had a CNS relapse during the observation period.[101] These 2 patients were rescued with high dose chemotherapy plus autologous stem cell transplantation. With a median follow-up of 56.43 (range 8–132) months all patients were alive and in CR.

Discussion

This study presents data (diagnosis, histology, treatment and outcome) retrieved from 107 papers published from 2000 to 2020 on PPL.

As compared to pancreatic adenocarcinoma, which usually manifests in the 60- to 80-year-old age group,[125] PPL is usually diagnosed in younger adults (mean age 53 years). As previously described, the presentation of PPL may overlap with the onset of other neoplastic or inflammatory pancreatic diseases.[126,127]

Interestingly, in spite of symptoms and radiological findings (pancreatic head involvement) overlapping those of pancreatic ductal adenocarcinoma and autoimmune pancreatitis, some findings may indicate a diagnosis of PPL. For instance, a relatively large tumor size (>60 mm) together with the presence of distant lymph nodes at radiological assessment may suggest a diagnosis of lymphoma.[114] Unfortunately, few reports reported laboratory data, so it is not clear whether a elevate LDH value associated with CA 19–9 within the normal range may suggest a diagnosis of PPL, as previously indicated by our group.[51] Concerning the diagnostic approach, in more than half of cases reported in literature, tumor samples were collected through a noninvasive procedure: transcutaneous biopsy in 30.3%, endoscopic in 17.4% and FNA in 14.8%. However, in a significant number of patients, a surgical biopsy (11.6%) or a demolition surgery (21.9%) were performed. Worth of note, demolitive surgery was more frequently performed in the earlier time frame of our analysis, while noninvasive methods were preferred in most recent years (Figure 2). Finally, in 4.0% the diagnosis was obtained post-mortem.

In regard to histology, PPL was mostly classified as DLBCL (53.6%), FL (9.8%) and BL (7.5%). However, other types of lymphoma were described, including a non-negligible number of T-NHL (6.7%) and 5.2% of high-grade B cell lymphomas. Therefore, an accurate histological diagnosis should be obtained in order to provide patients with the best available treatment. It is interesting to note that among the 21 (7.9%) patients aged <18 years, BL was the prevalent histological type (52.4%). Unfortunately, a detailed histological classification according to the new 2016 WHO classification of lymphomas[128] was difficult to establish based on data reported by most of the articles here revised.

The lack of large PPL study series hampers definitive conclusion about the optimal treatment, which should rely on the histological subtype. According to our revision, chemotherapy with or without rituximab was the standard of care in most patients (53.6%), sometimes associated to RT (14.0%) or following diagnostic debulking surgery (18.9%). Overall, 26.1% of patients underwent a probably unnecessary surgical treatment.

Similarly to what has been described in the literature for other extranodal lymphomas,[129] the recurrence rate was high, at least according to the larger case series (23.52% and 34.21%, respectively).[10,51] Extranodal lymphomas may have different patterns of relapse depending on the tissue/organ involved. The CNS-International Prognostic Index (IPI) considers some primitive lymphoma sites (kidney and adrenal gland) as at increased risk of CNS relapse without taking into account pancreas.[130] Importantly, according to this literature search, in agreement with our previous findings,[51] patients affected by PPL had a relatively high incidence of CNS relapses (20% of the relapses reported). Obviously, no definitive conclusions can be drawn given the relatively small number of patients considered, and their heterogeneity in regard to histology and therapy received.

In conclusion, PPL represents a rare and challenging disease with relatively non-specific symptoms. Most PPLs are DLBCL in adults, BL in children. According to literature, a noninvasive approach should be the preferred diagnostic method. The diagnosis of PPL should be suspected also in pediatric cases. Because reported studies have been retrospective, used undersized patient groups and involved heterogenous regimens, no conclusions can be drawn for an optimal regimen, that should be tailored on histological subtype. The high CNS relapse rate reported in available literature, suggests that patients with PPL may benefit from a CNS directed prophylaxis.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Baylor SM, Berg JW. Cross-classification and survival characteristics of 5,000 cases of cancer of the pancreas. *J Surg Oncol*. 1973;5 (4):335–358. doi:10.1002/jso.2930050410
2. Freeman C, Berg JW, Cutler SJ. Occurrence and prognosis of extranodal lymphomas. *Cancer*. 1972;29(1):252–260.
3. Behrns KE, Sarr MG, Strickler JG. Pancreatic lymphoma: is it a surgical disease? *Pancreas*. 1994;9(5):662–667. doi:10.1097/00006676-199409000-00019
4. Dawson IM, Cornes JS, Morson BC. Primary malignant lymphoid tumours of the intestinal tract. Report of 37 cases with a study of factors influencing prognosis. *Br J Surg*. 1961;49:80–89. doi:10.1002/bjs.18004921319
5. Fléjou JF. [WHO Classification of digestive tumors: the fourth edition]. *Ann Pathol*. 2011;31(5 Suppl):S27–31. France. doi:10.1016/j.annpat.2011.08.001
6. Jones WF, Sheikh MY, McClave SA. AIDS-related non-Hodgkin's lymphoma of the pancreas. *Am J Gastroenterol*. 1997;92(2):335–338.
7. Rad N, Khafaf A, Alizadeh AHM. Primary pancreatic lymphoma: what we need to know. *J Gastrointest Oncol*. 2017;8(4):749–757. doi:10.21037/jgo.2017.06.03

8. Manning MA, Paal EE, Srivastava A, Mortele KJ. Nonepithelial neoplasms of the pancreas, part 2: malignant tumors and tumors of uncertain malignant potential from the radiologic pathology archives. *RadioGraphics*. 2018;38(4):1047–1072. doi:10.1148/rg.2018170201

9. Mishra MV, Keith SW, Shen X, Ad VB, Champ CE, Biswas T. Primary pancreatic lymphoma. *Am J Clin Oncol*. 2013;36(1):38–43. doi:10.1097/COC.0b013e3182354bbb

10. Sadot E, Yahalom J, Do RKG, et al. Clinical features and outcome of primary pancreatic lymphoma. *Ann Surg Oncol*. 2015;22(4):1176–1184. doi:10.1245/s10434-014-4176-6

11. Mohamadnejad M, Khosravi P, Khani M, Nikmanesh A, Eloubeidi MA. Primary pancreatic Hodgkin's lymphoma diagnosed on EUS-guided FNA. *Gastrointest Endosc*. 2016;83(4):844–845. doi:10.1016/j.gie.2015.09.028

12. Matsubayashi H, Takagaki S, Otsubo T, et al. Pancreatic T-cell lymphoma with high level of soluble interleukin-2 receptor. *J Gastroenterol*. 2002;37(10):863–867. doi:10.1007/s005350200143

13. Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ*. 2009;339:b2700. doi:10.1136/bmj.b2700

14. Boni L, Benevento A, Dionigi G, Cabrini L, Dionigi R. Primary pancreatic lymphoma. *Surg Endosc*. 2002;16(7):1107–1108. doi:10.1007/s00464-001-4247-1

15. Bozzoli V, Tisi MC, Pianese L, et al. Primary pancreatic lymphoma in a patient with maturity onset diabetes of the young type 3. *Mediterr J Hematol Infect Dis*. 2012;4(1):e2012005. doi:10.4084/MJHID.2012.005

16. Ghobakhlou M, Mohammad alizadeh AH, Naderi N, et al. A patient with chronic hepatitis C and a pancreatic mass in endoscopic ultrasound. *Case Rep Gastroenterol*. 2012;6(2):387–393. doi:10.1159/000339693

17. Li Z, Zhang S, Vasdani N, Castillo E. Clues for diagnosing primary pancreatic lymphoma. *Case Rep Gastroenterol*. 2012;6(2):438–445. doi:10.1159/000339968

18. Liu W, Hua R, Zhang JF, Huo YM, Liu DJ, Sun YW. First report of primary pancreatic natural killer/T-cell nasal type lymphoma. *Eur Rev Med Pharmacol Sci*. 2013;17(3):318–322.

19. Carbonetti F, Iannicelli E, Federici M, et al. Primary pancreatic burkitt lymphoma presenting as acute pancreatitis. *J Gastrointest Cancer*. 2014;45(1):265–269. doi:10.1007/s12029-014-9657-0

20. Abedi SH, Ahmadzadeh A, Nikmanesh A, et al. The role of endoscopic ultrasound in primary pancreatic lymphoma presented with acute pancreatitis: a case report. *JOP*. 2014;15(5):493–496. doi:10.6092/1590-8577/2637

21. Nakaji S, Hirata N, Shiratori T, et al. A case of primary pancreatic lymphoblastic lymphoma diagnosed by endoscopic ultrasound-guided fine-needle aspiration. *Clin J Gastroenterol*. 2014;7(2):180–184. doi:10.1007/s12328-014-0462-x

22. Knibbeler-van Rossum CTAM, Peters FJP, Erdkamp FLG, Bos LP. Unusual presentation of Hodgkin's lymphoma. *Ann Oncol*. 2002;13(4):637. doi:10.1093/annonc/mdf148

23. Levy MJ, Wiersema MJ. Endoscopic removal of a Biliary Wallstent with a suture-cutting device in a patient with primary pancreatic lymphoma. *Endoscopy*. 2002;34(10):835–837. doi:10.1055/s-2002-34258

24. Kurosawa H, Matsunaga T, Shimaoka H, et al. Burkitt lymphoma associated with large gastric folds, pancreatic involvement, and biliary tract obstruction. *J Pediatr Hematol Oncol*. 2002;24(4):310–312. doi:10.1097/00043426-200205000-00018

25. Cohen Y, Libster D, Amir G, et al. Primary ALK positive anaplastic large cell lymphoma of the pancreas. *Leuk Lymphoma*. 2003;44(1):205–207. doi:10.1080/1042819021000054715

26. Yoshida N, Nakamura M, Yamada H. Primary pancreatic lymphoma forming a giant mass in a short period: a case report. *J Med Ultrason*. 1999;26(12):1205–1210. doi:10.1007/bf02481373

27. Reddy D, Gumaste V, Benisovich V. Primary pancreatic lymphoma presenting as acute pancreatitis. *Pancreatology*. 2003;3(5):403–405. doi:10.1159/000073656

28. Nayer H, Weir EG, Sheth S, Ali SZ. Primary pancreatic lymphomas: a cytopathologic analysis of a rare malignancy. *Cancer*. 2004;102(5):315–321. doi:10.1002/cncr.20488

29. Volmar KE, Routbort MJ, Jones CK, Xie HB. Primary pancreatic lymphoma evaluated by fine-needle aspiration: findings in 14 cases. *Am J Clin Pathol*. 2004;121(6):898–903. doi:10.1309/UAD9PYFUA82X9R9U

30. Lee ACW, Li CH. Burkitt lymphoma presenting as acute pancreatitis: report of 3 cases and review of the literature. *J Pediatr Hematol Oncol*. 2020;42(8):e830–e834. doi:10.1097/MPH.0000000000001630

31. Wang P, Cheng X, Huo L, Li F. Primary pancreatic lymphoma on FDG PET/CT. *Clin Nucl Med*. 2020;45(10):830–832. doi:10.1097/RLU.0000000000003205

32. Chon HK, Choi KH, Kim TH. Primary pancreatic natural killer/T-cell lymphoma. *Pancreas*. 2020;49(3):E21–E23. doi:10.1097/MPA.0000000000001508

33. Pezzilli R, De Giorgio R, Ceciliato R, et al. A case of primary pancreatic lymphoma. *JOP*. 2004;5(2):105–106.

34. Koniaris LG, Lillemoe KD, Yeo CJ, et al. Is there a role for surgical resection in the treatment of early-stage pancreatic lymphoma? *J Am Coll Surg*. 2000;190(3):319–330. doi:10.1016/S1072-7515(99)00291-4

35. Fraser CJ, Chan YF, Heath JA. Anaplastic large cell lymphoma of the pancreas: a pediatric case and literature review. *J Pediatr Hematol Oncol*. 2004;26(12):840–842.

36. Yoon SN, Lee MH, Yoon JK. F-18 FDG positron emission tomography findings in primary pancreatic lymphoma. *Clin Nucl Med*. 2004;29(9):574–575. doi:10.1097/01.rlu.0000135269.00531.f8

37. Turkish A, Levy J, Kato M, et al. Pancreatitis and probable paraneoplastic cholestasis as presenting manifestations of pancreatic lymphoma in a child. *J Pediatr Gastroenterol Nutr*. 2004;39(5):552–556. doi:10.1097/00005176-200411000-00020

38. Arcari A, Anselmi E, Bernuzzi P, et al. Primary pancreatic lymphoma. A report of five cases. *Haematologica*. 2005;90(1):7–9.

39. Shahar KH, Carpenter LS, Jorgensen J, Truong L, Baker K, Teh BS. Role of radiation therapy in a patient with primary pancreatic lymphoma. *Clin Lymphoma Myeloma*. 2005;6(2):143–145. doi:10.3816/CLM.2005.n.042

40. Chim CS, Ho J, Ooi GC, Choy C, Liang R. Primary anaplastic large cell lymphoma of the pancreas. *Leuk Lymphoma*. 2005;46(3):457–459. doi:10.1080/1042819040007474

41. Ji Y, Kuang TT, Tan YS, Chen Y, Zeng H-Y, Jin DY. Pancreatic primary lymphoma: a case report and review of the literature. *Hepatobiliary Pancreat Dis Int*. 2005;4(4):622–626.

42. Savopoulos CG, Tsesmeli NE, Kaiafa GD, et al. Primary pancreatic anaplastic large cell lymphoma, ALK negative: a case report. *World J Gastroenterol*. 2005;11(39):6221–6224. doi:10.3748/wjg.v11.i39.6221

43. Mimery A, De Clercq S. Primary pancreatic lymphoma presenting as recurrent idiopathic pancreatitis: a diagnostic dilemma. *ANZ J Surg*. 2020;90(4):E81–E82. doi:10.1111/ans.15309

44. Tikue A, Bedanie G, Brandi L, Islam S, Nugent K. Primary pancreatic large B-cell lymphoma presenting as acute pancreatitis. *Cureus*. 2020;12(8). doi:10.7759/cureus.9583

45. Ueda K, Nagayama Y, Narita K, Kusano M, Mernyei M, Kamiya M. Pancreatic involvement by non-Hodgkin's lymphoma. *J Hepatobiliary Pancreat Surg*. 2000;7(6):610–613. doi:10.1007/s005340070013

46. Kopel J, Swarup K, Thein K, Swarup S. Primary B cell lymphoma of the pancreas. *J Gastrointest Cancer*. 2020;51 (3):1077–1080. doi:10.1007/s12029-020-00400-4

47. McCullagh D, McNeice A, Rice P, Mainie I. Primary pancreatic lymphoma. Ulster Med J. 2020 Jan;89(1):41-42. Available from: https://pubmed.ncbi.nlm.nih.gov/32218630/. Accessed January 13, 2021.

48. Alzerwi NAN. Primary pancreatic lymphoma masquerading as carcinoma. *Case Rep Oncol Med*. 2020;2020:1–6. doi:10.1155/2020/5160545

49. Dunphy L, Abbas SH, Al Shoek I, Al-Salti W. Primary pancreatic lymphoma: a rare clinical entity. *BMJ Case Rep*. 2020;13(1): e231292. doi:10.1136/bcr-2019-231292

50. Savari O, Al-Duwal Z, Wang Z, Ganesan S, Danan-Rayes R, Ayub S. Pancreatic lymphoma: a cytologic diagnosis challenge. *Diagn Cytopathol*. 2020;48(4):350–355. doi:10.1002/dc.24349

51. Facchinelli D, Sina S, Boninsegna E, et al. Primary pancreatic lymphoma: clinical presentation, diagnosis, treatment, and outcome. *Eur J Haematol*. 2020;105(4):468–475. doi:10.1111/ejh.13468

52. Lin H, Li SD, Hu XG, Li ZS. Primary pancreatic lymphoma: report of six cases. *World J Gastroenterol*. 2006;12 (31):5064–5067. doi:10.3748/wjg.v12.i31.5064

53. Grimison PS, Chin MT, Harrison ML, Goldstein D. Primary pancreatic lymphoma - pancreatic tumours that are potentially curable without resection, a retrospective review of four cases. *BMC Cancer*. 2006;6:1–9. doi:10.1186/1471-2407-6-117

54. Battula N, Srinivasan P, Prachalias A, Rela M, Heaton N. Primary pancreatic lymphoma: diagnostic and therapeutic dilemma. *Pancreas*. 2006;33(2):192–194. doi:10.1097/01.mpa.0000227910.63579.15

55. Meier C, Kapellen T, Tröbs RB, et al. Temporary diabetes mellitus secondary to a primary pancreatic Burkitt lymphoma. *Pediatr Blood Cancer*. 2006;47(1):94–96. doi:10.1002/pbc.20752

56. Islam S, Callery MP. Primary pancreatic lymphoma—a diagnosis to remember. *Surgery*. 2001;129(3):380–382. doi:10.1067/msy.2001.113284

57. Saif MW. Primary pancreatic lymphomas. *JOP*. 2006;7 (3):262–273.

58. Lee MK, Jeon SW, Lee YD, et al. A case of primary pancreatic non-Hodgkin's lymphoma. *Korean J Intern Med*. 2006;21 (2):123–126. doi:10.3904/kjim.2006.21.2.123

59. Wang YJ, Jeng CM, Wang YC, Chang PP, Wang TH. Primary pancreatic Burkitt's lymphoma mimicking carcinoma with obstructive jaundice and very high CA19-9. *Eur J Gastroenterol Hepatol*. 2006;18(5):537–540. doi:10.1097/00042737-200605000-00014

60. Alldinger I, Peiper M, Diallo R, et al. Primary pancreatic lymphoma - A rare cause of cholestasis leading to surgical treatment [1]. *Ann Hematol*. 2006;85(7):485–486. doi:10.1007/s00277-006-0102-8

61. Dyckmans K, Lerut E, Gillard P, et al. Post-transplant lymphoma of the pancreatic allograft in a kidney-pancreas transplant recipient: a misleading presentation. *Nephrol Dial Transplant*. 2006;21 (11):3306–3310. doi:10.1093/ndt/gfl382

62. Sallapan S, Abu Bakar NZ, Jarmin R, et al. Primary follicular lymphoma of the pancreas: a rare tumour mimicking pancreatic carcinoma. Malaysian J Pathol .2018; 40(3) : 359 – 371. Available from: https://pubmed.ncbi.nlm.nih.gov/30580370/. Accessed January 13, 2021.

63. Ito H, Hiraiwa SI, Sugiyama T, et al. An autopsy case of primary extranodal NK/T cell lymphoma (extranodal NK/T-cell lymphoma) of the bile duct. *Clin J Gastroenterol*. 2019;12 (3):209–212. doi:10.1007/s12328-018-00931-1

64. Rodríguez-Infante A, Fernández-Martínez D, Iglesias-García E, García-Flórez LJ. Linfoma pancreático primario como causa de ictericia obstructiva. *Rev Gastroenterol Mex*. 2018;4–5. doi:10.1016/j.rgmx.2018.01.003

65. Badrinath M, Tambe A, Mandru R, Saleem S, Heisig D. Large pancreatic mass with chylous ascites. *Baylor Univ Med Cent Proc*. 2020;33(1):53–54. doi:10.1080/08998280.2019.1668661

66. Huang Z, Li M, He D, et al. Two-dimensional texture analysis based on CT images to differentiate pancreatic lymphoma and pancreatic adenocarcinoma: a preliminary study. *Acad Radiol*. 2019;26(8):e189–e195. doi:10.1016/j.acra.2018.07.021

67. Eisenhuber E, Schoefl R, Wiesbauer P, Bankier AA. Primary pancreatic lymphoma presenting as acute pancreatitis in a child. *Med Pediatr Oncol*. 2001;37(1):53–54. doi:10.1002/mpo.1163

68. Hughes B, Habib N, Chuang KY. Primary anaplastic large cell lymphoma of the pancreas. *ACG Case Reports J*. 2019;6(10): e00231. doi:10.14309/crj.0000000000000231

69. Zafar Y, Kaur A, Banno F, Anuj S. Primary pancreatic lymphoma: an uncommon presentation in the pancreatic tail. *Cureus*. 2019;11 (8). doi:10.7759/cureus.5479

70. Kim GE, Bingham S, Gariepy C. Pancreatic Non-Hodgkin lymphoma presenting as pancreatitis and duodenal polyps in a pediatric patient. *J Pediatr Gastroenterol Nutr*. 2019;69(5): e146–e147. doi:10.1097/MPG.0000000000002463

71. Yu L, Chen Y, Xing L. Primary pancreatic lymphoma: two case reports and a literature review. *Onco Targets Ther*. 2017;10:1687–1694. doi:10.2147/OTT.S121521

72. Bhagat VH, Sepe T. Pancreatic lymphoma complicating early stage chronic hepatitis C. *BMJ Case Rep*. 2017;2017: bcr2016216698. doi:10.1136/bcr-2016-216698

73. Amine B, Najat L, Ilias T. Epigastralgia revealing primary pancreatic large B-cell lymphoma in a young patient: about a case. *Pan Afr Med J*. 2018;31:161. doi:10.11604/pamj.2018.31.161.16850

74. Blouhos K, Boulas KA, Paraskeva A, Kariotis I, Barettas N, Hatzigeorgiadis A. Obstructive jaundice as primary presentation of a stage IIE Non-Hodgkin lymphoma: a decision making process between advanced lymphoma and locally advanced/metastatic pancreatic adenocarcinoma. *Int J Surg Case Rep*. 2018;44:226–229. doi:10.1016/j.ijscr.2018.02.038

75. Konjeti VR, Hefferman GM, Paluri S, Ganjoo P. Primary pancreatic Burkitt's lymphoma: a case report and review of the literature. *Case Rep Gastrointest Med*. 2018;2018:5952315. doi:10.1155/2018/5952315

76. Yoshizawa N, Inoue H, Yamada R, Takei Y. Pancreatic Burkitt's lymphoma presenting as an unusual cause of obstructive jaundice. *J Dig Dis*. 2018;19(8):508–510. doi:10.1111/1751-2980.12601

77. Cagle BA, Holbert BL, Wolanin S, Tappouni R, Lalwani N. Knife wielding radiologist: a case report of primary pancreatic lymphoma. *Eur J Radiol Open*. 2018;5:141–146. doi:10.1016/j.ejro.2018.08.007

78. Nishimura R, Tsujimoto M, Nakao K, et al. Usefulness of intraoperative cytology for the diagnosis of primary pancreatic lymphoma. *Acta Cytol*. 2001;45(1):104–108. doi:10.1159/000327196

79. Magalhães-Costa P, Brito MJ, Pinto-Marques P. A diffusely enlarged pancreas: the (un)usual suspect? *Rev Esp Enfermedades Dig*. 2016;108(12):809–811.

80. Inoue A, Sawada Y, Ohmori S, et al. Erythema papulosa semicircularis recidivans associated with primary pancreas B cell lymphoma. *Eur J Dermatol*. 2016;26(3):306–307. doi:10.1684/ejd.2016.2749

81. Lachter J, Balanson S, Ilivitzki A. Endoscopic ultrasound-guided fine-needle aspiration for diagnosis of Burkitt's lymphoma in the pancreas of a 3 year old. *Dig Endosc*. 2016;28(5):613. doi:10.1111/den.12648

82. Shapira G, Fisher Y, Ilivitzki A. Bifocal primary pancreatic Burkitt's lymphoma in a 4-year-old child. *J Clin Ultrasound*. 2017;45(3):171–174. doi:10.1002/jcu.22372

83. Zheng S-M, Zhou D-J, Chen Y-H, et al. Pancreatic T/histiocyte-rich large B-cell lymphoma: a case report and review of literature. *World J Gastroenterol*. 2017;23(24):4467. doi:10.3748/wjg.v23.i24.4467

84. Araújo J, Sampaio Macedo C, Sousa L. Pancreas Burkitt primary lymphoma in pediatric age. *Rev Esp Enferm Dig*. 2017;109(6):451.

85. Qiu T, Li W, Geng H, Shi S. Clinicopathological characteristics of primary pancreatic lymphoma: report of two cases. *Int J Clin Exp Pathol*. 2017;10(11):10941–10946.

86. Gleeson FC, Zhang L, Levy MJ. Primary pancreatic lymphoma: endoscopic ultrasound-guided Trucut biopsy to the rescue! *Endoscopy*. 2008;40(Suppl 2):E23–E24. doi:10.1055/s-2007-966960

87. Fernández-Plaza S, Sevilla J, Diaz MA, Madero L, Tamariz-Martel A. Dyspnea as the first manifestation of primary pancreatic lymphoma. *Pediatr Blood Cancer*. 2008;50(2):434. doi:10.1002/pbc.21242

88. Sağlam M, Yilmaz MI, Mas MR, et al. A case of pancreatic Burkitt lymphoma: radiological findings. *Diagn Interv Radiol*. 2009;15(1):39–42.

89. Nishimura R, Takakuwa T, Hoshida Y, Tsujimoto M, Aozasa K. Primary pancreatic lymphoma: clinicopathological analysis of 19 cases from Japan and review of the literature. *Oncology*. 2001;60(4):322–329. doi:10.1159/000058528

90. Nistala SS, Sawalakhe NR, Thiruvengadam NR, Rathi PM. A rare case of primary pancreatic Burkitt lymphoma in a young Indian male. Case report and review of the literature. *JOP*. 2009;10(6):686–689.

91. Haji AG, Sharma S, Majeed KA, Vijaykumar DK, Pavithran K, Dinesh M. Primary pancreatic lymphoma: report of three cases with review of literature. *Indian J Med Paediatr Oncol*. 2009;30(1):20–23. doi:10.4103/0971-5851.56331

92. Francisco B, Lucas M, Del Mar AM, Maria FNJ, Manuel FS, Amparo V. Abdominal pain as the first manifestation of primary pancreatic lymphoma. *J Pediatr Hematol Oncol*. 2009;31(3):222–223. doi:10.1097/MPH.0b013e3181979e28

93. Miwa I, Maruyama Y, Kageoka M, et al. A case of pancreatic mucosa-associated lymphoid tissue (MALT) lymphoma. *Japanese J Gastroenterol*. 2008;105(12):1794–1801. doi:10.11405/nisshoshi.105.1794

94. Baysal B, Kayar Y, Ince AT, Arici S, Türkmen I, Şentürk H. Primary pancreatic lymphoma is a rare cause of pancreatic mass: a case report. *Oncol Lett*. 2015;10(3):1701–1703. doi:10.3892/ol.2015.3412

95. Shirai Y, Okamoto T, Kanehira M, et al. Pancreatic follicular lymphoma presenting as acute pancreatitis: report of a case. *Int Surg*. 2015;100(6):1078–1083. doi:10.9738/INTSURG-D-14-00132.1

96. Koca T, Aslan N, Dereci S, Akcam M. Burkitt lymphoma with unusual presentation: acute pancreatitis. *Pediatr Int*. 2015;57(4):775–777. doi:10.1111/ped.12640

97. Fukuba N, Moriyama I, Ishihara S, et al. Primary pancreatic malignant lymphoma diagnosed from endoscopic ultrasound-guided fine-needle aspiration findings. *Intern Med*. 2016;55(1):31–35. doi:10.2169/internalmedicine.55.5749

98. Abe Y, Tamura K, Sakata I, et al. Unique intense uptake demonstrated by 18F-FDG positron emission tomography/computed tomography in primary pancreatic lymphoma: a case report. *Oncol Lett*. 2010;1(4):605–607. doi:10.3892/ol_00000107

99. Sugishita H, Watanabe Y, Yamamoto Y, et al. Primary pancreatic lymphoma: the role of surgical treatment. *Case Rep Gastroenterol*. 2010;4(1):104–110. doi:10.1159/000283405

100. Pietsch JB, Shankar S, Ford C, Johnson JE. Obstructive jaundice secondary to lymphoma in childhood. *J Pediatr Surg*. 2001;36(12):1792–1795. doi:10.1053/jpsu.2001.28840

101. Yoon WJ, Yoon YB, Kim YJ, Ryu JK, Kim YT. Primary pancreatic lymphoma in Korea-a single center experience. *J Korean Med Sci*. 2010;25(4):536–540. doi:10.3346/jkms.2010.25.4.536

102. Rossi ED, Larghi A, Verna EC, et al. Endoscopic ultrasound-guided fine-needle aspiration with liquid-based cytologic preparation in the diagnosis of primary pancreatic lymphoma. *Pancreas*. 2010;39(8):1299–1302. doi:10.1097/MPA.0b013e3181dc694e

103. Kav T, Soyuoz AG, Altundag K, Bayraktar Y. Primary pancreatic lymphoma or secondary involvement: what is the difference? *JOP*. 2010;11:482–483. doi:10.6092/1590-8577/3442

104. Ravi K, Sanchez W, Sweetser S. Primary pancreatic follicular lymphoma mimicking adenocarcinoma. *Clin Gastroenterol Hepatol*. 2010;8(10):e101–e102. doi:10.1016/j.cgh.2010.05.011

105. Alexander RE, Nakeeb A, Sandrasegaran K, et al. Primary pancreatic follicle center-derived lymphoma masquerading as carcinoma. *Gastroenterol Hepatol*. 2011;7(12):834–838.

106. Federico E, Falconi M, Zuodar G, Falconieri G, Puglisi F. B-cell lymphoma presenting as acute pancreatitis. *Pancreatology*. 2011;11(6):553–556. doi:10.1159/000332038

107. Rehbinder B, Wullstein C, Bechstein WO, et al. Epstein-barr virus-associated posttransplant lymphoproliferative disorder of donor origin after simultaneous pancreas-kidney transplantation limited to pancreas allograft. *Am J Transplant*. 2006;6(10):2506–2511. doi:10.1111/j.1600-6143.2006.01464.x

108. Sata N, Kurogochi A, Endo K, Shimura K, Koizumi M, Nagai H. Follicular lymphoma of the pancreas: a case report and proposed new strategies for diagnosis and surgery of benign or low-grade malignant lesions of the head of the pancreas. *JOP*. 2007;8(1):44–49.

109. Basu A, Patil N, Mohindra P, et al. Isolated non-Hodgkin's lymphoma of the pancreas: case report and review of literature. *J Cancer Res Ther*. 2007;3(4):236–239. doi:10.4103/0973-1482.39000

110. Jayanthi V, Randhir J, Rajesh N. Problems in diagnosing lymphoma of the pancreas with computed tomography. A case report. *J Gastrointest Liver Dis*. 2007;16(1):101–103.

111. Piesman M, Forcione DG, Carr-Locke DL. A case of pancreatic lymphoma. *Med Gen Med Medscape Gen Med*. 2007;9(3).

112. Liakakos T, Misiakos EP, Tsapralis D, Nikolaou I, Karatzas G, Macheras A. A role for surgery in primary pancreatic B-cell lymphoma: a case report. *J Med Case Rep*. 2008;2:1–6. doi:10.1186/1752-1947-2-167

113. Nakamura T, Ito T, Abe Y, et al. Primary pancreatic low-grade mucosa-associated lymphoid tissue lymphoma presenting with multiple masses. *Clin J Gastroenterol*. 2008;1(4):168–173. doi:10.1007/s12328-008-0028-x

114. Boninsegna E, Zamboni GA, Facchinelli D, et al. CT imaging of primary pancreatic lymphoma: experience from three referral centres for pancreatic diseases. *Insights Imaging*. 2018;9(1):17–24. doi:10.1007/s13244-017-0585-y

115. Mukhija D, Nagpal SJS, Sohal DPS. Epidemiology, tumor characteristics, and survival in patients with primary pancreatic lymphoma: a large population-based study using the seer database. *Am J Clin Oncol Cancer Clin Trials*. 2019;42(5):454–458. doi:10.1097/COC.0000000000000544

116. Puli SR, Bechtold ML, Buxbaum JL, Eloubeidi MA. How good is endoscopic ultrasound-guided fine-needle aspiration in diagnosing the correct etiology for a solid pancreatic mass?: A meta-analysis and systematic review. *Pancreas*. 2013;42(1):20–26. doi:10.1097/MPA.0b013e3182546e79

117. Ramesh J, Hebert-Magee S, Kim H, Trevino J, Varadarajulu S. Frequency of occurrence and characteristics of primary pancreatic lymphoma during endoscopic ultrasound guided fine needle aspiration: a retrospective study. *Dig Liver Dis*. 2014;46(5):470–473. doi:10.1016/j.dld.2013.12.016

118. Johnson EA, Benson ME, Guda N, Pfau PR, Frick TJ, Gopal DV. Differentiating primary pancreatic lymphoma from adenocarcinoma using endoscopic ultrasound characteristics and flow cytometry: a case-control study. *Endosc Ultrasound*. 2014;3 (4):221–225. doi:10.4103/2303-9027.144530

119. Khashab M, Mokadem M, Dewitt J, et al. Endoscopic ultrasound-guided fine-needle aspiration with or without flow cytometry for the diagnosis of primary pancreatic lymphoma a case series. *Endoscopy*. 2010;42(3):228–231. doi:10.1055/s-0029-1243859

120. Anand D, Lall C, Bhosale P, Ganeshan D, Qayyum A. Current update on primary pancreatic lymphoma. *Abdom Radiol*. 2016;41 (2):347–355. doi:10.1007/s00261-015-0620-8

121. Webb TH, Lillemoe KD, Pitt HA, Jones RJ, Cameron JL. Pancreatic lymphoma. Is surgery mandatory for diagnosis or treatment? *Ann Surg*. 1989;209(1):25–30. doi:10.1097/00000658-198901000-00003

122. Zucca E, Roggero E, Bertoni F, Cavalli F. Primary extranodal non-Hodgkin's lymphomas. Part 1: gastrointestinal, cutaneous and genitourinary lymphomas. *Ann Oncol*. 1997;8(8):727–737. doi:10.1023/A:1008282818705

123. Ng YY, Healy JC, Vincent JM, Kingston JE, Armstrong P, Reznek RH. The radiology of non-Hodgkin's lymphoma in childhood: a review of 80 cases. *Clin Radiol*. 1994;49(9):594–600. doi:10.1016/S0009-9260(05)81874-4

124. Vade A, Blane CE. Imaging of Burkitt lymphoma in pediatric patients. *Pediatr Radiol*. 1985;15(2):123–126. doi:10.1007/BF02388718

125. Klöppel G, Solcia E, Sobin LH, et al. *Histological Typing of Tumours of the Exocrine Pancreas*. Springer Science & Business Media; 1996. doi:10.1007/978-3-642-61024-0_2

126. Kanno A, Masamune A, Hanada K, Kikuyama M, Kitano M. Advances in early detection of pancreatic cancer. *Diagnostics*. 2019;9(1):18. doi:10.3390/diagnostics9010018

127. de Pretis N, Amodio A, Frulloni L. Updates in the field of autoimmune pancreatitis: a clinical guide. *Expert Rev Gastroenterol Hepatol*. 2018;12(7):705–709. doi:10.1080/17474124.2018.1489230

128. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390. doi:10.1182/blood-2016-01-643569

129. Vitolo U, Seymour JF, Martelli M, et al. Extranodal diffuse large B-cell lymphoma (DLBCL) and primary mediastinal B-cell lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2016;27(suppl5):mdw175. doi:10.1093/annonc/mdw175

130. Ollila TA, Olszewski AJ. Extranodal diffuse large B cell lymphoma: molecular features, prognosis, and risk of central nervous system recurrence. *Curr Treat Options Oncol*. 2018;19(8):38. doi:10.1007/s11864-018-0555-8

Prevalence of Leucopenia and Associated Factors before and after Initiation of ART among HIV-Infected Patients, North East Ethiopia: Cross-Sectional Study

Feredegn Talargia, Yonas Teshome, Yared Asmare Aynalem & Adisu Asefa

ORIGINAL RESEARCH

Prevalence of Leucopenia and Associated Factors before and after Initiation of ART among HIV-Infected Patients, North East Ethiopia: Cross-Sectional Study

Feredegn Talargia [ID] [1]
Yonas Teshome [ID] [1]
Yared Asmare Aynalem [ID] [2]
Adisu Asefa [ID] [1]

[1]Department of Biomedical Science, College of Medicine, Debre Berhan University, Debre Berhan, Ethiopia; [2]Department of Pediatrics Nursing, College of Health Science, Debre Berhan University, Debre Berhan, Ethiopia

Background: Leucopenia is the commonest hematological abnormaly that occurs in patients with human immune deficiency virus (HIV) infection. The magnitude and related factors of leucopenia during the time of ART are not characterized in Ethiopia. This study aimed to assess the prevalence of leucopenia before and after the initiation of ART among HIV patients attending Debre Berhan Referral Hospital (DBRH), North East Ethiopia.

Methods: A cross-sectional study was conducted from September to December 2020 in DBRH, North-East Ethiopia. A total of 272 patients on ART were selected by simple random sampling techniques. Socio-demographic and clinical characteristics of the study participants were collected by standard questionnaires. Measurements of leucocyte count and CD4 counts were made by Sysmex XT 2000i hematology analyzer and BD FACS count CD4 analyzer, respectively. Statistical analysis of data was done by SPSS version 23. Logistic regression was done and a P-value<0.05 was taken as statistically significant.

Results: The prevalence of leucopenia, neutropenia, and lymphopenia were 20.9%, 7.0%, and 6.6% before initiation of ART and 15.4%, 1.1, and 4.4% after initiation of ART, respectively. There was a significant difference in total white blood cell count, absolute neutrophil count, and total lymphocyte count between patients on ART and ART naïve patients. HIV patients whose cluster of differentiation (CD4) counts were <200 cells/µL and patients on a zidovudine (AZT)-based regimen were more likely to have leucopenia than HIV patients whose CD4 counts were ≥ 200 cells/µL and on a tenofovir (TDF)-based regimen.

Conclusion: In this study, the prevalence of leucopenia, neutropenia, and lymphopenia has shown a significant decrement after the initiation of ART. HIV patients with low CD4 count and on an AZT-based regimen are more likely to have leucopenia, neutropenia, and lymphopenia. Based on our findings, we recommend that the health care professional routinely investigate and should treat leucopenia.

Keywords: ART, leucopenia, HIV/AIDS, Ethiopia

Background

Hematologic disorders are the most common complications of HIV AIDS, which include anemia, leucopenia, and thrombocytopenia.[1] Among these hematological abnormalities; leucopenia is the common hematological abnormality that occurs in patients with HIV infection. The cause of HIV-related leucopenia is multifactorial, which includes a direct effect of HIV, autoimmune disease, neoplasm and ART

Correspondence: Feredegn Talargia
P.O.Box: 445
Tel +251921792491
Email ftalarge@gmail.com

drugs (especially zidovudine-based regimen), cotrimoxazole, and opportunistic infection.[2–4] The prevalence of leucopenia is higher among treatment naïve people than treated ones in most studies.[3–5] The use of antiretroviral therapy (ART) improves total white blood cell count by preventing suppression of bone marrow cells from the HIV virus and it blocks the interference of virus in the hematopoiesis process.[6] Leucopenia occurs in HIV-infected patients. Even though low leukocyte count mainly indicates the toxicities of drugs for HIV or associated conditions, studies of untreated patients have also shown a high magnitude of leucopenia.[7] The most common form of leucopenia usually encountered is neutropenia, occurring in 10–30% of HIV- infected individuals.[8] Neutropenia is caused by decreased production of cells because HIV suppresses the bone marrow by changing the marrow microenvironment and by altering the cytokine expression and it is also common among HIV-infected patients and presents in approximately 10–50% of cases.[8,9] In the study conducted in different parts of the United States, worsening of HIV disease parameters, like lower CD4 counts and higher HIV viral load, was found to be associated with the development of neutropenia.[10] ART showed a statistically significant increment in the mean WBC (White blood cells) count in different studies.[11–15] On the other hand, some studies reported that patients on ART had low WBC count compared to treatment naïve patients.[16,17] Patients on ART showed a significant reduction in the prevalence of leucopenia and neutropenia compared to their ART-naïve groups.[6,18] The improvement in these hematological abnormalities after ART initiation was due to increased production of Granulocyte- Macrophage Colony Stimulating Factor (GM-CSF) and Granulocyte Colony Stimulating Factor (G-CSF), which play an important role in the activation of granulocytes.[19] Studies indicated that low CD4 count, advanced clinical stage of HIV disease, and zidovudine-based regimen are associated with an increased risk of leucopenia.[8,16,20–22] Although hematologic abnormalities have been widely reported in HIV-related infections, there are a few data related with the prevalence of leucopenia and associated factors of leucopenia among HIV-infected patients before and after initiation of ART in Ethiopia. This study provides further information on HIV-associated leucopenia, and it can serve as a baseline for future studies. The aim of this study is to determine the prevalence of leucopenia and associated factors before and after the initiation of ART

among HIV patients who attended the ART clinic at DBRH, North-East Ethiopia.

Methods and Materials
Study Period and Area
This study was conducted at the ART clinic of DBRH, North East Ethiopia from September to December 2020.

Study Design
A hospital-based cross-sectional study was conducted.

Source Population
All adult HIV-1 infected people receiving ART at the DBRH.

Study Population
All adult HIV-1 infected people receiving ART for at least 6 months in the ART clinic of DBRH.

Inclusion and Exclusion Criteria
Age greater or equal to 18-years old HIV-positive individuals who received ART for at least 6 months in DBRH were included in this study. Pregnant women, patients referred from other health institutions, patients with hematological disorders, severely sick patients with any other medical conditions, and patients who had taken other medications were excluded from the study.

Sample Size Determination
The sample size was determined by using a statistical for a single population (n=z2 p (1-p)/d2), taking p=19% (prevalence of leucopenia from previous study [8]5% level of precision (d) with 95% confidence interval plus 15% non-response rate were added.

n= (z2 p (1-p)/d2), substitute the value, n=1.962*0.19*0.81/0.052=236.48, when we added a 15% non-response rate we found approximately 272 sample sizes.

Sampling Procedures and Techniques
The samples of this study were collected by simple random sampling technique until the desired sample size was achieved from the subjects who fulfilled the inclusion criteria during the study period.

Study Variables
Dependent Variables
Prevalence of leucopenia, neutropenia, and lymphopenia

Independent Variables

Age, sex, stage of HIV disease, CD4 count, and type of ART regimen.

Data Collection and Procedures

The standard structured questionnaire was adapted after the review of different literature, and the data was collected by trained ART nurses. Data about socio-demographic, clinical characteristics, and pre-ART information of the study participants were collected by face-to-face interview and a review of medical registration books. After that, a blood sample was collected by laboratory technicians and sent to a hematology analyzer. Based on the standard procedures, WBC count and CD4 cell counts were determined by using the Sysmex XT 2000i hematology analyzer and BD FACS count system, respectively. In order to keep the quality of data, standard procedures were followed in every laboratory aspect of the procedures and the quality of CD4 and hematology analyzer was checked by running quality control samples along with the patient sample. In addition, there was training for data collectors, a pretest of the questioner was made and the data collection process was done two times a day by the principal investigators.

Leucopenia: Defined as WBC counts <4,000 cells/μL.

Neutropenia: Absolute neutrophil count (ANC) less than 1,000 cells/μL.

Lymphopenia: Total lymphocyte count (TLC) of less than 800 cells/μL.

Data Processing and Analysis

The data were coded, checked and entered in the EPI data software version 3.1, and exported to SPSS software version 23 for analysis. The results of descriptive statistics were expressed as frequency and percentage. Continuous variables were presented as mean ± standard deviation. Univariate logistic regression was performed to examine the association between dependent and independent variables using crude odds ratio (COR) with a 95% confidence interval (CI). Those independent variables with a P-value<0.2 in univariate analysis were included in multivariate logistic regression models. A P-value<0.05 on multivariate logistic regression was considered a statistically significant association.

Result

General Characteristics

From the total 272 HIV-positive patients, 110 (40.4%) were male and 162 (59.6%) were female. The mean age

of the study participants was 40.94±10.88 years, ranging from 18–78 years. Most of the study participants were within the WHO stage one category at the baseline.

The WBC Count, ANC, TLC, and CD4 Counts of the Study Participants

The mean WBC count, ANC, and TLC at baseline were 4.65±1.53 X 103/μL, 2.24±0.9 X 103/μL, 1.48±0.6 X 103/μL, and 5.58±1.82 X 103/μL, 2.92±1.38 X 103/μL, 1.81 ±0.72 X 103/μL after initiation of ART (P<0.01). Similarly, the mean CD4 counts showed an increment from 264.75±184.5 cells/μL at the baseline to 544.0 ±261.3 cells/μL after the initiation of ART (P<0.01). The most commonly used ART regimen in the current was 1j (TDF-3TC-DTG) (Table 1).

Prevalence of Leucopenia, Neutropenia, and Lymphopenia and Associated Factors before Initiation of ART

The prevalence of leucopenia, neutropenia, and lymphopenia before ART was 20.9%, 7.0%, and 6.6%, respectively. The prevalence of leucopenia in males was 24.5% and 17.9% in females, whereas the prevalence of neutropenia and lymphopenia were 8.2%, 7.3% and 5.6%, 5.6% in males and females, respectively. In this study, most of the leucopenia cases (27.12%) were observed in the age groups ≥50 years. However, the majority of neutropenia cases were observed in the age range between 40–49 years and lymphopenia falls between the age group 18–19 years. Patients whose CD4 counts were <200 were strongly associated with a high prevalence of leucopenia and lymphopenia with a P-value<0.05. Lymphopenia was significantly associated with the advanced stage of HIV disease with a P-valve<0.03 (Table 2).

Prevalence of Leucopenia, Neutropenia, and Lymphopenia, and Associated Factors after the Initiation of ART

The prevalence of leucopenia, neutropenia, and lymphopenia after ART was 15.4%, 1.1, and 4.4%, respectively. The prevalence of leucopenia, neutropenia, and lymphopenia after the initiation of ART was decreased by 5.5%, 5.9%, and 2.2%, respectively. The prevalence of leucopenia, neutropenia, and lymphopenia in males was 24.5%, 0.9%, and 6.4% and in females 17.9, 1.2, and 3.1, respectively (Table 3). The prevalence of leucopenia was higher (21.6%) among patients whose age group was ≥50 years. The prevalence of

Table I Socio-Demographic and Clinical Characteristics of HIV Positive Patients Taking ART at DBRH, North East Ethiopia, 2020

Variables	Frequency	Percentage
Age in years		
18–29	34	12.5
30–39	97	35.9
40–49	82	30.0
≥50	59	21.6
Sex		
Male	110	40.4
Female	162	59.6
Marital status		
Single	23	49.8
Divorced	39	27.5
Married	135	8.4
Widowed	75	14.3
Educational status		
Illiterate	74	27.1
Primary school	92	33.7
High school	63	23.1
Certificate and above	44	16.1
WHO clinical stages		
Stage I	144	52.9
Stage II	36	13.2
Stage III	87	31.9
Stage IV	5	1.8
Types of ART regimen		
1c	14	5.1
1d	10	3.7
1e	75	27.6
1j	130	47.8
2f	19	7.0
2h	18	6.6
Not registered	6	2.2

Notes: 1c=AZT-3TC-NVP, 1d=AZT-3TC-EFV, 1e=TDF-3TC-EFV, 1f=TDF-3TC-NVP, 1j=TDF-3TC-DTG, 2f=AZT-3TC-ATV/r, 2h=TDF-3TC-ATV/r

leucopenia, neutropenia, and lymphopenia was high in patients with low CD4 count (<200). However, there was no significant association observed between the low CD4 count (<200) and that of leucopenia, neutropenia, and lymphopenia. HIV patients on an AZT-based regimen are more likely to have leucopenia and lymphopenia than HIV patients on a TDF-based regimen with a significant association at a *P*-value <0.02 and 0.01, respectively (Table 3).

Discussion

It's well documented that hematological abnormalities are common in HIV-infected patients.[1] Leucopenia, for instance, occurs in patients with HIV infection. The cause of HIV-associated leucopenia is multifactorial, including a direct consequence of HIV infection, autoimmune disorders, malignancies, and drugs used to treat HIV.[3] This study revealed that the prevalence of leucopenia, neutropenia, and lymphopenia were 20.9%, 7.0%, and 6.6% at baseline, and 15.4%, 1.1%, and 4.4% after the initiation of ART. A study conducted in Ghana reported that the prevalence of leucopenia, neutropenia, and lymphopenia were 13%, 72.5%, and 6.5% in pre-ART patients and 6.5%, 85%, and 18% in post-ART patients.[4] Another study conducted in Gondar, Ethiopia reported that the prevalence of leucopenia, neutropenia, and lymphopenia were 35.9%, 28.3, and 2.1 on ART patients and 16.9%, 14.5, and 2.1 ART naïve patients.[16] The difference in results seen from the present study might be due to the difference in the definition of leucopenia, neutropenia, and lymphopenia, study design and size of the study population. The decrease in the prevalence of leucopenia, neutropenia, and lymphopenia after ART initiation might be due to; disorders of hematopoiesis, opportunistic infections, and immune causes related to HIV leading to low white blood cell count could be reverted after ART initiation.[23] In the present study the prevalence of leucopenia was elevated in patients whose age is ≥50 years old, which is in agreement with another study.[2] The increase in the prevalence of leucopenia with age might be due to a higher incidence of myelodysplasia in older patients.[9] However, leucopenia did not show a statistically significant difference with sex and age. This was in line with previous studies.[2,21]

According to the present study, the prevalence of leucopenia, neutropenia, and lymphopenia were increased with decreasing in CD4 count both before and after the initiation of ART. Leucopenia, neutropenia, and lymphopenia were more prevalent among HIV-positive patients whose CD4 count was <200 cells/μL. These findings were similar to several studies that reported the leucopenia, neutropenia, and lymphopenia was more prevalent among patients with CD4 count <200 cells/μL.[2,3,16] The prevalence of leucopenia and lymphopenia with decreased CD4 count was significantly associated before initiation of ART, but it did not significantly associate after ART initiation, this may be due to ART bringing a statistical increment into the WBC count.[11,13] The present study revealed that patients on AZT-based ART regimen had a higher prevalence of leucopenia, neutropenia, and lymphopenia compared to TDF-based ART regimen. Similar to the

Table 2 Leucopenia, Neutropenia, and Lymphopenia and Associated Factors before ART Initiation in HIV Positive Patients Attended at DBRH, North East Ethiopia, 2020

Variables	Leucopenia, N (%)	COR (95% CI)	AOR (95% CI)	Neutropenia, N %	COR (95% CI)	AOR (95% CI)	Lymphopenia, N (%)	COR (95% CI)	AOR (95% CI)
Age groups									
18–29	7 (20.6)	1.4 (0.52–3.94)	1.3 (0.42–3.96)	1 (2.9)	3.7 (0.43–32.4)	3.7 (0.42–32.2)	3 (8.8)	0.6 (0.11–2.91)	0.6 (0.09–2.92)
30–39	18 (18.4)	1.7 (0.77–3.57)	1.2 (0.49–2.76)	3 (3.1)	3.6 (0.86–14.92)	5.4 (1.04–27.6)	5 (5.1)	0.9 (0.23–4.33)	0.9 (0.18–4.27)
40–49	16 (19.5)	1.5 (0.69–3.39)	1.1 (0.45–2.65)	9 (11.9)	0.9 (0.31–2.74)	0.9 (0.31–2.87)	7 (8.5)	0.6 (0.14–2.32)	0.4 (0.09–1.87)
≥50	16 (27.1)	1	1	6 (10.2)	1	1	3 (5.1)	1	1
Sex									
Male	27 (24.5)	0.7 (0.37–1.21)	0.9 (0.47–1.8)	9 (8.2)	0.7 (0.25–1.72)	0.8 (0.3–2.1)	8 (7.3)	0.8 (0.28–2.01)	1.1 0.39–3.3
Female	9 (17.9)	1	1	10 (5.6)	1	1	10 (5.6)	1	1
Clinical stage									
Stage I & II	29 (16)	3.6 (1.17–11.27)**	2.3 (1.26–4.16)	9 (5.0)	1.6 (0.81–3.12)	2.3 (0.91–5.96)		2.6 (0.97–6.93)	4.4 (1.58–12.07) *
Stage III & IV	28 (34.4)	1	1	10 (10.9)	1	1		1	1
CD4 count cells/μL									
<200	48 (41.0)	0.1 (0.02–0.21)*	0.1 (0.02–0.26)**	19 (16.2)	–	–	16 (13.7)	0.1 (0.01–0.68)*	0.1 (0.02–0.9)**
200–349	6 (7.2)	0.6 (0.13–2.28)	0.6 (1.15–2.59)	0 (0.0)	–	–	1 (1.2)	1.1 (0.07–18.54)	1.32 (0.08–22.39)
≥350	3 (4.1)	1	1	0 (0.0)	1	1	1 (1.4)	1	1

Notes: *Indicates statistically significant association by univariate analysis, **Statistically significant association by multivariate analysis.
Abbreviations: COR, crude odds ratio; AOR, adjusted odd ratio; CI, confidence interval.

Table 3 Leucopenia, Neutropenia and Lymphopenia and Associated Factors after Initiation of ART in HIV Positive Patients Attended at DBRH, North East Ethiopia, 2020

Variables	Leucopenia, N (%)	COR (95% CI)	AOR (95% CI)	Neutropenia, N %	COR (95% CI)	AOR (95% CI)	Lymphopenia, N (%)	COR (95% CI)	AOR (95% CI)
Age groups									
18–29	4 (11.8)	1.9 (0.57–6.49)	2.2 (0.62–7.6)	1 (2.9)	–	–	1(2.9)	1.8 (0.18–17.69)	1.9 (0.17–20.81)
30–39	12 (12.2)	1.83 (0.76–4.39)	1.9 (0.78–4.68)	2 (2.0)	–	–	5(5.1)	0.9 (0.23–4.33)	1.0 (0.21–4.65)
40–49	14 (17.3)	1.22 (0.52–2.88)	1.1 (0.45–2.62)	0 (0.0)	–	–	3 3.7	1.4 (0.27–7.16)	1.3 (0.24–7.34)
≥50	12 (20.3)	1	1	0 (0.0)	–	–	3 (5.1)	1	1
Sex									
Male	17 (15.6)	0.7 (0.37–1.21)	0.9 (0.47–1.8)	1 (0.9)	1.3 (0.12–14.94)	3.1 (0.18–49.86)	7 (6.4)	0.5 (0.14–1.5)	0.6 (0.16–2.06)
Female	25 (15.4)	1	1	2 (1.2)	1	1	5 (3.1)	1	1
ART regimen									
AZT based	13 (30.2)	0.4 (0.16–0.74)*	0.3 (0.14–0.65)**	2 (4.8)	0.1 (0.01–1.02)	0.1 (0.01–1.52)	6 (14.0)	0.2 (0.1–0.56)*	0.2 (0.06–0.7)**
TDF based	29 (13.0)	1	–	1 (0.1)	1	–	6 (2.7)	1	1
CD4 count cells/µL									
<200	3 (23.1)	0.6 (0.17–2.45)	–	1 (8.3)	0.5 (0.01–0.87)	0.1 (0.02–1.52)	2 (15.4)	0.2 (0.03–0.98)	0.3 (0.05–1.72)
200–349	4 (9.5)	1.8 (0.61–5.44)		1 (2.4)	0.2 (0.01–3.1)	0.3 (0.14–4.32)	3 (7.1)	0.4 (0.12–1.75)	0.1 (0.13–2.53)
≥350	35 (16.1)	1		1 (0.5)	1	1	7 (3.2)	1	1

Notes: *Indicates statistically significant association by univariate analysis, **Statistically significant association by multivariate analysis.
Abbreviations: COR, crude odds ratio; AOR, adjusted odd ratio; CI, confidence interval.

present study conducted in Ethiopia and Ghana, it revealed an increment in the prevalence of leucopenia, neutropenia, and lymphopenia after initiation of AZT.[2,4] However, neutropenia did not show a statistically significant difference with the AZT-based ART regimen. The high prevalence of leucopenia, neutropenia, and lymphopenia may be due to the suppression of bone marrow by Zidovudine-based therapy.[16]

Conclusion

In this study, the prevalence of leucopenia, neutropenia, and lymphopenia had shown a significant decrement after the initiation of ART. HIV patients with low CD4 count and on AZT-based regimen are more likely to have leucopenia, neutropenia, and lymphopenia. Based on our findings, we recommend that health care professionals routinely investigate and should treat leucopenia.

Abbreviations

AIDS, acquired immunodeficiency syndrome; ANC, absolute neutrophil count; ART, anti-retroviral therapy; AZT, zidovudine; CD4, cluster of differentiation; HIV, human immunodeficiency virus; TLC, total lymphocyte count; TDF, tenofovir.

Data Sharing Statement

The datasets used and analyzed during the present study are available from the corresponding author on reasonable requests.

Ethics Approval and Consent to Participate

This study was conducted after ethical letters obtained from the institute of research ethics and review board (IRB) of college of Medicine, Debre Berhan University, Ethiopia complied with the Declaration of Helsinki. The institute of research and ethics board committee (IRB) had reviewed and looed originality, feasibility, laboratory setting, and ethical aspects of the study. Following thorough discussion the committee approved the research proposal by authors with ethical approval using reference number med/219/2019. Then permission was taken from hospital higher management and data was collected after obtaining informed consent from the study participants. To keep confidentiality, codes were used and unauthorized persons did not have access to the data.

Acknowledgments

Author Contributions

All authors had valuable contributions to the conception and design, data collection, analysis of data and interpretation; involved in drafting of the article or revising it critically for important intellectual content; agreed on journal to which the article will be submitted; gave final approval of the version to be published; agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors declare that they have no competing of interests in this work.

References

1. Shen Y, Wang J, Wang Z, et al. A cross-sectional study of leukopenia and thrombocytopenia among Chinese adults with newly diagnosed HIV/AIDS. *Biosci Trends*. 2015;9(2):91–96. doi:10.5582/bst.2015.01024
2. Gebreweld A, Fiseha T, Girma N, Haileslasie H, Gebretsadik D. Prevalence of cytopenia and its associated factors among HIV infected adults on highly active antiretroviral therapy at Mehal Meda Hospital, North Shewa Zone, Ethiopia. *PLoS One*. 2020;15(9):e0239215. doi:10.1371/journal.pone.0239215
3. Fekene TE, Juhar LH, Mengesha CH, Worku DK. Prevalence of cytopenias in both HAART and HAART naïve HIV infected adult patients in Ethiopia: a cross sectional study. *BMC Hematol*. 2018;18:8.
4. Afari S, Blay E. Prevalence of haematological and serum biochemical abnormalities in hiv infected patients in Ghana, before and after anti-retroviral therapy. *Int J Virol AIDS*. 2018;5(1):039.
5. Tamir Z, Seid A, Haileslassie H. Magnitude and associated factors of cytopenias among antiretroviral therapy naïve Human Immunodeficiency Virus infected adults in Dessie, Northeast Ethiopia. *PLoS One*. 2019;14(2):e0211708.
6. Choi SY, Kim I, Kim NJ, et al. Hematological manifestations of human immunodeficiency virus infection and the effect of highly active anti-retroviral therapy on cytopenia. *Korean J Hematol*. 2011;46(4):253–257. doi:10.5045/kjh.2011.46.4.253
7. Akinbami A, Oshinaike O, Adeyemo T, et al. Hematologic abnormalities in treatment-naive HIV patients. *Infect Dis Res Treat*. 2010;3 (IDRT):S6033.

8. Addis Z, Yitayew G, Tachebele B. Prevalence of some hematological abnormalities among HIV positive patients on their first visit to a tertiary health institution in ethiopia; a cross sectional study. *Int Blood Res Rev.* 2014;2:270–278. doi:10.9734/IBRR/2014/11137

9. Dikshit B, Wanchu A, Sachdeva RK, Sharma A, Das R. Profile of hematological abnormalities of Indian HIV infected individuals. *BMC Hematol.* 2009;9(1):1–6. doi:10.1186/1471-2326-9-5

10. Levine AM, Karim R, Mack W, et al. Neutropenia in human immunodeficiency virus infection: data from the women's interagency HIV study. *Arch Intern Med.* 2006;166(4):405–410. doi:10.1001/archinte.166.4.405

11. Amegor O, Bigila D, Oyesola O, Oyesola T, Buseni S. Hematological changes in HIV patients placed on anti retroviral therapy in Markurdi, Benue State of Nigeria. *Asian J Epidemiol.* 2009;2 (4):97–103. doi:10.3923/aje.2009.97.103

12. Zama II, Amina A, Abdulrahaman Y, Yeldu MH. Some haematological profile of HIV/AIDS patients on Highly Active Antiretroviral Therapy (HAART) in Usamanu Danfodiyo University Teaching Hospital Sokoto, North-Western Nigeria.*J Sci Technol.* 2014;2(1):27–32.

13. Ako SE, Njunda LA, Akum EA, et al. Immuno-hematological Profile Trends of HIV/AIDs Patients on HAART in the South west region of cameroon: retrospective medical report review for possible stratified follow-up pattern in low income settings. *Am J Microbiol Res.* 2018;6 (2):47–56. doi:10.12691/ajmr-6-2-3

14. Servais J, Nkoghe D, Schmit J-C, et al. HIV-associated hematologic disorders are correlated with plasma viral load and improve under highly active antiretroviral therapy. *J Acquired Immune Deficiency Syndromes.* 2001;28(3):221–225. doi:10.1097/00042560-200111010-00003

15. Odunukwe N, Idigbe O, Kanki P, et al. Haematological and biochemical response to treatment of HIV-1 infection with a combination of nevirapine+ stavudine+ lamivudine in Lagos Nigeria. *Turkish J Haematol.* 2005;22(3):125–131.

16. Enawgaw B, Alem M, Addis Z, Melku M. Determination of hematological and immunological parameters among HIV positive patients taking highly active antiretroviral treatment and treatment naïve in the antiretroviral therapy clinic of Gondar University Hospital, Gondar, Northwest Ethiopia: a comparative cross-sectional study. *BMC Hematol.* 2014;14 (1):1–7.

17. Wanjari A, Acharya S, Singh AP, Rathi C. A study of hematological profile in HIV/AIDS. *AIDS.* 2013;8:9.

18. Idowu C, Oke O, Afolayan D, Olaniran O, Akinloye O, Awodumila O. Effect of HAART on some haematological parameters, correlations between total lymphocyte and CD4 counts of HIV clients attending ssh, ikole-ekiti. *Int J Biol Med Res.* 2013;4(3):3265–3270.

19. Ijeoma OL, Ifeoma UE, Emmanuel O, Ifeanyi AU, Andrew A. Comparative study of Cd8+ T-Cell count and leukopoietin levels in human immunodeficiency virus infection in Umuahia, Nigeria. *IOSR J Dental Med Sci.* 2014;13:102–110. doi:10.9790/0853-1392102110

20. Katemba C, Muzoora C, Muwanguzi E, Mwambi B, Atuhairwe C, Taremwa IM. Hematological abnormalities in HIV-antiretroviral therapy naïve clients as seen at an immune suppression syndrome clinic at Mbarara Regional Referral Hospital, southwestern Uganda. *J Blood Med.* 2018;9:105. doi:10.2147/JBM.S157148

21. Tamir Z, Seid A, Haileslassie H. Magnitude and associated factors of cytopenias among antiretroviral therapy naive Human Immunodeficiency Virus infected adults in Dessie, Northeast Ethiopia. *PLoS One.* 2019;14(2):e0211708. doi:10.1371/journal.pone.0211708

22. Forna F, Moore D, Mermin J, et al. Hematologic changes associated with Zidovudine following single-drug substitution from stavudine in a home-based AIDS care program in rural Uganda. *J Int Assoc Physicians AIDS Care.* 2009;8(2):128–138. doi:10.1177/1545109709333081

23. Fekene TE, Juhar LH, Mengesha CH, Worku DK. Prevalence of cytopenias in both HAART and HAART naïve HIV infected adult patients in Ethiopia: a cross sectional study. *BMC Hematol.* 2018;18 (1):1–11. doi:10.1186/s12878-018-0102-7

Murine Leukemia-Derived Extracellular Vesicles Elicit Antitumor Immune Response

Alejandro Pando, Loren Fast, Patrycja M Dubielecka, Anna Chorzalska, Sicheng Wen & John Reagan

Published online: 17 May 2021.

Submit your article to this journal

Article views: 130

View related articles

View Crossmark data

Citing articles: 4 View citing articles

ORIGINAL RESEARCH

Murine Leukemia-Derived Extracellular Vesicles Elicit Antitumor Immune Response

Alejandro Pando
Loren Fast
Patrycja M Dubielecka
Anna Chorzalska
Sicheng Wen [iD]
John Reagan

Division of Hematology/Oncology, Department of Medicine, Rhode Island Hospital and the Warren Alpert School of Medicine at Brown University, Providence, RI, USA

Background: Extracellular vesicles (EVs) are heterogeneous lipid bilayer particles secreted by cells. EVs contain proteins, RNA, DNA and other cargo that can have immunomodulatory effects. Cancer-derived EVs have been described as having immunomodulating effects in vivo with immunosuppressive and pro-tumor growth capabilities. However, cancer-derived EVs have also been harnessed and utilized for anti-cancer potential.

Methods: To assess the immunomodulatory effect of EVs produced by acute myeloid leukemia (AML) cells, we isolated vesicles secreted by the murine AML cell line, C1498, and investigated their effect on in vitro and in vivo immune responses.

Results: These leukemia-derived EVs were found to induce increased proliferation of CD3+ cells and enhanced cytolytic activity of CD3+ cells directed toward leukemic cells in vitro. Injection of leukemia-derived EVs into syngeneic naïve mice induced T cell responses in vivo and resulted in enhanced immune responses upon T cell re-stimulation in vitro.

Conclusion: These findings indicate that C1498-derived EVs have immunomodulatory effects on cell-mediated immune responses that could potentially be utilized to facilitate anti-leukemia immune responses.

Keywords: extracellular vesicles, acute myeloid leukemia, immune responses

Introduction

There is an increasing emphasis on harnessing the patient's immune system to achieve anti-cancer responses. A variety of approaches have been tested including activating the patient's immune system directly using various stimuli. Alternative adoptive immunotherapy approaches involve removing the patient's lymphocytes and then expanding, activating and modifying the lymphocytes in vitro prior to reinfusing them back into the patient. Our group has developed a cellular immunotherapy approach in which a large number of G-CSF mobilized haploidentical donor CD3+ cells ($1 - 2$ x 10^8 CD3+ cells/kg) are infused into patients who had received 100 cGy of total body irradiation prior to the infusion.[1] This approach was shown to induce responses in about half of patients with refractory hematological malignancies (14/26, 5 complete remissions). The course of the treatment following infusion included rapid development (a median time of 14 hours) of a cytokine storm characterized by very high levels of IL-6 and high fevers. This response could be controlled by administration of corticosteroids, if needed. In almost all patients, no remaining donor cells could be detected in the patient at two weeks.

While effective, the mechanism(s) responsible for the anti-cancer responses in this therapeutic protocol have not been determined. One potential mechanism is that the

Correspondence: Loren Fast
Division of Hematology/Oncology, Department of Medicine, Rhode Island Hospital and the Warren Alpert School of Medicine at Brown University, 31 S Killingly Road, Foster, RI, 02825, USA
Tel +1 401 533-8658
Email Loren_Fast@brown.edu

activation of the alloreactive effector cells responsible for eliminating the haploidentical donor cells cross-react with tumor cells. A number of studies have shown that alloreactive effector cells cross-react with cancer cells.[2–7] To explore this possibility further, we obtained blood from newly diagnosed leukemic patients, isolated the CD3+ cells, stimulated them with allogeneic stimulator cells and then tested the ability of alloreactive effector cells that were generated to lyse cells in a syngeneic population of cells containing leukemic cells.[8] It was observed that about half of the patient's CD3+ cells were able to lyse syngeneic leukemic cell containing populations when stimulated with allogeneic cells. In trying to identify characteristics of the responders and non-responders, we found that lack of response was associated with increased expression of cytolytic CD4+ cells, increased expression of CD39 by CD8+ cells and an increased number of γδ T cells.

There has been an increased understanding of the extracellular vesicles (EVs) and the functional implications of the cargo that is carried by them. EVs are nano-sized heterogeneous lipid bilayer particles released by almost all cells and secreted at higher numbers in cancer cells.[9] Initially thought to be cellular debris, over the last decade EVs have been shown to be involved in numerous biological processes such as intercellular communication, antigen presentation, protein secretion, and RNA shuttling.[10] EVs carry DNA, proteins, RNA, bioactive lipids, and other cargo that is typically representative of the parent cell.[11,12] Depending on the cell of origin, EVs can modulate physiological and pathological processes including proliferation, differentiation, inhibition, quiescence, and/or cellular death. EVs produced by cancer cells have been shown to enhance malignancy by transmission of regulatory factors to normal cells. EVs have also been shown to enhance anti-tumor immune responses by inducing immunity to antigens that are carried by tumor EVs. This raised the question of whether EVs were contributing to responses or lack of responses in the cellular immunotherapy protocol. To begin to test the role of EVs in this protocol, we isolated EVs from C1498, a murine AML cell line, and examined their effect on in vivo and in vitro immune responses directed toward C1498 cells. The results showed that the EVs derived from the C1498 cell line enhanced in vitro and in vivo anti-tumor responses.

Materials and Methods
Mice
C57BL/6J female mice (#000664 Jackson Laboratory, Bar Harbor, ME), 7 to 9 weeks of age (20–25g) were housed one week before the experiments. Mice were injected intraperitoneally with C1498-derived EVs or C1498 cells. Control mice were injected with PBS. All studies were approved by the Lifespan Institutional Animal Care and Use Committee.

Cell Lines and Reagents
The murine AML cell line (C1498) was obtained from ATCC. C1498 arose spontaneously in a C57BL/6J mouse. C1498 cells grow aggressively in C57BL/6J mice, when injected intravenously into syngeneic mice resulting in the development of acute leukemia.[13] C1498 cells were cultured at 37°C in 5% CO_2 in DMEM (ATCC) containing 10%fetal calf serum (FCS, Atlanta Biologicals) that had been depleted of extracellular vesicles by 2 centrifugation steps at 100,000 x×g for 70 minutes plus 100 U/mL penicillin/streptomycin (Gibco).

Isolation and Characterization of EVs
EVs were isolated from C1498 culture medium as previously described.[14] Conditioned culture medium was obtained by expanding the C1498 cells until sixteen 175 cm^2 flasks were obtained each containing 120 mL of medium at a cell density of approximately $2×10^6$ cells/mL. Briefly, C1498 conditioned cell culture medium was subjected to a series of centrifugation steps starting at 3300 g for 5 minutes and 2000 g for 10 minutes at room temperature followed by 10,000 g for 1 hour at 4 °C and 100,000 g at 4 °C for 70 minutes with collection of the 1100,000 g pellet. The higher speed centrifugation steps were done using a Surespin 630 rotor and a Thermo WX ULTRA ultracentrifuge. The EVs pellet was then resuspended in PBS and centrifuged at 100,000 ×g for 70 minutes at 4 °C. The freshly prepared C1498 EVs were directly used for experiments.

Characterization of mouse C1498 derived EVs was carried out by electron microscopy as previously described.[14] The representative images of C1498-derived EVs are shown in Supplemental Figure S.4A. Number and size distribution of vesicles was determined on a NanoSight NS500 (Malvern Instruments, Malvern, UK) (Supplemental Figure S.4B).

Proliferation and Cytolytic Analyses

C57BL/6J mice were injected ip with 200 uL PBS (i), or PBS containing C1498-derived EVs (ii), or C1498 cells (iii). At 7- or 14-days post injection, spleens were obtained from these mice and a ssingle-cellsuspension was prepared. The cells were washed twice with MLC medium (RPMI 1640 containing 4% EV depleted FCS, 2 mg/mL glucose, 2 mM glutamine, Pen/Strep and 10μM 2-mercaptoethanol). The splenocytes were resuspended in MLC medium and counted, and then stimulated with C1498-derived EVs or mitomycin C treated C1498 cells. C1498 cells (25×10^6 cells/mL MLC medium) and 25ug mitomycin C/mL were incubated for 30 minutes at 37°C. Following three washes with MLC medium, the treated cells were resuspended in MLC medium and counted. 1×10^5 responder splenocytes were co-incubated with mitomycin treated C1498 cells (1×10^4 cells), C1498 derived EVs, or PBS in triplicate wells in a 96 well flat bottom plate in a final volume of 200 μL MLC medium. High EV in vitro conditions (C1) was defined as 15 μg of EV protein added per 1×10^6 responder cells, and low EV condition (C2) was defined as adding 3 μg of EV protein per 1×10^6 responder cells. On the fourth day, 1 μCi of ^{3}H thymidine (Perkin Elmer Health Sciences, Shelton, CT, USA) was added to each well, incubated for 4 hours at 37° and the DNA was collected on filter paper to measure thymidine incorporation and assess the proliferation of stimulated splenocytes.

To measure cytolytic activity, cultures were set up by adding 4×10^6 responder spleen cells to 1×10^6 mitomycin C treated C1498 cells, or C1498 derived-EVs in a final volume of 2 mL of MLC medium per well in a 24 well plate. On day 5 of culture, the cells were collected, centrifuged, and aliquots of the supernatants were stored at −20°C. The cells were then resuspended in RHG medium (RPMI 1640, 20 mM HEPES, 4% FCS and pen/strep). The cells were counted and used as effectors against ^{51}Cr labeled target cells (C1498 cells). ^{51}Cr labeled cells were prepared by incubating 5×10^6 target cells with 15 μCi ^{51}Cr (Perkin Elmer Health Sciences, Shelton, CT, USA) in 200 μL of medium for 45 minutes at 37°C with frequent agitation. After three washes with RHG medium the cells were counted. Each set of effector cells was added in duplicate to wells in 96 well V-bottom plates. Responder cell numbers ranged from 1×10^6 cells per well in 100 μL RHG medium in 2-fold serial dilutions to 1.25×10^5 cells per well. ^{51}Cr-labeled target cells at 1×10^4 cells/100 μL were added to all wells. Control wells contained 100 μL RHG medium or 100 μL 1N HCl in addition to the target cells. After four hours

incubation at 37°C, 100 μL of the supernatant was collected from each well and the ^{51}Cr present in each sample measured using a gamma counter. Percent specific lysis was determined by subtracting the counts obtained from the medium containing wells from the experimental value divided by the counts obtained in the wells containing 1 N HCl minus the counts obtained from the medium containing wells, and then multiplied by 100. The results are presented as lytic units/10^6 cells in which 1 lytic unit (LU) is defined as the number of cells required to achieve 30% lysis of 1×10^4 target cells. All experiments were replicated four times.

Cytokine Measurements

Aliquots of the supernatants collected on day 5 of the mixed lymphocyte cultures were thawed on the same day and tested for cytokine levels using a multiplex CBA assay measuring IL-2, IL-4, IL-6, TNF-α, IFN-γ, IL-10, and IL-17A levels according to manufacturer's instructions (BD Biosciences, San Jose, CA) using the LSRII flow cytometer. FACSARRAY software was used for analysis.

Immunophenotypic Analysis

Cell phenotypes were evaluated by multiparameter flow cytometry. Cells were incubated with a panel of labeled monoclonal antibodies (mAbs) anti-CD3 AF700, CD4 BV605, CD8 APC-H7, CD25 BV786, PD-1 APC, TIGIT BV421, and LAG3 BV711 (BD Biosciences) in staining buffer on ice in the dark for 20 min. Cells were then washed in the staining buffer and re-suspended in staining buffer and analyzed on an LSRII flow cytometer. Isotype controls were used for each experiment. Analysis of the results used FlowJo 10 software.

CFSE staining was conducted according to manufacturer's instructions (Thermo Fisher Scientific). Briefly, cells were labeled with CFSE by adding 1 mL of freshly prepared CFSE (2 μM in PBS containing 2% EV free FCS) to cells (up to 1×10^8 cells) in 1 mL of PBS 2% EV free FCS. The tube containing this mixture was covered with foil and incubated at 337°Cfor 5 minutes. Cells were pelleted, washed twice with 10 mL of PBS 2% EV free FCS, resuspended, counted and used for experiments.

Statistical Analysis

Statistical analysis was performed utilizing GraphPad Prism 7.0 (GraphPad Software). Student's *t*-test, unpaired, two-tail testing was applied to populations to determine the *p* values indicated in the figures. *p* values <0.05 were

determined to be statistically significant. All data are represented as mean ± SD.

Results

Leukemia-Derived EVs Induce Increased Splenocyte Proliferation in vitro

To investigate the immunomodulatory ability of leukemia-derived EVs, we assessed the impact of culturing splenocytes obtained from naïve C57BL/6J mice with cells from the C1498 AML cell line, with two different concentrations of purified C1498-derived-EVs or when C1498 cells and C1498-EVs were combined. Results showed that the addition of C1498 cells or C1498-derived EVs alone to splenocytes induced a significant proliferative response and that combining C1498 cells and C1498-derived EVs provided an additive effect (Figure 1A, Supplemental Fig. S.1A). Increased C1498-derived EVs concentrations (C1 vs C2) trended toward increased proliferative responses (Figure 1A, Supplemental Fig. S.1A).

Immunizing Naïve Mice of Leukemia-Derived EVs Enhances Immune Responses

To assess the effect of immunization on splenocyte responses, C57BL/6J mice were injected with, C1498-derived EVs, or PBS (Supplemental Fig. S.1B). Splenocytes were obtained at 7 days or 14 days post injection and cultured with C1498 cells, EVs from C1498 cells, or a combination of the two. Results demonstrated overall significantly increased proliferation responses from splenocytes isolated from EV immunized mice over splenocytes isolated from the naïve mice (Figure 1A, Supplemental Fig. S.1).

To investigate which cell sub-populations were undergoing proliferation, splenocytes obtained from mice injected with PBS, C1498-derived EVs, or C1498 AML cells were labeled with CFSE prior to culture, and CFSE positive CD3+ cells were assessed on day 4 of culture. Gating strategy is found in Supplemental Figure S.2. For all three in vitro stimuli, the proliferating splenocytes (CFSE^{lo}) from PBS injected mice exhibited a preferential proliferation of CD3– cells(35.5± 8.6% CD3+ cells) while splenocytes from EV immunized mice (59.1 ± 1.1% CD3+ cells) or AML cell immunized mice (77.1 ± 14.3% CD3+ cell) exhibited preferential proliferation of CD3+ cells (Figure 1B and C). To investigate which subpopulations of CD3+ cells proliferated in response to EVs, the

frequencies of CD4+ and CD8+ cells in the CFSE^{lo} CD3 + cells were determined. CD8+ T cells from all three groups of mice comprised a significantly greater fraction of the proliferating cells than CD4+ T cells (Figure 2A and B).

To investigate the effect of exposure to leukemia-derived EVs on cytolytic activity, immune splenocytes were co-cultured with either PBS, leukemia-derived EVs, or C1498 cells for 5 days and then tested for their ability to lyse C1498 target cells. These immune splenocytes cultured for 5 days in medium exhibited the ability to lyse C1498 cells and this lytic activity was enhanced by co-culturing the immune splenocytes with leukemia-derived EVs or mitomycin C treated C1498 cells (Figure 2C). Increased levels of the inflammatory cytokines IL-6, IL-10, TNF-α, and IFN-γ were observed in the supernatants of immune splenocytes cultured in medium (Figure 3A–D). An additional increase in production of TNF-α and IFN-γ was seen when the splenocytes from the immunized mice were cultured with leukemia derived EVs and/or C1498 cells.

CD3+ cells from immune mice upregulated the expression of activation induced inhibitory receptors PD-1, TIGIT, and LAG3 when analyzed after collection (Supplemental Fig. S.3). The increased expression of these markers suggests that EVs can induce activation in CD3+ cells but the increased expression of these inhibitory receptors by these activated CD3+ cells could result in inhibited responses if the ligands for these inhibitory receptors are present in the environment (Supplemental Fig. S.3).

Discussion and Conclusion

Tumor cells secrete increased numbers of extracellular vesicles and have the ability to both suppress and enhance immune activity depending on the underlying pathologic state, parental cell derivation, and absolute EV number. These two seemingly juxtaposed notions likely depend on the microenvironment context and complexity of the interactions amongst the components within that environment. In this study, we tested the impact of EVs obtained from a murine acute myeloid leukemic cell line, C1498, on in vivo and in vitro immune responses. Immune responses that were enhanced by immunization compared to responses by spleen cells from control unimmunized mice included increased proliferation, increased lytic effector function, a bias toward enhanced CD8+ cell proliferation and proinflammatory cytokine production. In addition, T lymphocytes from these immunized mice
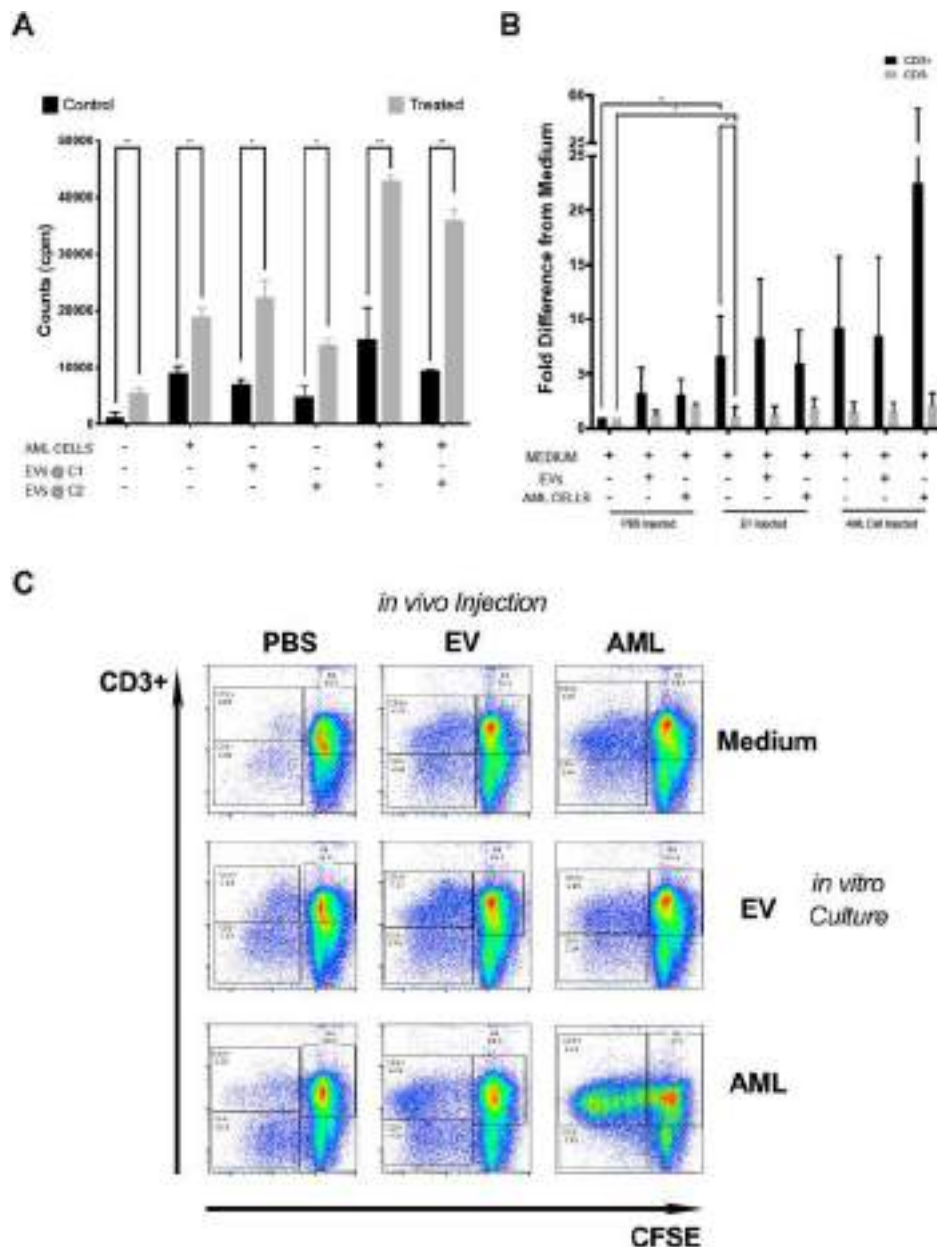
Figure 1 C1498 derived EVs induce splenocyte and CD3+ proliferation. Splenocytes (1×10^5 cells) were cultured with PBS, C1498 cells, different concentrations of C1498 derived EVs (C1 = high, C2 = low), or both C1498 cells and C1498 derived EVs. (**A**) Comparison of tritiated thymidine incorporation (mean ± SD) of combined splenocyte proliferation activity from splenocytes isolated from naïve mice (control) and splenocytes isolated from EV immunized mice (treated) (mean ± SD). (**B**) CD3+ splenocytes from PBS injected, C1498 derived EV immunized, or C1498 cell immunized mice were labeled with CFSE and co-cultured in vitro with medium, C1498 derived EVs, or C1498 cells. On day 4 the splenocytes were analyzed by flow cytometry and the number of CFSE low cells (proliferating cells) were measured as fold difference from PBS injected naïve mice cultured with medium (mean ± SD). (**C**) Representative flow cytometry plots of CFSE labeled splenocytes stained with anti-CD3 after four days of culture. CD3+ expression on the vertical axis and CFSE expression on the horizontal axis. Column 1: cells underwent in vivo injection of PBS, column 2 cells underwent in vivo injection of C1498 derived EVs, column 3: cells underwent in vivo injection of C1498 cells. Row 1: cells underwent in vitro culture with medium, row 2: cells underwent in vitro culture with C1498 derived EVs, row 3: cells underwent in vitro culture with C1498 cells. Left upper quadrant represents CD3+ CFSE^{lo} population. Left bottom quadrant represents CD3- CFSE^{lo} population. Asterisks indicate significant differences (*$p < 0.05$, **$p<0.01$).

exhibited increased expression of activation markers including the inhibitory receptors PD-1, TIGIT, and Lag3. These findings suggest that EVs may be used instead of cells to induce immune responses and raises the possibility that EVs could be used as a component of immunotherapy protocols.[15] Notably, observed increased expression of inhibitory receptors could possibly represent an inhibitory hurdle for the responses of these cells if the ligands for these receptors are present in the environment.

A

	in vivo Injection		
CD8+ CFSE^{lo} cells vs. CD4+ and CD8+ CFSE^{lo} cells	PBS	EV	AML CELL
in vitro Culture			
PBS	57.1%	81.3%	92.6%
EV	85.4%	93.0%	94.3%
AML CELL	56.1%	79.6%	84.2%

B

C

Lytic Effector Function
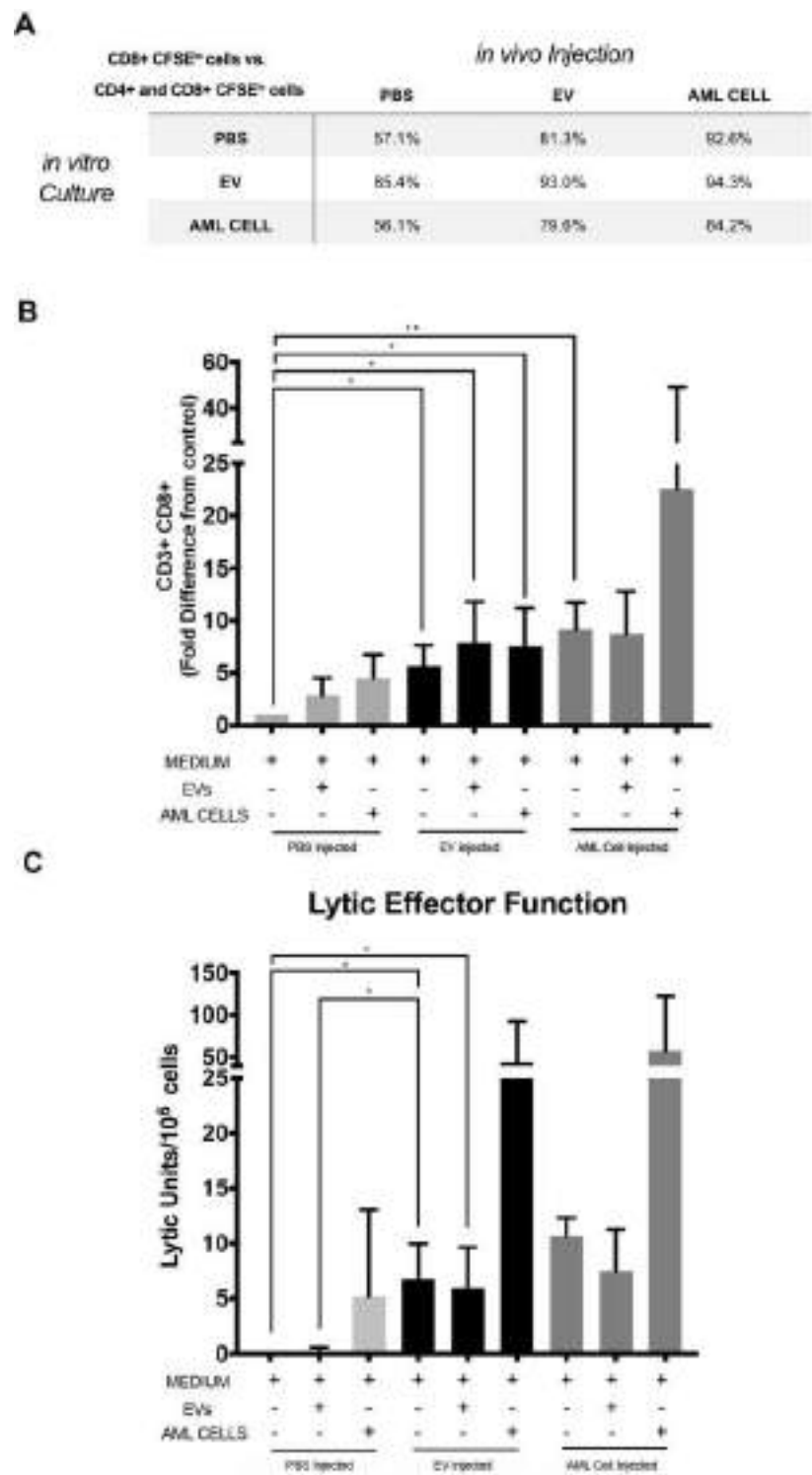
Figure 2 C1498 derived EVs preferentially activate CD8+ T cells and C1498 derived EV immunization increases lytic effector function. CFSE labeled splenocytes from in vivo PBS injected, C1498 derived EV immunized, or C1498 cell immunized mice were co-cultured in vitro with medium, C1498 derived EVs, or C1498 cells. On day 4 the cultures were harvested and stained with a panel of antibodies including anti-CD3, anti-CD8, anti-CD4, and underwent immunophenotypic analysis. (**A**) Table shows the fraction of CD8+ and CD4+ CFSE^{lo} population that are CD8+ cells. (**B**) Flow cytometry bar graphs of measured CD3+ CD8+ proliferation of each condition. Experiment repeated at least 3 times. Data is presented as fold difference in CFSE low cells (proliferating cells) from PBS injected naïve mice cultured with medium (mean ± SD). (**C**) Splenocytes isolated from C57BL/6J mice 7- and 14-days following injection with PBS, C1498 derived EVs, or AML cells were co-cultured in vitro with PBS, C1498 derived EVs, or AML cells for five days and the responding cells were tested for their ability to lyse ^{51}Cr labeled C1498 cells. Data is presented as LU/10^6 effector cells and represents replicates from 4 experiments. Asterisks indicate significant differences (*$p < 0.05$, **$p<0.01$).

282

Journal of Blood Medicine 2021:12
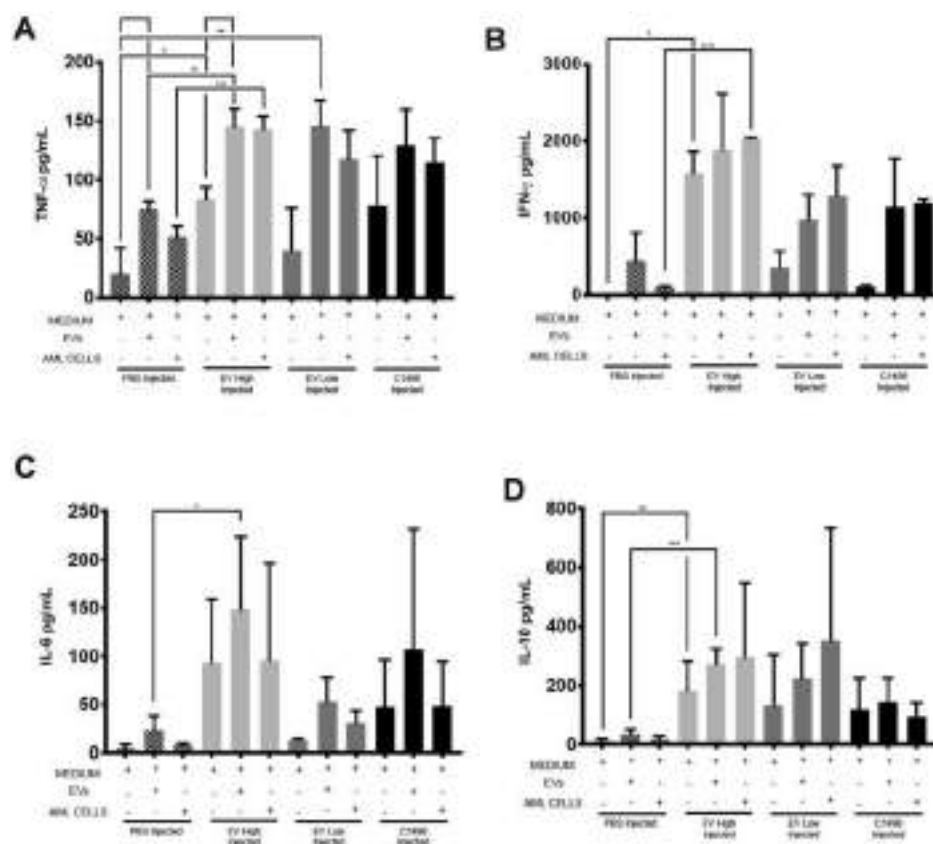
Figure 3 C1498 derived EVs increase cytokine production. Splenocytes obtained from PBS injected, C1498 derived EV immunized, or C1498 cell immunized mice co-cultured in vitro with medium, C1498 derived EVs, or with C1498 cells were incubated for five days at 37°C. Supernatants were then collected and stored at −20°C. These supernatants were tested for cytokine levels using the mouse Th1/Th2/Th17A CBA assay. The cytokines shown are as follows: Panel (**A**), TNFα; Panel (**B**), IFNγ; Panel (**C**) IL6, Panel (**D**), IL10. Error bars indicate standard deviation of of triplicate samples from a representative of three independent experiments. Asterisks indicate significant differences (*$p < 0.05$, **$p<0.01$, ***$p<0.001$).

The possibility also exists that the EVs could directly present antigen. The ability of EVs to present antigen was supported with the findings that EVs express multiple different H-2 class I histocompatibility antigen chains and MHC class 1b proteins, indicating that EVs are able to present antigens to the adaptive immune system. These antigens can be recognized by immune cells and elicit a proliferative immune response. EVs also expressed the antigen CD160 on their plasma membrane which has broad specificity for binding to classical and nonclassical MHC class I molecules.[16] CD160 is a ligand for Herpesvirus entry mediator (HVEM), that is considered a proposed immune checkpoint inhibitor with anti-tumor activity, further suggesting that EVs may harbor antigenic presentation potential.[17] This may be important if patients are infused with haploidentical CD3+ cells. These CD3+ cells could respond in a restricted or alloreactive manner to EVs circulating in the cancer patient and the responses to the EVs could influence whether the hematological malignant cells are attacked.

Eliciting effective T cell responses has been a major component in producing anti-leukemia responses. Therefore, inducing the effective antigen-specific cytolytic CD8+ T cell is vital to producing anti-leukemia responses. Others demonstrated that leukemia-derived exosomes can induce proliferation of CD4+ T cells and suggested that tumor-derived EVs can partially suppress T cell activation.[18,19] One of the components found in EVs produced by AML cells are microRNAs (miRNA).[20,21] Characterization of these EVs have identified a selected subset of these miRNAs[21] Some of these miRNAs have been found to modulate immune responses. MiR21 has

been shown to inhibit immune function by causing premature apoptosis of T lymphocytes while miR145 and miR448 have been shown to enhance anti-tumor responses.[21,22] In our study, we found that leukemia-derived EVs produced significant proliferation and activation of CD8+ T cells in mice immunized with EVs. Perhaps this is due to preferential expression of specific miRNA. Another possibility is the preferential expression of MHC class I molecules on EVs relative to the expression of MHC class II molecules. Interestingly, splenocytes from mice injected with leukemia derived EVs responded to leukemia derived EV re-exposure at a significantly higher level than splenocytes exposed to EVs for the first time, suggesting that EVs are able to prime murine splenocytes.

The finding that EVs isolated from a leukemic cell line are able to induce immune responses could suggest a potential role of leukemic cell derived EVs for generating anti-leukemic responses in the cellular immunotherapy protocol. A study by Lulla et al showed that activating and expanding leukemia antigen-specific T cells from stem cell donor lymphocytes enhanced anti-leukemic responses and minimized graft-versus-host disease when infused into recipients following a hematopoietic stem cell transplant.[23] Because CD3+ cells from a non-tumor bearing haploidentical donor are infused into the patient with refractory hematological malignancy, one possibility is that the circulating EVs isolated from the patient could be used to activate leukemia-specific donor CD3+ cells prior to infusion. The EVs could induce effector cells restricted to peptides presented by syngeneic MHC molecules or alloreactive effector cells able to recognize and lyse the patient's leukemic cells. The EVs could also potentially be used to test the responses of potential donors to identify which donor would generate the strongest anti-leukemic responses.

Our study suggests that tumor-derived EVs should be considered a component of possible anti-leukemia vaccines and that modification of them could be a possible path to a reproducible personalized medicine. Tumor-derived EVs have the advantage of containing unique tumor-associated antigens from the parental cell which they derive from and also contain immune stimulatory proteins. Tumor-derived EVs also have the advantage of not containing cell organelles and are highly reproducible and storable. Tumor-derived EV effects on target cells involve complex interactions, but harnessing these vesicles for anti-leukemia purposes is an important possibility warranting further studies.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

These studies were approved by the Lifespan Hospital Institutional Review Board. The studies followed the "Guide for the Care and Use of Laboratory Animals. 8th edition."

Acknowledgments

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

Funding

Disclosure

The authors declare that they have no conflicts of interest.

References

1. Colvin GA, Berz D, Ramanathan M, et al. Nonengraftment haploidentical cellular immunotherapy for refractory malignancies: tumor responses without chimerism. *Biol Blood Marrow Transplant*. 2009;15(4):421–431. doi:10.1016/j.bbmt.2008.12.503
2. Burdach S, Kolb HJ. The vigor of defense against non-self: potential superiority of allorestricted T cells in immunotherapy of cancer? *Front Oncol*. 2013;3:100. doi:10.3389/fonc.2013.00100
3. Kumari S, Walchli S, Fallang LE, et al. Alloreactive cytotoxic T cells provide means to decipher the immunopeptidome and reveal a plethora of tumor-associated self-epitopes. *Proc Natl Acad Sci U S A*. 2014;111(1):403–408. doi:10.1073/pnas.1306549111
4. Paciucci PA, Macphail S, Zarling JM, Bach FH. Lysis of syngeneic solid tumor cells by alloantigen stimulated mouse T and non-T cells. *J Immunol*. 1980;124(1):370–375.

5. Schirrmacher V, Hubsch D, Garrido F. Syngeneic tumor cells can induce alloreactive T killer cells: a biological role for transplantation antigens. *Proc Natl Acad Sci U S A*. 1980;77(9):5409–5413. doi:10.1073/pnas.77.9.5409

6. Sensi ML, Parenza M, Parmiani G. Alloreactivity and tumor antigens: generation of syngeneic antilymphoma killer lymphocytes by alloimmunization of mice with normal cells. *J Natl Cancer Inst*. 1983;70(2):291–297.

7. Stauss HJ, Xue S, Gillmore R, et al. Exploiting alloreactivity for tumour immunotherapy. *Vox Sang*. 2004;87(Suppl 2):227–229. doi:10.1111/j.1741-6892.2004.00493.x

8. Pando A, Reagan JL, Nevola M, Fast LD. Induction of anti-leukemic responses by stimulation of leukemic CD3+ cells with allogeneic stimulator cells. *Exp Hematol Oncol*. 2018;7:25. doi:10.1186/s40164-018-0118-5

9. Pando A, Reagan JL, Quesenberry P, Fast LD. Extracellular vesicles in leukemia. *Leuk Res*. 2018;64:52–60. doi:10.1016/j.leukres.2017.11.011

10. Mahaweni NM, Kaijen-Lambers ME, Dekkers J, Aerts JG, Hegmans JP. Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma. *J Extracell Vesicles*. 2013;2. doi:10.3402/jev.v2i0.22492

11. Simons M, Raposo G. Exosomes–vesicular carriers for intercellular communication. *Curr Opin Cell Biol*. 2009;21(4):575–581. doi:10.1016/j.ceb.2009.03.007

12. Quesenberry PJ, Aliotta J, Deregibus MC, Camussi G. Role of extracellular RNA-carrying vesicles in cell differentiation and reprogramming. *Stem Cell Res Ther*. 2015;6:153. doi:10.1186/s13287-015-0150-x

13. Mopin A, Driss V, Brinster C, Detailed A. Protocol for characterizing the murine C1498 cell line and its associated leukemia mouse model. *J Vis Exp*. 2016;116.

14. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol*. 2002;2(8):569–579. doi:10.1038/nri855

15. Yao Y, Wang C, Wei W, et al. Dendritic cells pulsed with leukemia cell-derived exosomes more efficiently induce antileukemic immunities. *PLoS One*. 2014;9(3):e91463. doi:10.1371/journal.pone.0091463

16. Tu TC, Brown NK, Kim TJ, et al. CD160 is essential for NK-mediated IFN-gamma production. *J Exp Med*. 2015;212 (3):415–429. doi:10.1084/jem.20131601

17. Vigano S, Banga R, Bellanger F, et al. CD160-associated CD8 T-cell functional impairment is independent of PD-1 expression. *PLoS Pathog*. 2014;10(9):e1004380. doi:10.1371/journal.ppat.1004380

18. Whiteside TL. Immune modulation of T-cell and NK (natural killer) cell activities by TEXs (tumour-derived exosomes). *Biochem Soc Trans*. 2013;41(1):245–251. doi:10.1042/BST20120265

19. Huang F, Wan J, Hao S, Deng X, Chen L, Ma L. TGF-beta1-silenced leukemia cell-derived exosomes target dendritic cells to induce potent anti-leukemic immunity in a mouse model. *Cancer Immunol Immunother*. 2017;66(10):1321–1331. doi:10.1007/s00262-017-2028-5

20. Wallace JA, O'Connell RM. MicroRNAs and acute myeloid leukemia: therapeutic implications and emerging concepts. *Blood*. 2017;130:11. doi:10.1182/blood-2016-10-697698

21. Agha DM, Rouas R, Najar M, et al. Identification of acute myeloid leukemia bone marrow circulating MicroRNAs. *Int J Mol Sci*. 2020;21(19).

22. Agha DM, Rouas R, Najar M, et al. Impact of bone marrow miR-21 expression on acute myeloid leukemia T lymphocyte fragility and dysfunction. *Cells*. 2020;9:9. doi:10.3390/cells9071640

23. Lulla P, Naik S, Vasileiou S, et al. Clinical effects of administering leukemia-specific donor T cells to patients with AML/MDS post-allogeneic transplant. *Blood*. 2020. doi:10.1182/blood.2020009471

Heterogeneity in Hematological Parameters of High and Low Altitude Tibetan Populations

Nipa Basak, Tsering Norboo, Mohammed S Mustak & Kumarasamy Thangaraj

ORIGINAL RESEARCH

Heterogeneity in Hematological Parameters of High and Low Altitude Tibetan Populations

Nipa Basak [1,2]
Tsering Norboo [3]
Mohammed S Mustak [4]
Kumarasamy Thangaraj [1,2,5]

[1]CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India; [2]Academy of Scientific and Innovative Research, Ghaziabad, India; [3]Ladakh Institute of Prevention, Leh, India; [4]Mangalore University, Mangalore, India; [5]DBT-Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India

Introduction: High altitude hypoxia is believed to be experienced at elevations of more than 2500 meters above sea level. Several studies have shed light on the biochemical aspects of high altitude acclimatization, where participants were sojourners to the high altitude from low altitude areas. However, information regarding the difference between the high altitude adapted Tibetans living at high altitude and their counterparts who reside at low altitude are lacking. To understand this, we have measured various hematological parameters in the Tibetan populations, who are residing in both high and low altitudes in India.

Methods: A total of 168 individuals (79 from high altitude ($\geq$4500 meters) and 89 from low altitude (~850 meters) were recruited for this study. Hematological parameters such as red blood cells (RBC) count, hematocrit (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured from the individuals from high and low altitudes. Serum erythropoietin (EPO) was measured by ELISA. Statistical analyses were performed to compare data from both of the altitudes. Gender-wise comparison of data was reported. Correlation analysis was performed within relevant parameters.

Results: Highly significant differences ($p < 0.0001$) between high and low altitude Tibetans were detected in RBC count, HCT, Hb, MCHC in both males and females and in MCV in females. In the case of MCHC, however, age and BMI were potential confounders. Nominally significant differences ($p < 0.05$) were detected in MCV and MCH within males. No significant difference in serum EPO level was found between altitude groups, in any gender. No significant correlation was found between serum EPO with Hb as well as serum EPO with HCT.

Discussion: Our study explores significantly lower RBC count, HCT, Hb, MCH, MCHC and higher MCV in long-term Tibetan residents living at low altitude compared to their high altitude counterparts, which is likely due to the outcome of hematological adaptation to a relatively hyperoxic environment in low altitude areas.

Keywords: high altitude, low altitude, Tibetans, hematological parameters, erythropoietin

Introduction

High altitude is clinically defined as altitudes $\geq$2500 meters above sea level, where physiological changes start appearing in vulnerable people.[1] The distinct environmental features of the high altitude area, that are, extreme cold and hypobaric hypoxia, solar radiation and aridity, make it difficult for sea-level residents to cope, at least for a few days after arrival until they acclimatize sufficiently. Often, travelers develop acute mountain sickness (AMS), high-altitude pulmonary edema (HAPE) or high-altitude cerebral edema (HACE); in contrast, native high altitude

Correspondence: Kumarasamy Thangaraj
CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad, 500 007, Telangana, India
Tel +91-40-27192634
Fax +91-40-27160591
Email thangs@ccmb.res.in

dwellers undergo adaptation and natural selection that give them survival benefits in the harsh environment.[2] Tibetans, Andeans and Ethiopians are among the most studied native highlanders; among them, Tibetans are the oldest. Initial colonization of the high altitude Tibetan plateau dates back 25,000–30,000 years.[1,3,4] Prolonged exposure allows the populations to experience physiological and genetic adaptations, as revealed by multiple studies.[5–15] Compared to the Andeans, Tibetans have lower hemoglobin concentration at high altitude.[16] The Tibetans have lower hemoglobin concentration and hematocrit compared to ethnically non-highlander, acclimatized Han migrants at high altitude.[17,18]

Several studies have looked at the genetic aspects of high altitude adaptation and physiological changes.[5–15] However, studies on physiological aspects of high altitude acclimatization or adaptation performed to date have focused on individuals traveling to a high altitude from a low altitude or compared high altitude natives with non-native residents of high altitude areas to a large extent.[11,17,19–22] Studies on ethnically high altitude adapted populations living at low altitude for a long period are also very limited.[23,24] Adaptation is a slow process and might take thousands of years to integrate genetic changes, whereas acclimatization can happen in hours/days to combat the stress in an urgent manner. Therefore, if high altitude adapted populations reside at low altitude for several years, it is likely that they will experience the impact of this environmental shift as the partial pressure of oxygen is higher at low altitude compared to at high altitude.

According to Gustavo Zubieta-Calleja, high altitude residents experience "relative hyperoxia" at sea level, which is an apparent aggressive environment to the high altitude hypoxia-adapted people.[25] This results in their experiencing lower ventilation, probably to combat "relative hyperoxia". Zubieta-Calleja et al reported lower hematocrit after descent from high altitude to low altitude.[26] Decreased hemoglobin has been reported in the Andean natives after arrival at sea level.[27] Studies of people belonging to the same ethnic group but inhabiting high and low altitudes could be interesting as those are rare and could be insightful to understand the impact of the environmental shift. In this study, hence, we have included Tibetan people from high and low altitudes from India. Migration of ethnically Tibetan populations in India can be classified into two distinct categories: (1) migration of the Tibetans to Ladakh (sometimes described as "India's little Tibet") that started apparently during the 9th–10th centuries and continued for centuries thereafter (known as native Ladakhis also), and (2) migration of the Tibetans taking shelter in India during or after 1959 following subdual of the Tibetan uprising in 1959 by the People's Liberation Army of China. The later migrants reside in various Tibetan settlements across India, including places at high altitude, Ladakh, and low altitude, Karnataka.[28–31] Therefore, in our study, the high altitude group comes from different regions of Ladakh (altitude ≥4500 meters), and the low altitude group comes from Karnataka state (altitude ~850 meters). In the current study, we aimed to investigate how hematological parameters change in response to an extreme environmental shift (altitude) in the Tibetan population.

Materials and Methods
Participants and Study Design
Our study includes a total of 79 high altitude Tibetans from Samad Rakchan village and adjacent pastures (Norchen, Dipling, Kumlung), having an average elevation of 4500–4900 meters above sea level (barometric pressure <430 mmHg) in the Rupsho valley (part of the Changtang region of the Tibetan plateau, extending to Southeastern Ladakh) of the union territory of Ladakh, India. Another set of samples includes 89 low altitude Tibetans from a small town, Bylakuppe, having an average elevation of ~850 meters above sea level (barometric pressure ~690 mmHg), in Karnataka state, India, (Figure 1). People of non-Tibetan descent were excluded from our study.

High altitude Tibetans in our study belong to semi-nomadic and pastoral communities and low altitude Tibetans are dependent on agriculture, handicraft and trade.[32] In Ladakh, we organized health camps during July, 2017 and August, 2018 at the above-mentioned sites. In Karnataka, camps were organized during February−April, 2018. The current study was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants after explaining the purpose of the study. Consent was obtained from the father of a lone participant who was under the age of 18 years. The Institutional Ethical Committee (IEC) of the CSIR - Centre for Cellular and Molecular Biology, Hyderabad, the Institutional Review Board of the Ladakh Institute of Prevention and the District Ethical Committee, Leh, Ladakh approved the study.

About 3.0 mL of venous blood was collected in EDTA vacutainers (BD, New Jersey, USA) for the
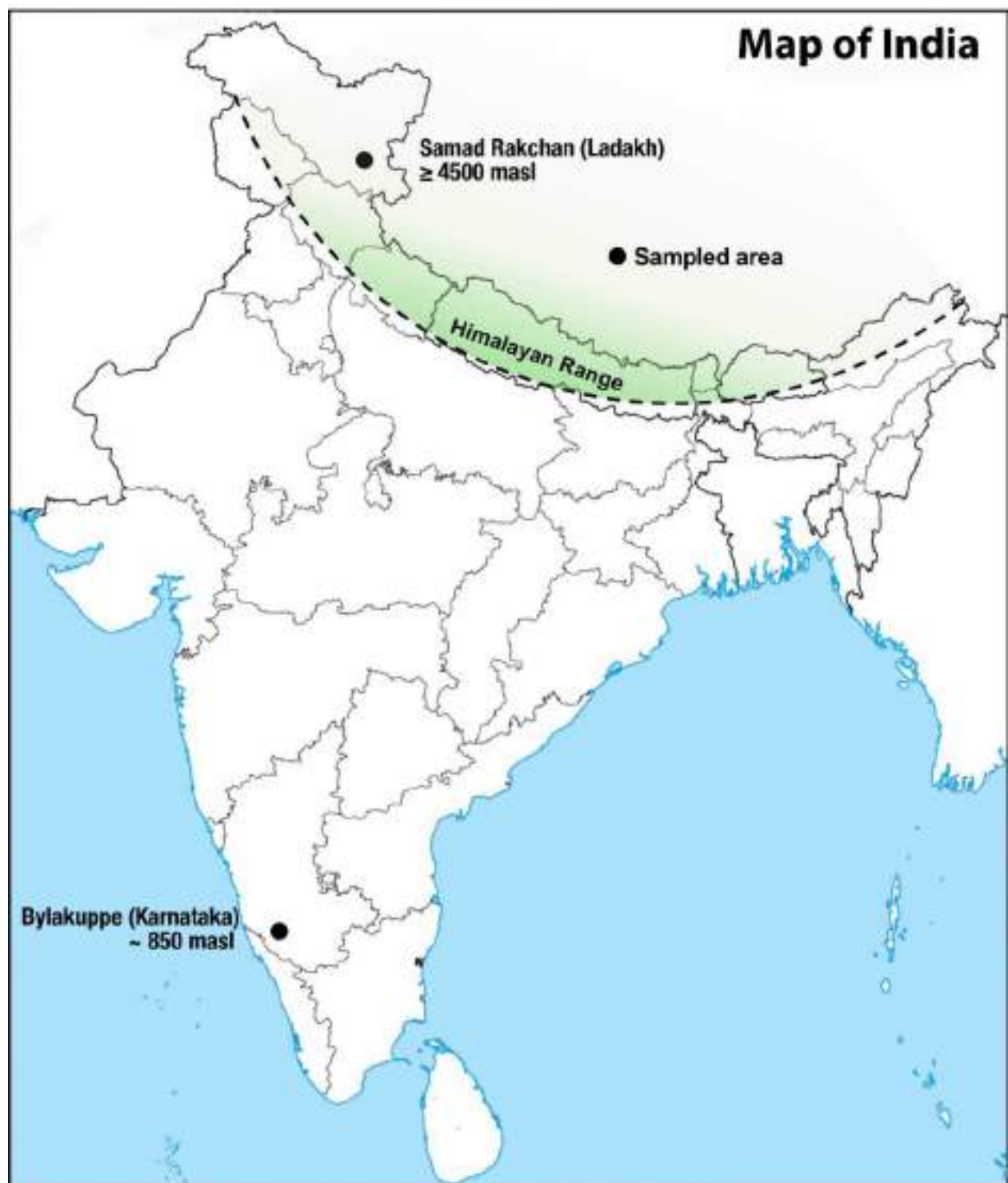
Figure 1 India map showing sampled area. The distance between Samad Rakchan and Bylakuppe is ~2320 km.
Abbreviation: masl, meters above sea level.

assessment of hematological parameters. About 3.0 mL of blood was also collected in vacutainer serum tubes (BD, New Jersey, USA) and were kept at room temperature for half an hour. Clots were removed by centrifuging at 2000 x g for 10 minutes. Clear serum was immediately transferred into clean polypropylene tubes, which were kept in dry ice temporarily and stored at −80 °C for further experiments.

Anthropometric and Hematological Data

Age and gender data were collected from all participants. Height and weight were measured. Body mass index (BMI) was calculated by dividing the weight in kilograms of the individuals by their height, in meters squared. Hematological data were acquired using ADVIA 2120i automated hematology analyzer (Siemens AG, Erlangen, Germany) for the low altitude participants. For high altitude participants, hematological data were obtained using manual techniques, where WBC and RBC were counted by microscopic examination of Leishman-stained blood smears within 3 days of sample collection. HCT was measured using Wintrobe hematocrit tubes.[33] Hemoglobin was measured using Sahli's method on the spot.[34] RBC cellular indices were calculated accordingly.[35] We analyzed WBC, RBC, HCT, MCV, MCH, MCHC and Hb parameters. A quantikine human erythropoietin kit from R & D Systems (Minneapolis, MN, USA) was used to measure serum EPO levels in high and low altitude Tibetans.

Data Analysis

GraphPad Prism trial version 8.4.3 (San Diego, CA) was utilized for statistical analysis. The distribution of the variables was checked using the D'Agostino-Pearson omnibus normality test. For comparison between the two groups, unpaired *t*-tests with Welch's correction were performed where residuals followed Gaussian distribution and Mann–Whitney tests were performed where residuals did not follow Gaussian distribution. Spearman correlation coefficients were calculated to assess the correlation between parameters. Univariate and multiple linear regression analyses were performed in R (version 3.6.1). P-values of less than 0.05 were considered to be significant.

Results
Anthropometric and Hematological Parameters

Our study includes a total of 168 individuals, of whom 79 were from high altitude and 89 were from low altitude areas, with ages ranging from 24 to 80 years and 17 to 92 years, respectively. The median age was 58 years. The age distribution of participants is provided in Figures 2A and B. BMI of the participants was in the range 14.24–34.48 kg/m^2 and median BMI was 23.82 kg/m^2. Table 1 shows that high

altitude participants had lower mean age and BMI compared to low altitude participants.

Highly significant differences (p <0.0001) in RBC, HCT and hemoglobin concentration were detected between the high and low altitude Tibetans (Figures 3A–C). Mean values of RBC and HCT were higher in high altitude males and females compared to low altitude males and females, respectively. Mean values of RBC in males from high altitude and low altitude were 6.21 ± 0.74 and 4.45 ± 0.49 million cells/µL, respectively. In females, these were 5.4 ± 0.40 and 4.19 ± 0.40 million cells/µL, respectively. Mean values of HCT in high altitude males, low altitude males, high altitude females and low altitude females were 59.83 ± 6.89, 44.57 ± 4.56, 50.09 ± 3.6, 42.5 ± 4.06%, respectively. Hemoglobin concentration was significantly (p <0.0001) higher in high altitude individuals, irrespective of gender, compared to low altitude individuals. Mean hemoglobin concentrations in males from high and low altitudes were 20.01 ± 1.91 and 13.42 ± 1.75 g/dL, respectively. In females, these were 16.97 ± 1.74 and 12.56 ± 1.19 g/dL, respectively. MCV was significantly (p <0.05 in males and p <0.0001 in females) lower in both genders at high altitude compared to their low altitude counterparts, with mean values of 96.6 ± 8.06 and 100.5 ± 6.97 fL in males and 92.7 ± 6.17 and 101.5 ± 4.67 fL in females from high and low altitudes, respectively (Figure 3D). MCH showed a nominally significant difference (p <0.05) only in males, with mean values of 32.29 ± 2.51 and 30.27 ± 3.40 pg/cell in high altitude and low altitude males, respectively (Figure 3E). A significant difference was also detected in MCHC in individuals of both genders; however, age and gender were potential confounders (Figure 3F). Details of the tests performed and statistical parameters are given in Tables 2 and 3.

Univariate linear regression analysis was performed separately with age, gender, altitude and BMI for each blood parameter. Out of these (age, gender, altitude and BMI), whichever showed significant association with the blood parameter, were considered for multiple linear regression analysis as potential confounding factors. Complete details of univariate and multiple linear regression analyses are provided in Supplementary Tables S1–S6. In multiple regression models, age and BMI did not show significant associations with most of the blood parameters (p >0.05) except MCHC and hemoglobin concentration. Both age and BMI showed a significant association with MCHC (p-values 0.0200 and 0.0232, respectively). However, only BMI (but not age) was significantly associated with hemoglobin concentration (p-value 0.0386) in the model and had a very
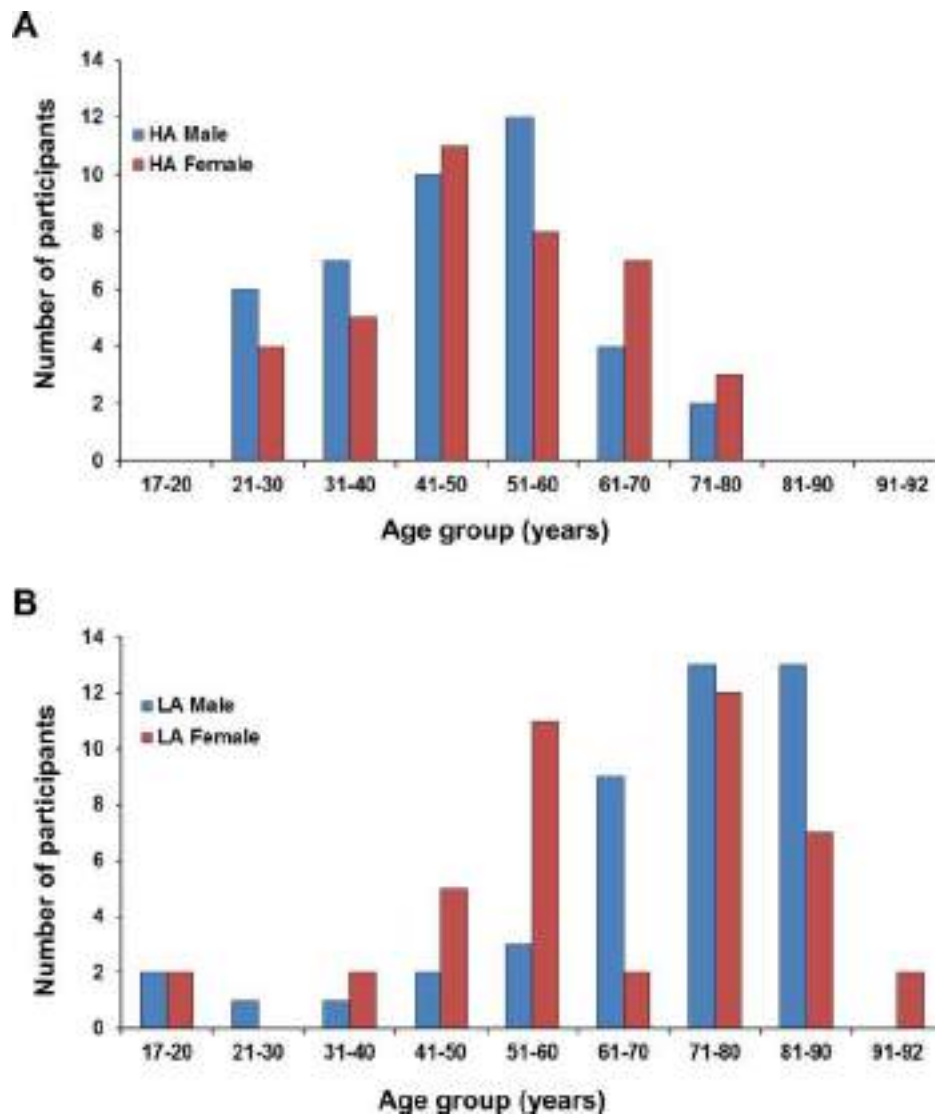
Figure 2 Age distribution among (**A**) high altitude and (**B**) low altitude participants.
Abbreviations: HA, high altitude; LA, low altitude.

small regression coefficient (0.0723), after correcting for confounding factors.

Additionally, we categorized our participants (up to 80 years, maximum age of high altitude participants) into three age categories, and checked the differences in hemoglobin concentrations between them. Details of BMI in these age categories are provided in Table 4. Hemoglobin concentration was significantly different in high altitude

Table I Description of Anthropometric Parameters of the Subjects (n=168); Data is Expressed in Mean ± SD Format

Parameter	High Altitude	Low Altitude	Test, p-value
Age (years)	49.32 ± 13.70	67.15 ± 18.42	Mann-Whitney test, <0.0001
BMI (kg/m^2)	22.75 ± 3.67	25.28 ± 4.78	Unpaired *t*-test with Welch's correction, 0.0004
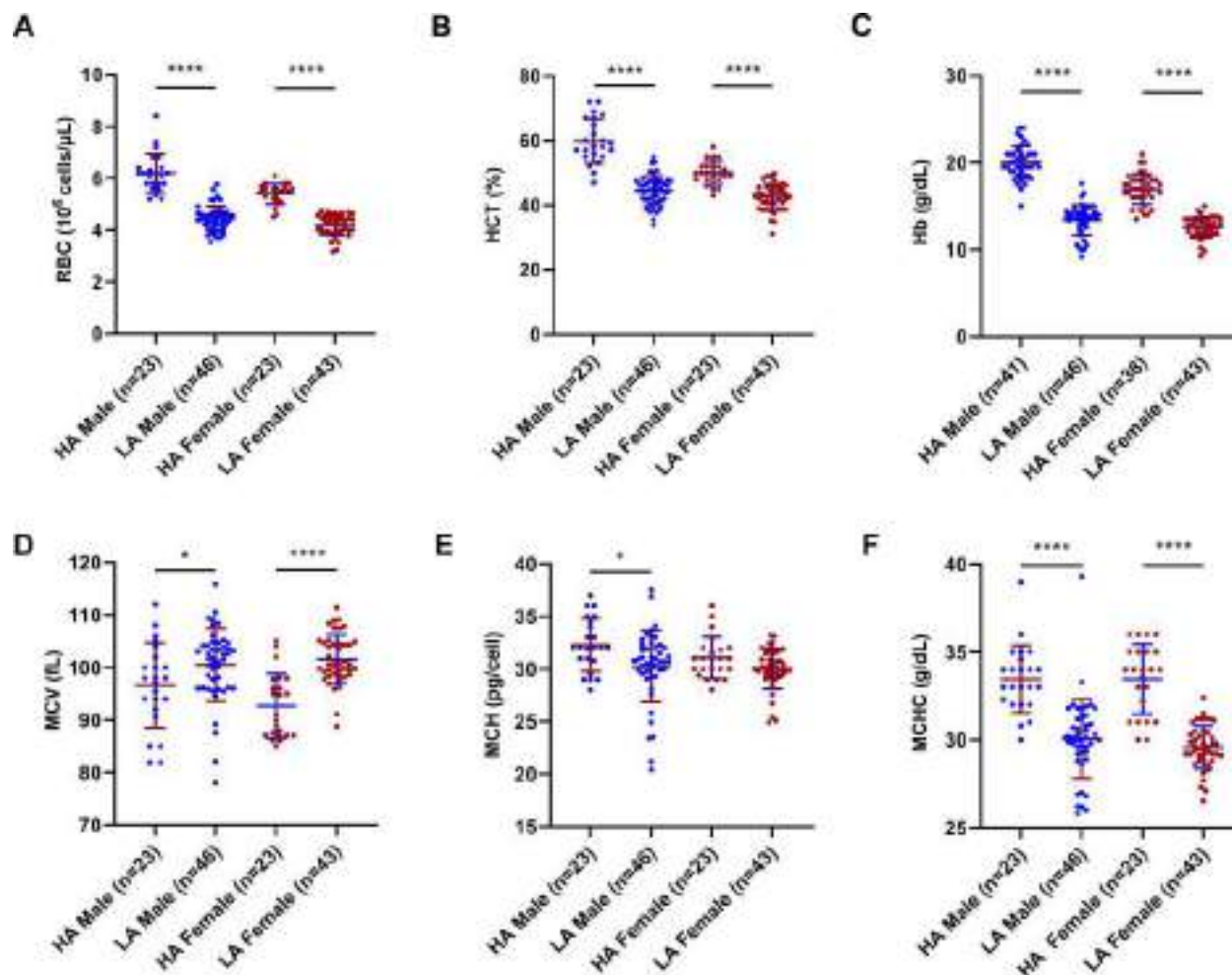
Figure 3 Various hematological parameters of high and low altitude Tibetan males and females: (**A**) RBC, (**B**) HCT, (**C**) Hb, (**D**) MCV, (**E**) MCH and (**F**) MCHC. Blue and maroon horizontal lines represent mean ± SD. ****p <0.0001, *p = 0.01–0.05.
Abbreviations: HA, high altitude; LA, low altitude; RBC, red blood cell; HCT, hematocrit; Hb, hemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

and low altitude participants, after categorization of age as well (Figures 4A–C; Table 5). For other parameters, age categorization could not be performed, as the number of individuals in each age category was very low or absent.

Serum EPO

Serum EPO is one of the prominent outcomes of altitude-induced hypoxia that becomes elevated in people traveling to high altitudes. We checked serum EPO levels within high and low altitude Tibetan males and females. No significant difference was observed in either males or females, with mean values of 13.3 ± 11.61, 11.32 ± 7.77 mIU/mL in males and 13.21 ± 6.98 and 11.31 ± 7.63 mIU/mL in females from high and low altitudes, respectively (Figure 5; Table 3).

In an earlier study, serum EPO was shown to be correlated with HCT and hemoglobin concentration in children

and young adults from Ladakh by Yanamandra et al.[36] Since one group of our study participants is also from Ladakh, we checked the correlation of serum EPO with hemoglobin concentration and HCT of the participants. Both serum EPO and hemoglobin concentration data were available from 130 participants and both serum EPO and HCT data were available from 107 participants. Interestingly, none of these parameters showed any significant correlation (Spearman r = −0.03984, p-value = 0.6526 for serum EPO and Hb) with serum EPO in our study (Spearman r = 0.1562, p-value = 0.1081 for serum EPO and HCT).

Discussion

Change of hematological parameters has been shown to be associated with various pathological conditions like myocardial infarction, leukemia, coronary heart disease,

Table 2 Description of Various Hematological Parameters in the Subjects; Data is Expressed in Mean ± SD Format

Parameter	HA Male (n=23)	LA Male (n=46)	HA Female (n=23)	LA Female (n=43)	Test, p-value (HA Male vs LA Male); Test, p-value (HA Female vs LA Female)
WBC (cells/μL)	6957 ± 1788	6744 ± 1682	6743 ± 1938	7277 ± 1472	Mann-Whitney test, 0.7022; Unpaired t-test with Welch's correction, 0.2565
RBC (10⁶ cells/μL)	6.21 ± 0.74	4.45 ± 0.49	5.4 ± 0.40	4.19 ± 0.40	Mann-Whitney test, <0.0001; Unpaired t-test with Welch's correction, <0.0001
HCT (%)	59.83 ± 6.89	44.57 ± 4.56	50.09 ± 3.6	42.5 ± 4.06	Unpaired t-test with Welch's correction, <0.0001; Unpaired t-test with Welch's correction, <0.0001
MCV (fL)	96.6 ± 8.06	100.5 ± 6.97	92.7 ± 6.17	101.5 ± 4.67	Mann-Whitney test, 0.0280; Unpaired t-test with Welch's correction, <0.0001
MCH (pg/cell)	32.29 ± 2.51	30.27 ± 3.40	31.07 ± 1.97	30.04 ± 1.92	Mann-Whitney test, 0.0212; Mann Whitney test, 0.1347
MCHC (g/dL)	33.47 ± 1.92	30.07 ± 2.26	33.47 ± 1.99	29.58 ± 1.23	Mann-Whitney test, <0.0001; Unpaired t-test with Welch's correction, <0.0001

cardiovascular disease and so on.[37–40] Our study shows differences in hematological parameters between Tibetans residing at high and low altitudes for the first time, to the best of our knowledge. We observed significantly higher RBC, HCT and hemoglobin concentration and significantly lower MCV in high altitude males and females compared to their low altitude counterparts. Though BMI showed a significant association with hemoglobin concentration (p-value = 0.0386) after adjusting for age, gender, BMI and altitude, the coefficient of its association was too small (0.0723) to be considered a strong confounder, considering apparently small mean BMI differences (2.53 kg/m^2) and apparently high mean hemoglobin concentration differences between high and low altitude

male (6.59 g/dl) and female (4.41 g/dl) Tibetans. In our regression model for hemoglobin concentration (Hemoglobin concentration + Age + Gender + BMI + Altitude), the coefficient of altitude (low) was −5.5356 (p-value <2.25×10^{-7}), which suggests that, on average, hemoglobin concentration is ~5.5 g/dl lower in low altitude individuals compared to high altitude individuals. Our observed values of hemoglobin concentration were a little higher than some studies conducted with high altitude Tibetans; however, it was reported that, at an elevation of more than 4500 meters, hemoglobin concentration in Tibetans also showed higher mean values.[16,18,41–43] The hemoglobin concentration observed in our study for the low altitude Tibetans was on a par with another study with Tibetans at sea
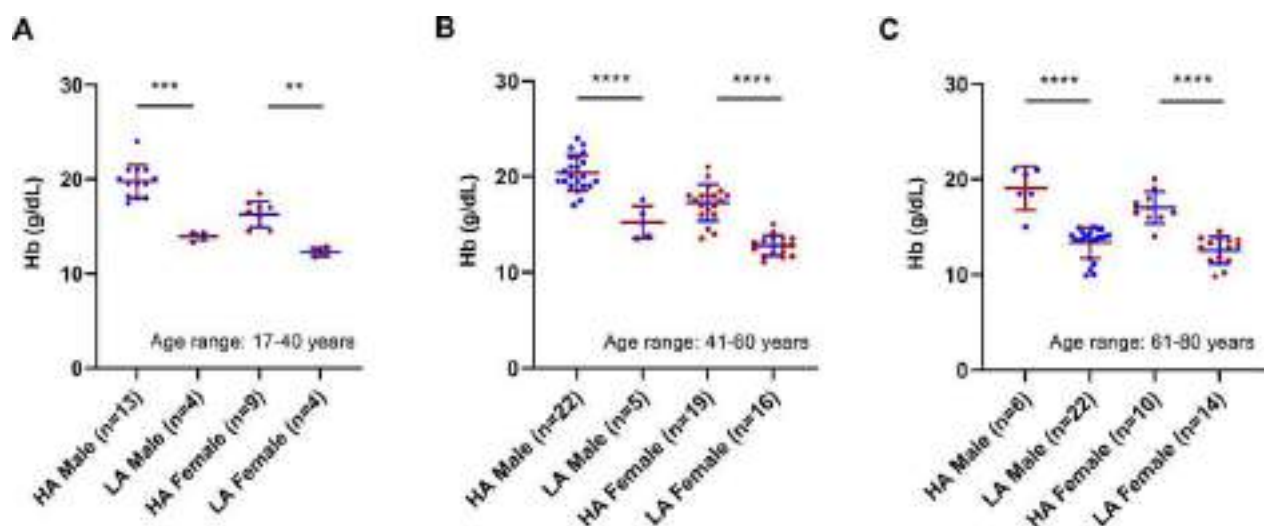
Figure 4 Hemoglobin concentration (Hb) among age-categorized participants: (**A**) in the age range of 17–40 years, (**B**) 41–60 years, (**C**) 61–80 years. ****p <0.0001, ***p = 0.0001–0.001, **p = 0.001–0.01. Blue and maroon horizontal lines represent mean ± SD.
Abbreviations: HA, high altitude; LA, low altitude.
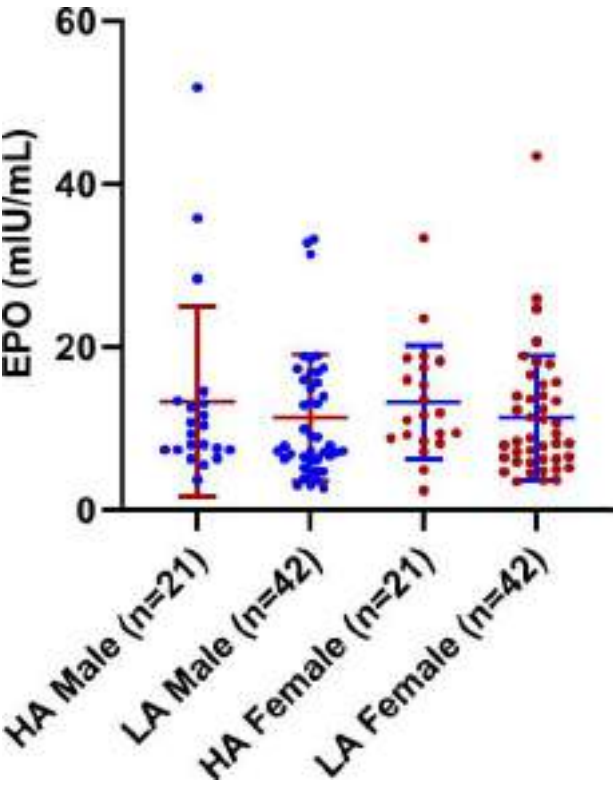
Figure 5 Distribution of serum erythropoietin (EPO) level among participants.
Abbreviations: HA, high altitude; LA, low altitude.

level.[44] Since mean hemoglobin concentration was apparently high in our high altitude participants, we checked their chronic mountain sickness (CMS) scores, which had been recorded during the current health camp or a previous health camp organized in similar places. Data were available for the CMS scores from 41 male and 36 female participants. Among them, 12 males and 5 females had mild CMS, having scores of between 6 to 10 as per the Qinghai scoring system, resulting in a 22.07% CMS prevalence (unpublished data, Basak/Norboo et al).[45] Earlier studies have reported a CMS prevalence of 13.73–28.7% in high altitude natives of the neighboring state, Himachal Pradesh.[46,47] The Tibetans are known to have a lower prevalence of CMS compared to the

Andeans.[48–50] Though our participants had a comparatively higher prevalence of CMS, all of them had mild CMS. Though RBC, HCT and hemoglobin were significantly higher in the high altitude Tibetans, interestingly, serum EPO was only insignificantly higher in high altitude individuals; this might be because the normal concentration of serum erythropoietin has an apparently broad range: 3.3–16.6 mIU/mL.[51] A similar observation was reported by Winslow et al, whereby HCT and hemoglobin were higher in Andean natives compared to Sherpas from Nepal living at the same altitude, yet serum erythropoietin was not significantly different.[52] Moreover, serum EPO becomes elevated upon acute hypoxic exposure, when low altitude residents visit high altitude areas. Within a few days, it returns to almost the pre-exposure level. Both our high altitude and low altitude groups comprise long-term residents of the respective altitudes. Our observation of an almost similar level of serum EPO in both groups is consistent with that as well. A recent study showed that blood cell phenotypes are ancestry-dependent and selective pressure can give rise to different blood cell traits.[53] Our study additionally shows that different exposure of the same population to different environments can also alter blood cell traits. These changes of hematological parameters are possibly part of the hematological adaptation mechanism, to cope with the "relative hyperoxia" that is evidently an aggressive environment to the high altitude hypoxia-adapted Tibetans when they migrate to low altitude.

It has previously been hypothesized that hemoglobin concentration might be modulated by environmental context.[54] Our study reveals differences in various hematological parameters including hemoglobin concentration between the high and low altitude Tibetans, which fits the hypothesis well. Hemoglobin concentration has been shown to play an important role in reproductive fitness among Tibetan women, where lower hemoglobin favored reproductive fitness.[55] Similarly, Tibetan men, having lower hemoglobin concentrations are known to show better exercise capacity at high altitude.[41] It would be interesting to

Table 3 Description of Serum EPO and Hb in the Subjects; Data is Expressed in Mean ± SD Format

Parameter	HA Male	LA Male	HA Female	LA Female	Test, p-value (HA Male vs LA Male); Test, p-value (HA Female vs LA Female)
EPO (mIU/mL)	13.3 ± 11.61 (n=21)	11.32 ± 7.77 (n=42)	13.21 ± 6.98 (n=21)	11.31 ± 7.63 (n=42)	Mann-Whitney test, 0.4826; Mann-Whitney test, 0.1283
Hb (g/dL)	20.01 ± 1.91 (n=41)	13.42 ± 1.75 (n=46)	16.97 ± 1.74 (n=38)	12.56 ± 1.19 (n=43)	Unpaired t-test with Welch's correction, <0.0001; Unpaired t-test with Welch's correction, <0.0001

Table 4 Description of BMI Among Participants Belonging to Three Different Age Categories; Data is Expressed in Mean ± SD Format

Age Category	BMI				Test, p-value (HA Male vs LA Male); Test, p-value (HA Female vs LA Female)
	HA Male	**LA Male**	**HA Female**	**LA Female**	
17–40 years	20.46 ± 2.18 (n=13)	23.83 ± 7.25 (n=3)	24.34 ± 3.74 (n=9)	28.08 ± 5.35 (n=4)	Mann-Whitney test, 0.5643; Mann-Whitney test, 0.3119
41–60 years	21.66 ± 3.01 (n=22)	24.44 ± 5.18 (n=5)	23.83 ± 3.78 (n=19)	25.98 ± 3.86 (n=10)	Mann-Whitney test, 0.1413; Mann-Whitney test, 0.0915
61–80 years	24.33 ± 4.89 (n=6)	23.35 ± 4.50 (n=22)	23.7 ± 4.08 (n=10)	28.88 ± 4.24 (n=8)	Mann-Whitney test, 0.7745; Mann-Whitney test, 0.0143

Table 5 Description of Hemoglobin Concentration Among Participants Belonging to Three Different Age Categories; Data is Expressed in Mean ± SD Format

Age Category	Hemoglobin Concentration (g/dl)				Test, p-value (HA Male vs LA Male); Test, p-value (HA Female vs LA Female)
	HA Male	**LA Male**	**HA Female**	**LA Female**	
17–40 years	19.78 ± 1.7 (n=13)	13.9 ± 0.49 (n=4)	16.28 ± 1.39 (n=9)	12.3 ± 0.44 (n=4)	Mann-Whitney test, 0.0008; Mann-Whitney test, 0.0028
41–60 years	20.4 ± 1.87 (n=22)	15.24 ± 1.69 (n=5)	17.26 ± 1.90 (n=19)	12.79 ± 1.04 (n=16)	Mann-Whitney test, <0.0001; Mann-Whitney test, <0.0001
61–80 years	19.08 ± 2.30 (n=6)	13.34 ± 1.59 (n=22)	17.05 ± 1.69 (10)	12.56 ± 1.41 (14)	Mann-Whitney test, <0.0001; Mann-Whitney test, <0.0001

investigate those events separately in the low altitude Tibetans to check whether the trend is true since hemoglobin concentration was strikingly lower in the Tibetans from low altitude areas in our study.

John B. West discussed "Barcroft's bold assertion" that assumes "All dwellers at high altitudes are persons of impaired physical and mental powers." This is a provocative statement; nonetheless, it does provide scope to study native highlanders at low altitude and to assess their health status.[56] Our study is a step in that direction, which would help to understand the physiological aspects better when native Tibetans are not present in their native high altitude environment. Simultaneously, these kinds of studies would help to understand the impact of environmental change, knowledge of which could be important for tourists and soldiers traveling to high altitude areas. It would be interesting to explore the molecular driving forces behind these kinds of differences. Epigenetic studies, important tools for exploring gene–environment interaction, could be fruitful in this context.

A limitation of our study is that we could not measure complete blood count (CBC) profiles of the high altitude and low altitude individuals using the same method because of geographical differences between the sampling sites, remote locations and limited resources (at high altitude). CBC profiles for the high altitude samples were obtained using manual techniques; while for the low altitude samples, these were obtained using an automated hematology analyzer. However, both of the methods are known to be well-correlated.[33,57,58] Harris et al reported good correlation between ADVIA 2120 (previous version of ADVIA 2120i) and the manual method in an international, multicenter clinical trial. Values reported for WBC differentials measured by the ADVIA 2120 analyzer and the manual method in that study were close. The within-run precision of CBC on the platform was also very good.[59] We used Sahli's method, one of the most commonly used methods for hemoglobin estimation where resources are limited, for estimating hemoglobin concentration in remote high altitude areas. This method has been proven to be efficient, resulting in an absolute difference between two measurements of less than 1 g/dl. Another study reported that Sahli's method provides lower values of hemoglobin concentration of 0.62gms/dl in capillary

blood and 1.1gms/dl in venous blood compared to a hemiglobincyanide (HiCN)-based reference method. Our observed differences in mean hemoglobin concentration between high and low altitude male and female Tibetans were 6.59 g/dl and 4.41 g/dl, respectively, which are far above these values. Moreover, Sahli's method was used to measure hemoglobin concentration in high altitude individuals, in whom we observed higher values than in low altitude individuals. On the other hand, technical specifications of ADVIA 2120i show that the precision of the instrument is good, with a coefficient of variation for MCV, Hb, RBC, WBC in the range of 0.78–2.7.[60]

Considering all these facts, it is highly unlikely that our observed differences in hematological parameters in high and low altitude individuals are due to artifacts. We needed to be a bit careful while selecting the parameters for analysis considering the gap between sample collection and hematological data collection. As the samples were collected from remote areas, due to logistic issues, hematological data collection (except hemoglobin measurement by Sahli's method) of the samples could be done within 48–72 hours after sample collection. A recent meta-analysis and systematic review reports that Hb, WBC, RBC count, HCT, MCH, MCHC are quite stable within the time range in which we collected and measured them.[61]

Conclusion

In conclusion, our study reveals that prolonged residence of native Tibetans at a low altitude (over 50 years) results in significantly lower RBC count, HCT, Hb, MCH, MCHC and higher MCV compared to their high altitude counterparts, which is likely due to the outcome of hematological adaptation to the relatively hyperoxic environment in low altitude areas.

Acknowledgments

Disclosure

The authors declared no conflicts of interest for this work.

References

1. Beall CM. Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proc Natl Acad Sci U S A*. 2007;104(Suppl 1):8655–8660. doi:10.1073/pnas.0701985104

2. Luks AM, Swenson ER, Bärtsch P. Acute high-altitude sickness. *Eur Respir Rev*. 2017;26(143):143. doi:10.1183/16000617.0096-2016

3. Aldenderfer MS. Moving up in the World: archaeologists seek to understand how and when people came to occupy the Andean and Tibetan plateaus. *Am Sci*. 2003;91(6):542–549. doi:10.1511/2003.6.542

4. Aldenderfer M. Peopling the Tibetan plateau: insights from archaeology. *High Alt Med Biol*. 2011;12(2):141–147. doi:10.1089/ham.2010.1094

5. Simonson TS, Yang Y, Huff CD, et al. Genetic evidence for high-altitude adaptation in Tibet. *Science*. 2010;329(5987):72–75. doi:10.1126/science.1189406

6. Yang J, Jin ZB, Chen J, et al. Genetic signatures of high-altitude adaptation in Tibetans. *Proc Natl Acad Sci U S A*. 2017;114 (16):4189–4194. doi:10.1073/pnas.1617042114

7. Jeong C, Alkorta-Aranburu G, Basnyat B, et al. Admixture facilitates genetic adaptations to high altitude in Tibet. *Nat Commun*. 2014;5 (1):3281. doi:10.1038/ncomms4281

8. Huerta-Sánchez E, Jin X, Bianba Z, et al. Altitude adaptation in Tibetans caused by introgression of Denisovan-like DNA. *Nature*. 2014;512(7513):194–197. doi:10.1038/nature13408

9. Zhuang J, Droma T, Sun S, et al. Hypoxic ventilatory responsiveness in Tibetan compared with Han residents of 3658 m. *J Appl Physiol*. 1993;74(1):303–311. doi:10.1152/jappl.1993.74.1.303

10. Bigham A, Bauchet M, Pinto D, et al. Identifying signatures of natural selection in Tibetan and Andean populations using dense genome scan data. *PLoS Genet*. 2010;6(9):e1001116. doi:10.1371/journal.pgen.1001116

11. Droma T, McCullough RG, McCullough RE, et al. Increased vital and total lung capacities in Tibetan compared to Han residents of Lhasa (3658 m). *Am J Phys Anthropol*. 1991;86(3):341–351. doi:10.1002/ajpa.1330860303

12. Moore LG. Measuring high-altitude adaptation. *J Appl Physiol*. 2017;123(5):1371–1385. doi:10.1152/japplphysiol.00321.2017

13. Brutsaert TD. Population genetic aspects and phenotypic plasticity of ventilatory responses in high altitude natives. *Respir Physiol Neurobiol*. 2007;158(2–3):151–160. doi:10.1016/j.resp.2007.03.004

14. Gassmann M, Muckenthaler MU. Adaptation of iron requirement to hypoxic conditions at high altitude. *J Appl Physiol*. 2015;119 (12):1432–1440. doi:10.1152/japplphysiol.00248.2015

15. West JB. Other Recent High-Altitude Studies. In: *High Life*. New York, NY: Springer; 1998:364–400.

16. Beall CM, Brittenham GM, Strohl KP, et al. Hemoglobin concentration of high-altitude Tibetans and Bolivian Aymara. *Am J Phys Anthropol*. 1998;106(3):385–400. doi:10.1002/(SICI)1096-8644-(199807)106:3<385::AID-AJPA10>3.0.CO;2-X

17. Garruto RM, Chin CT, Weitz CA, Liu JC, Liu RL, He X. Hematological differences during growth among Tibetans and Han Chinese born and raised at high altitude in Qinghai, China. *Am J Phys Anthropol*. 2003;122(2):171–183. doi:10.1002/ajpa.10283

18. Wu T, Wang X, Wei C, et al. Hemoglobin levels in Qinghai-Tibet: different effects of gender for Tibetans vs. Han. *J Appl Physiol*. 2005;98(2):598–604. doi:10.1152/japplphysiol.01034.2002

19. Basu M, Malhotra AS, Pal K, et al. Erythropoietin levels in lowlanders and high-altitude natives at 3450 m. *Aviat Space Environ Med*. 2007;78(10):963–967. doi:10.3357/ASEM.2085.2007

20. Brothers MD, Wilber RL, Byrnes WC. Physical fitness and hematological changes during acclimatization to moderate altitude: a retrospective study. *High Alt Med Biol*. 2007;8(3):213–224. doi:10.1089/ham.2007.8308

21. Akunov A, Sydykov A, Toktash T, Doolotova A, Sarybaev A. Hemoglobin changes after long-term intermittent work at high altitude. *Front Physiol*. 2018;9:1552. doi:10.3389/fphys.2018.01552

22. Moore LG, Young D, McCullough RE, Droma T, Zamudio S. Tibetan protection from intrauterine growth restriction (IUGR) and reproductive loss at high altitude. *Am J Hum Biol*. 2001;13(5):635–644. doi:10.1002/ajhb.1102

23. Petousi N, Robbins PA. Human adaptation to the hypoxia of high altitude: the Tibetan paradigm from the pregenomic to the postgenomic era. *J Appl Physiol*. 2014;116(7):875–884. doi:10.1152/japplphysiol.00605.2013

24. Gelfi C, De Palma S, Ripamonti M, et al. New aspects of altitude adaptation in Tibetans: a proteomic approach. *FASEB J*. 2004;18(3):612–614. doi:10.1096/fj.03-1077fje

25. Zubieta-Calleja G. *Human Adaptation to High Altitude and to Sea Level: Acid-Base Equilibrium, Ventilation and Circulation in Chronic Hypoxia*. 1st ed. Düsseldorf, German: VDM Verlag Dr. Müller; 2010.

26. Zubieta-Calleja GR, Paulev PE, Zubieta-Calleja L, Zubieta-Castillo G. Altitude adaptation through hematocrit changes. *J Physiol Pharmacol*. 2007;58 Suppl 5(Suppl Pt 2):811–818.

27. McKenzie DC, Goodman LS, Nath C, et al. Cardiovascular adaptations in Andean natives after 6 wk of exposure to sea level. *J Appl Physiol*. 1991;70(6):2650–2655. doi:10.1152/jappl.1991.70.6.2650

28. Prakash LTO. Tibetan refugees in India: the case of Bylakuppe in Karnataka. *Indian J Soc Dev*. 2011;11(2):503–515.

29. Jian C. The Tibetan Rebellion of 1959 and China's changing relations with India and the Soviet Union. *J Cold War Stud*. 2006;8(3):54–101. doi:10.1162/jcws.2006.8.3.54

30. Petech L. *The Kingdom of Ladakh: C. 950-1842 A.D*. Rome: Istituto italiano per il Medio ed Estremo Oriente; 1977.

31. Goldstein MC. *Tibet, China, and the United States: Reflections on the Tibet Question*. Washington, D.C.: The Atlantic Council of the United States; 1995.

32. Tamang R, Chaubey G, Nandan A, et al. Reconstructing the demographic history of the Himalayan and adjoining populations. *Hum Genet*. 2018;137(2):129–139. doi:10.1007/s00439-018-1867-2

33. Mondal H, Budh DP. Hematocrit; 2020 July 10. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. 2020 Jan. Available from: https://www.ncbi.nlm.nih.gov/books/NBK542276/. Accessed March 03, 2021.

34. van Lerberghe W, Keegels G, Cornelis G, Ancona C, Mangelschots E, van Balen H. Haemoglobin measurement: the reliability of some simple techniques for use in a primary health care setting. *Bull World Health Organ*. 1983;61(6):957–965.

35. Maakaron JE, Taher AT, Conrad ME. Anemia Workup; 2019. Avialable from: https://emedicine.medscape.com/article/198475-workup#c8. Accessed March 03, 2021.

36. Yanamandra U, Senee H, Yanamadra S, et al. Erythropoietin and ferritin response in native highlanders aged 4–19 years from the Leh-Ladakh region of India. *Br J Haematol*. 2019;184(2):263–268. doi:10.1111/bjh.15553

37. Lee G, Choi S, Kim K, et al. Association of hemoglobin concentration and its change with cardiovascular and all-cause mortality. *J Am Heart Assoc*. 2018;7(3).

38. Smith M, Arthur D, Camitta B, et al. Uniform approach to risk classification and treatment assignment for children with acute lymphoblastic leukemia. *J Clin Oncol*. 1996;14(1):18–24. doi:10.1200/JCO.1996.14.1.18

39. Horne BD, Anderson JL, John JM, et al. Which white blood cell subtypes predict increased cardiovascular risk? *J Am Coll Cardiol*. 2005;45(10):1638–1643. doi:10.1016/j.jacc.2005.02.054

40. Ensrud K, Grimm RH Jr The white blood cell count and risk for coronary heart disease. *Am Heart J*. 1992;124(1):207–213. doi:10.1016/0002-8703(92)90942-O

41. Simonson TS, Wei G, Wagner HE, et al. Low haemoglobin concentration in Tibetan males is associated with greater high-altitude exercise capacity. *J Physiol*. 2015;593(14):3207–3218. doi:10.1113/JP270518

42. Wagner PD, Simonson TS, Wei G, et al. Sea-level haemoglobin concentration is associated with greater exercise capacity in Tibetan males at 4200 m. *Exp Physiol*. 2015;100(11):1256–1262. doi:10.1113/EP085036

43. Beall CM, Goldstein MC. Hemoglobin concentration of pastoral nomads permanently resident at 4850–5450 meters in Tibet. *Am J Phys Anthropol*. 1987;73(4):433–438. doi:10.1002/ajpa.1330730404

44. Petousi N, Croft QP, Cavalleri GL, et al. Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *J Appl Physiol*. 2014;116(7):893–904. doi:10.1152/japplphysiol.00535.2013

45. León-Velarde F, Maggiorini M, Reeves JT, et al. Consensus statement on chronic and subacute high altitude diseases. *High Alt Med Biol*. 2005;6(2):147–157. doi:10.1089/ham.2005.6.147

46. Sahota IS, Panwar NS. Prevalence of chronic mountain sickness in high altitude districts of Himachal Pradesh. *Indian J Occup Environ Med*. 2013;17(3):94–100. doi:10.4103/0019-5278.130839

47. Negi PC, Asotra S, Marwah R, et al. Epidemiological study of chronic mountain sickness in natives of Spiti Valley in the Greater Himalayas. *High Alt Med Biol*. 2013;14(3):220–229. doi:10.1089/ham.2012.1127

48. Villafuerte FC, Corante N. Chronic mountain sickness: clinical aspects, etiology, management, and treatment. *High Alt Med Biol*. 2016;17(2):61–69. doi:10.1089/ham.2016.0031

49. Monge C, León-Velarde F, Arregui A. Increasing prevalence of excessive erythrocytosis with age among healthy high-altitude miners. *N Engl J Med*. 1989;321(18):1271.

50. Hancco I, Bailly S, Baillieul S, et al. Excessive erythrocytosis and chronic mountain sickness in dwellers of the highest city in the world. *Front Physiol*. 2020;11:773. doi:10.3389/fphys.2020.00773

51. Ahmed P, Chaudhry QU, Satti TM, Raza S, Mahmood SK. Erythrocytosis following allogeneic hemopoietic SCT in three cases of aplastic anemia. *Bone Marrow Transplant*. 2011;46(8):1163–1165. doi:10.1038/bmt.2010.265

52. Winslow RM, Chapman KW, Gibson CC, et al. Different hematologic responses to hypoxia in Sherpas and Quechua Indians. *J Appl Physiol*. 1989;66(4):1561–1569. doi:10.1152/jappl.1989.66.4.1561

53. Chen MH, Raffield LM, Mousas A, et al. Trans-ethnic and ancestry-specific blood-cell genetics in 746,667 individuals from 5 global populations. *Cell*. 2020;182(5):1198–1213.e1114. doi:10.1016/j.cell.2020.06.045

54. Beall CM, Cavalleri GL, Deng L, et al. Natural selection on EPAS1 (HIF2alpha) associated with low hemoglobin concentration in Tibetan highlanders. *Proc Natl Acad Sci U S A*. 2010;107 (25):11459–11464. doi:10.1073/pnas.1002443107

55. Cho JI, Basnyat B, Jeong C, et al. Ethnically Tibetan women in Nepal with low hemoglobin concentration have better reproductive outcomes. *Evol Med Public Health*. 2017;2017(1):82–96. doi:10.1093/emph/eox008

56. West JB. Barcroft's bold assertion: all dwellers at high altitudes are persons of impaired physical and mental powers. *J Physiol*. 2016;594 (5):1127–1134. doi:10.1113/JP270284

57. Mokhtari M, Najafi S. Evaluation of the correlation of automated and manual results of complete blood count in oncologic patients. *Comp Clin Path*. 2016;25(6):1151–1154. doi:10.1007/s00580-016-2319-9

58. Ike SO, Nubila T, Ukaejiofo EO, Nubila IN, Shu EN, Ezema I. Comparison of haematological parameters determined by the Sysmex KX - 2IN automated haematology analyzer and the manual counts. *BMC Clin Pathol*. 2010;10(1):3. doi:10.1186/1472-6890-10-3

59. Harris N, Jou JM, Devoto G, et al. Performance evaluation of the ADVIA 2120 hematology analyzer: an international multicenter clinical trial. *Lab Hematol*. 2005;11(1):62–70. doi:10.1532/LH96.04064

60. ADVIA 2120i Hematology System with Autoslide. Erlangen: Siemens Healthineers; 2021. Available from: https://www.siemens-healthineers.com/en-us/hematology/systems/advia-2120-hematology-system-with-autoslide. Accessed March 03, 2021.

61. Wu DW, Li YM, Wang F. How Long can we Store Blood Samples: A Systematic Review and Meta-Analysis. *EBioMedicine*. 2017;24:277–285.

A Third of Pregnant Women are Affected by Anemia in Eastern Ethiopia: A Facility-Based Study

Adera Debella, Merga Dheresa, Biftu Geda, Getahun Tiruye & Sagni Girma Fage

ORIGINAL RESEARCH

A Third of Pregnant Women are Affected by Anemia in Eastern Ethiopia: A Facility-Based Study

Adera Debella[1]
Merga Dheresa [1]
Biftu Geda [2]
Getahun Tiruye[1]
Sagni Girma Fage [1]

[1]School of Nursing and Midwifery, College of Health and Medical Sciences, Haramaya University, Harar, Ethiopia; [2]Department of Nursing, College of Health and Medical Sciences, Meda Welabu University, Bale-Robe, Ethiopia

Background: Although it is a public health problem of pregnant women in low, middle and high-income countries, the highest prevalence rate of anemia is found among pregnant women of low-income countries, particularly in Africa. Therefore, this study has aimed to determine the magnitude of the anemia and its associated factors among pregnant women admitted to labor wards of public hospitals in eastern Ethiopia.

Methods and Materials: A health facility-based cross-sectional study was conducted among 405 pregnant women admitted to labor wards in four public hospitals of Harar town and Dire Dawa City Administration. A systematic random sampling technique was used to select the study participants. Data were collected through an interview using a structured questionnaire. The hemoglobin level was measured for each study participant. Binary and multivariable logistic regression models were fitted and statistical significance was declared at $P<0.05$.

Results: The magnitude of anemia was 33.1% (95% CI: 28.4%, 37.8%). Being from rural areas (AOR: 3.8; 95% CI: 1.81, 7.94), no antenatal care (ANC) follow-up (AOR: 3.4; 95% CI: 1.34, 8.79), a habit of drinking milk with tea after meals (AOR: 2.8; 95% CI: 1.48, 5.61), taking a meal only 1–2 times per day (AOR: 3.9; 95% CI: 1.69, 8.97), experiencing no blood loss in the current pregnancy (AOR: 0.25; 95% CI: 0.06, 0.96) and a habit of eating leafy vegetables (AOR: 0.12; 95% CI: 0.06, 0.24) were significantly associated with anemia.

Conclusion: About one in three pregnant women were anemic and anemia was a moderate public health problem in the study settings. Interventions targeting reducing or preventing anemia should focus on pregnant women in rural areas, promoting the benefits of ANC follow-up, reducing the habit of drinking milk with tea after meals, enhancing the consumption of leafy vegetables and increasing meal frequency per day to minimize or prevent anemia and its consequences during pregnancy.

Keywords: anemia, pregnant women, eastern Ethiopia

Introduction

Anemia – a pathologic state, in which the number of red blood cells or their oxygen carrying capacity is insufficient to meet physiologic needs – is a common medical problem during pregnancy. It is a global public health problem with major consequences for human health as well as socio-economic development.[1]

Among 1.62 billion globally anemic people, pregnant women constitute 41.8% and thus, anemia remains a significant public health problem. The highest prevalence rate (61.3%) is found among pregnant women from Africa.[2] Rates of anemia are highest in low-income countries, especially in Central and West Africa (48% of reproductive-age women and 56% of pregnant women) and in South Asia (47% of reproductive-age women and 52% of pregnant women).[3] Overall, in low and middle-income countries,

Correspondence: Sagni Girma Fage
School of Nursing and Midwifery, College of Health and Medical Sciences, Haramaya University, P.O. Box 235, Harar, Ethiopia
Email giruu06@gmail.com

12% of low birth weight, 19% of preterm births and 18% of perinatal mortality were attributable to maternal anemia.[4]

Anemia is associated with morbidity and mortality of the growing fetus and neonates.[5,6] It leads to premature births, low birth weight, fetal cognitive impairment and death.[7–9] Similarly, maternal anemia increases the risk of preterm delivery and low birth weight, and iron-deficiency anemia underlies 115,000 maternal deaths and 591,000 perinatal deaths each year.[3] Although anemia is a common problem of pregnant women in developing countries, pregnancy outcomes vary depending on the level of maternal hemoglobin.[10]

The Ethiopian Demographic and Health Survey (EDHS) showed that prevalence of anemia among women aged 15–49 years declined from 27% in 2005 to 17% in 2011, but increased to 24% in 2016 of which pregnant women account for 29%. But, due to different socio-economic status, behavioral, geographical and methodological differences, the national figures from the EDHS could not represent the prevalence of anemia among pregnant women in different parts of Ethiopia.[11]

Despite the government's commitment and various stakeholders' interventions, the magnitude and the major risk factors of anemia remain unabated. Moreover, to the best knowledge of the investigators, there are few documented studies in eastern Ethiopia in general and no study on anemia among pregnant women in the study area. Therefore, this study has aimed to determine the magnitude of the anemia and its associated factors among pregnant women admitted to labor wards of public hospitals in eastern Ethiopia.

Methods and Materials
Study Design, Setting and Period
This hospital-based cross-sectional study was conducted from January 20 to February 19, 2018 in four public hospitals, of which one is a specialized university hospital, two are general hospitals and one is a primary hospital located at the Harari region and Dire Dawa City Administration in the eastern part of Ethiopia around 526 and 515 km from Addis Ababa respectively. The Harari region has a projected total population of 246,000 (124,000 males and 122,000 females), whereas the total population of Dire Dawa City Administration is projected to be 466,000 (234,000 males and 232,000 females).[12] In all four hospitals, there were trained maternal health care providers.

Study Participants
All pregnant women admitted for delivery in those four public hospitals were the source population while women with singleton pregnancy selected by a systematic random sampling technique were the study population. Pregnant women with a past history of preterm delivery, who neither know LNMP nor had ultrasound diagnosis for gestational age, had pregnancy induced hypertension and multiple pregnancies, were excluded from the study.

Sample Size and Sampling Procedure
We calculated the sample size by using a single population proportion formula with the assumptions of $Z_{\alpha/2}$=1.96, 95% confidence level, 5% margin of error, the prevalence of anemia, p=39.1% from a study conducted in Woldia Hospital,[13] 12% for the non-response rate, and included 410 laboring mothers.

We first reviewed monthly clients' flow to each hospital for delivery services in the previous six months from the registration book to estimate the expected number of women that could come for delivery in a month. Thus, the average numbers of women admitted for delivery per month in the previous six months were considered and the sample size 410 was then allocated to each hospital proportional to the total number of pregnant women expected to be admitted for delivery in each health facility in one-month period. A systematic random sampling technique was used to select the study participants and the first study subject for each hospital was randomly selected by a lottery method followed by enrolling every second woman.

Data Collection Tool and Procedure
The data were collected through an interview technique using a pretested structured questionnaire developed by reviewing the literature.[13–20] The questionnaires comprised questions about socio-economic and demographic information, obstetric, health and lifestyle-related variables. Each woman was interviewed during the intrapartum period while she was relatively in stable condition. Eight midwives and four laboratory technicians collected the data after getting training on the tools and survey methods.

Measurements
To diagnose anemia, the hemoglobin level of the laboring mother was measured using a Hemocue HB 301 analyzer. A blood sample was collected through a finger prick by sterile lancet and dropped on a micro-cuvette for analysis.

300

Journal of Blood Medicine 2021:12

Hemoglobin was adjusted for altitude and smoking before deciding women's status of anemia. To define anemia, WHO cut-off for hemoglobin values were used and hemoglobin <11 g/dL was considered as anemic while hemoglobin levels of 10–10.9 g/dL, 7–9.9 g/dL and <7 g/dL were considered as mild, moderate and severe anemia respectively.[21]

Data Quality Control

The questionnaire was initially prepared in English and translated to the local languages (Afaan Oromoo, Amharic and Somali). It was then translated back to English by language experts to check for its consistency. Training on the data collection tool and the procedures was provided to the data collectors and field supervisors. The questionnaire was pretested among 20 pregnant women in similar settings before the actual study. Regular supervision was done by experienced field research supervisors and the investigators.

Data Processing and Analysis

The collected data were checked for completeness, cleaned, coded and entered into EPI DATA version 3.1. Then, the data were exported to SPSS version 22 for analysis. Descriptive and summary statistics were done and the information was presented using tables and descriptive statements.

The outcome variable was recoded into binary as "anemia=1" and "no anemia=0". A binary logistic regression model was fitted to check for association between independent variables and anemia. The model fitness was checked by Hosmer–Lemeshow statistics and Omnibus tests. All variables with $p<0.25$ in the bivariate analysis were included in the final multivariate analysis to identify the true predictors of anemia. A multi-collinearity test was carried out to check the presence of correlation between independent variables using the standard error and collinearity statistics. The direction and strength statistical association was measured by odds ratio (OR) along with the 95% confidence interval (CI). *P* value <0.05 was used to declare statistical significance.

Results
Socio-Economic and Demographic Characteristics

A total of 405 pregnant women participated in this study yielding a response rate of 98.8%. The mean (±SD) age of the study participants was 26.6 (±6.15) years. About half, 206 (50.9%) of the participants, were in the age group of 25–34 years. Three hundred and twenty-one (79.2%), 248 (61.2%) and 131 (32.3%) women were married, urban residents and unable to read and write respectively. The majority of the participants, 178 (44%), were housewives while only 19 (4.7%) were daily laborers in their occupation (Table 1).

Obstetric, Health and Lifestyle of Participants

Of the total respondents, 327 (80.7%) had antenatal care (ANC) follow-up and half of them, 163 (40.2%), had less than four ANC visits. The majority of the women, 290 (71.6%), reported that they received iron/folic acid (IFA) in the current pregnancy. One hundred and eighty-two (44.9%) participants had history of contraceptive use and the birth interval between the current and the previous pregnancy was less than two years for 178 (44.0%) women. Among the participants, 38 (9.4%) had history of blood loss during the current pregnancy. Only 25 (6.2%) women reported having a habit of smoking cigarettes while food intake for the majority, 344 (84.9%), was three and above meals per day. About 33% of women reported that they have a habit of taking milk with tea after meals (Table 2).

Anemia and Its Associated Factors

The overall magnitude of anemia was 33.1% (95% CI: 28.4–37.8%). Among the anemic women, 10 (2.5%), 58 (14.3%) and 66 (16.3%) had severe, moderate and mild anemia respectively.

In bivariate analysis, participants' place of residence, marital status, ANC visit, birth interval, history of contraceptive use, IFA supplementation, blood loss in the current pregnancy, drinking alcohol, eating leafy vegetables, drinking milk with tea after meals and meal frequency per day were significantly associated with anemia among pregnant women. But, in the multivariable logistic regression, place of residence, ANC visit, blood loss in the current pregnancy, eating leafy vegetables, drinking milk with tea after meals and meal frequency per day were factors significantly associated with anemia.

Women who were from rural areas (AOR: 3.8; 95% CI: 1.81, 7.94), who had no ANC visit (AOR: 3.4; 95% CI: 1.34, 8.79), who had a habit of drinking milk with tea after meals (AOR: 2.8; 95% CI: 1.48, 5.61) and who had a meal frequency of 1–2 times per day (AOR: 3.9; 95% CI: 1.69, 8.97) were more likely to be anemic as compared to their counterparts. Pregnant women who did not experience

Table 1 Socio-Economic and Demographic Characteristics of Pregnant Women Admitted to Labor Wards of Public Hospitals in Eastern Ethiopia, 2018 (N=405)

Variables	Category	Frequency (n)	Percent (%)
Age in years	15–24	145	35.8
	25–34	206	50.9
	35–44	54	13.3
Place of residence	Urban	248	61.2
	Rural	157	38.8
Marital status	Married	321	79.2
	Divorced	21	5.2
	Single	34	8.4
	Separated	29	7.2
Educational status	Unable to read and write	131	32.3
	Read and write only	24	5.9
	Primary	77	19
	Secondary	88	21.7
	College and above	85	21
Occupation of women	Housewife	178	44
	Government employee	61	15
	Private employee	52	12.8
	Daily laborer	19	4.7
	Farmer	21	5.2
	Merchant	42	10.3
	Student	32	8

blood loss in the current pregnancy were 75% less likely to be anemic (AOR: 0.25; 95% CI: 0.06, 0.96) and those who had a habit of eating leafy vegetables were 88% less likely to be anemic (AOR: 0.12; 95% CI: 0.06, 0.24) than their counterparts (Table 3).

Discussion

This study assessed the magnitude of anemia among pregnant women admitted to labor wards of four public hospitals in eastern Ethiopia. It revealed that about one in three pregnant women were anemic. Women from rural areas, who had no ANC visit, who had a habit of drinking milk with tea after meals and who had meal frequency of 1–2 times per day were more likely to be anemic than their counterparts. Pregnant women who did not experience blood loss in their current pregnancy and who had a habit of eating leafy vegetables were less likely to be anemic.

In this study, the magnitude of anemia among pregnant women is in harmony with findings from similar studies in Arba Minch,[17] northwest Tigray[20] and Shalla-West Arsi.[22] However, it is higher than reports of other previous studies.[15,18,23–28] On the contrary, the current magnitude is

lower than similar previous studies.[13,14,19,29,30] These differences might be due to variations in the socio-economic characteristics, study settings and periods, dietary habits and health seeking behaviors across the towns with a community of relatively different lifestyle, feeding practices and social norms.

Pregnant women who lived in rural areas were more likely to be anemic as compared to those who lived in urban areas. This is in line with the study from Tigray.[20] The possible reason for this higher burden of anemia among pregnant women from rural areas could be related to inaccessibility of health care facilities and/or low health seeking behavior of the rural women, lack of information about causes of anemia, as well as accessibility and affordability of possible methods to prevent the risk factors of anemia. Additionally, women who had no ANC follow-up were more likely to be anemic than their counterparts. This result is consistent with previous findings.[18] This might be due to the fact that pregnant women who attend ANC follow-up are counseled about appropriate feeding practices and supported by the maternity care providers to take IFA supplementation to prevent or reduce the occurrence of anemia.

Furthermore, pregnant women who have a habit of drinking milk with tea after meals had higher likelihood

Table 2 Obstetric, Health and Lifestyle of Pregnant Women Admitted to Labor Wards of Public Hospitals in Eastern Ethiopia, 2018 (N=405)

Variables	Category	Frequency (n)	Percent (%)
ANC visits	Yes No	327 78	80.7 19.3
Number of ANC visits	<4 ≥4	163 164	40.2 40.4
Gravidity	1 2–4 ≥5	135 191 79	33.3 47.2 19.5
Parity	1 2–4 ≥5	160 181 64	39.5 44.7 15.8
Birth interval in years	<2 ≥2	178 227	44.0 56.0
History of contraceptive use	Yes No	182 223	44.9 55.1
IFA supplementation in current pregnancy	Yes No	290 115	71.6 28.4
Duration of IFA use in months	1–4 ≥4	232 58	57.3 14.3
Blood loss in the current pregnancy	Yes No	38 367	9.4 90.6
History of hypertension	Yes No	11 394	2.7 97.3
HIV/AIDS	Yes No	7 398	1.7 98.3
Smoking cigarettes	Yes No	25 380	6.2 93.8
Khat chewing	Yes No	88 317	21.7 78.3
Alcohol drinking	Yes No	36 369	8.9 91.1
Eating leafy vegetables	Yes No	233 172	57.5 42.5
Drinking milk after meals	Yes No	210 195	51.9 48.1
Drinking milk with tea after meals	Yes No	132 273	32.6 67.4
Drinking coffee after meals	Yes No	136 269	33.6 66.4
Meal frequency per day	1–2 times ≥ 3 times	61 344	15.1 84.9

Abbreviations: ANC, antenatal care; HIV/AIDS, human immune virus/acquired immune deficiency syndrome; IFA, iron folic acid.

Table 3 Factors Associated with Anemia Among Pregnant Women Admitted to Labor Wards of Public Hospitals in Eastern Ethiopia, 2018 (N=405)

Variables	Category	Anemia		COR (95% CI)	AOR (95% CI)
		No	**Yes**		
		n (%)	**n (%)**		
Place of residence	Urban	196 (79)	52 (21)	I	I
	Rural	75 (47.8)	82 (52.2)	4.12 (2.66,6.38)	3.79 (1.81,7.94)***
Marital status	Married	228 (71)	93 (29)	I	I
	Divorced	13 (61.9)	8 (38.1)	1.50 (0.60,3.76)	1.12 (0.24,5.10)
	Single	10 (29.4)	24 (70.6)	5.88 (2.70,12.78)	2.70 (0.78,9.28)
	Separated	20 (69)	9 (31)	1.10 (0.48,2.51)	0.73 (0.17,3.15)
ANC visit	Yes	248 (75.8)	79 (24.2)	I	I
	No	23 (29.5)	55 (70.5)	7.50 (4.33,12.99)	3.43 (1.34,8.79)*
Birth interval in years	<2	100 (56.2)	78 (43.8)	I	I
	≥2	171 (75.3)	56 (24.7)	0.42 (0.27,0.64)	0.67 (0.32,1.39)
History of contraceptive use	Yes	133 (73.1)	49 (26.9)	0.59 (0.39,0.91)	1.37 (0.64,2.95)
	No	138 (61.9)	85 (38.1)	I	I
IFA supplementation	Yes	208 (71.7)	82 (28.3)	0.47 (0.30,0.74)	0.56 (0.24,1.33)
	No	63 (54.8)	52 (45.2)	I	I
History of blood loss	Yes	10 (26.3)	28 (73.7)	I	I
	No	261 (71.1)	106 (28.9)	0.14 (0.06,0.30)	0.25 (0.06,0.96)*
Smoking cigarettes	Yes	8 (32)	17 (68)	I	I
	No	263 (69.2)	117 (30.8)	0.20 (0.08,0.49)	1.07 (0.23,4.86)
Alcohol drinking	Yes	12 (33.3)	24 (66.7)	4.70 (2.27,9.75)*	1.66 (0.44,6.15)
	No	259 (70.2)	110 (29.8)	I	I
Eating leafy vegetables	Yes	204 (87.6)	29 (12.4)	0.09 (0.05,0.14)	0.12 (0.06,0.24)***
	No	67 (39)	105 (61)	I	I
Drinking milk with tea after meals	No	49 (37.1)	83 (62.9)	I	I
	Yes	222 (81.3)	51 (18.7)	7.37 (4.62,11.75)	2.88 (1.48,5.61)*
Meal frequency per a day	1–2	19 (31.1)	42 (68.9)	6.05 (3.34,10.94)	3.90 (1.69,8.97)**
	≥3	252 (73.3)	92 (26.7)	I	I

Note: *p<0.05, **p<0.001, ***p<0.0000.
Abbreviations: ANC, antenatal care; IFA, iron folic acid.

of getting anemia than those who did not have such habit. This is similar to findings from a study in Addis Ababa[18] and this might be due to the inhibition effect of tea and calcium on iron absorption resulting in low bioavailability of iron.[31–34] The risk of anemia was higher among pregnant women who had a meal frequency of 1–2 times per day than those who had three or more times. This is in line with the finding from Tigray[20,28] and it might be because taking meals frequently helps to meet the increased demands for nutrients during pregnancy.

On the other hand, pregnant women who did not experience blood loss in the current pregnancy were 75% less likely to be anemic. This might be because blood loss would result in lower circulating serum iron with lower hemoglobin level. Similarly, women who had a habit of eating leafy vegetables were 88% less likely to experience anemia than those who did not. The possible reason for this might be feeding on different leafy vegetables could increase the folate/folic acid levels that are important to produce normal cells including red blood cells that aids in increasing the availability of hemoglobin.

We used standardized medical equipment and procedures to measure anemia. But, variables such as meal frequency and other lifestyle factors were prone to social desirability bias. In addition, the study was cross-sectional study and could not establish cause and effect relationship between anemia and the associated factors.

Conclusion

This study indicated that about one in three pregnant women were anemic and anemia was a moderate public health problem in the study settings. Interventions targeting reduction or prevention of anemia should focus on pregnant women in rural areas, promoting the use of ANC visits, reducing the habit of drinking milk with tea after meals, encouraging the consumption of leafy vegetables and increasing meal frequency per day to minimize the risk of anemia and its consequences.

Data Sharing Statement

The data sets used for this study are available from the corresponding author on reasonable request.

Ethical Approval

Ethical approval was obtained from the Institutional Health Research Ethics Review Committee (IHRERC) of Haramaya University, College of Health and Medical Sciences. Support letters from the College of Health and Medical Sciences were submitted to the selected hospitals where the study was conducted. After getting all permission letters from the responsible body, an informed voluntary written consent was signed by study participants. For study participants under the age of 18 years, we got informed written consent from parents or family before the data collection. Confidentiality was maintained by using codes instead of participant's name. Participants were also informed that they have full right to refuse participation or withdraw at any time from the research. This study was conducted in accordance with the Declaration of Helsinki.

Acknowledgment

The authors are very thankful to Haramaya University, heads and staff of the selected hospitals, the study participants, data collectors and field supervisors.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors have no competing interests to declare in this work.

References

1. WHO (World Health Organization). The global prevalence of anaemia in 2011; 2015.
2. WHO (World Health Organization). Worldwide prevalence of anaemia 1993–2005. WHO global database on anaemia; 2008.
3. Stevens GA, Finucane MM, De-Regil LM, et al. Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995–2011: a systematic analysis of population-representative data. *Lancet Glob Health*. 2013;1(1):e16–e25. doi:10.1016/S2214-109X(13)70001-9
4. Rahman M, Abe SK, Rahman S, et al. Maternal anemia and risk of adverse birth and health outcomes in low-and middle-income countries: systematic review and meta-analysis, 2. *Am J Clin Nutr*. 2016;103(2):495–504. doi:10.3945/ajcn.115.107896
5. Van DB, Nynke R, Letsky EA. Etiology of anemia in pregnancy in South Malawi. *Am J Clin Nutr*. 2000;72(1):247S–256S.
6. Crawley J. Reducing the burden of anemia in infants and young children in malaria-endemic countries of Africa: from evidence to action. *Am J Trop Med Hyg*. 2004;71(2_suppl):25–34. doi:10.4269/ajtmh.2004.71.25
7. Banhidy F, Acs N, Puho EH, et al. Iron deficiency anemia: pregnancy outcomes with or without iron supplementation. *Nutrition*. 2011;27(1):65–72. doi:10.1016/j.nut.2009.12.005
8. Kalaivani K. Prevalence & consequences of anaemia in pregnancy. *Indian J Med Res*. 2009;130(5):627–633.
9. Levy A, Fraser D, Katz M, et al. Maternal anemia during pregnancy is an independent risk factor for low birthweight and preterm delivery. *Eur J Obstet Gynecol Reprod Biol*. 2005;122(2):182–186. doi:10.1016/j.ejogrb.2005.02.015
10. Chang S, Zeng L, Brouwer ID, et al. Effect of iron deficiency anemia in pregnancy on child mental development in rural China. *Pediatrics*. 2013;131(3):e755–e763. doi:10.1542/peds.2011-3513
11. CSA-Ethiopia, I.C.F. *International. Ethiopia Demographic and Health Survey 2016: Key Indicators Report*. USA: CSA and ICF; 2016.
12. CSA. *Population Projection of Ethiopia for All Regions at Wereda Level from 2014–2017*. Addis Ababa: CSA; 2013.
13. Brhanie T, Sisay H. Prevalence of iron deficiency anemia and determinants among pregnant women attending antenatal care at Woldia Hospital, Ethiopia. *J Nutr Dis Ther*. 2016;6(4).
14. Getachew M, Yewhalaw D, Tafess K, et al. Anaemia and associated risk factors among pregnant women in Gilgel Gibe dam area, Southwest Ethiopia. *Parasit Vectors*. 2012;5(1):296. doi:10.1186/1756-3305-5-296

15. Getahun W, Belachew T, Wolide AD. Burden and associated factors of anemia among pregnant women attending antenatal care in southern Ethiopia: cross sectional study. *BMC Res Notes*. 2017;10(1):276. doi:10.1186/s13104-017-2605-x

16. Gupta A. Prevalence of maternal anemia and its impact on perinatal outcome in a rural area of Dakshina Kannada. *Int J Med Sci Public Health*. 2017;6(7):1209–1214. doi:10.5455/ijmsph.2017.041080 3052017

17. Bekele A, Tilahun M, Mekuria A. Prevalence of anemia and its associated factors among pregnant women attending antenatal care in health institutions of Arba Minch town, Gamo Gofa Zone, Ethiopia: a Cross-sectional study. *Anemia*. 2016;2016:1–9. doi:10.1155/2016/1073192

18. Jufar AH, Zewde T. Prevalence of anemia among pregnant women attending antenatal care at tikur anbessa specialized hospital, Addis Ababa Ethiopia. *J Hematol Thromboembolic Dis*. 2014.

19. Addis AK, Mohamed DA. Prevalence of anemia and associated factors among pregnant women in an urban area of Eastern Ethiopia. *Anemia*. 2014.

20. Gebre A, Mulugeta A. Prevalence of anemia and associated factors among pregnant women in North Western zone of Tigray, Northern Ethiopia: a cross-sectional study. *J Nutr Metab*. 2015;2015:1–7. doi:10.1155/2015/165430

21. WHO (World Health Organization). Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System; 2011. NMH/NHD/MNM/ 11.1. Available from: https://apps.who.int/iris/bitstream/handle/ 10665/85839/WHO_NMH_NHD_MNM_11.1_eng.pdf?sequence= 22&isAllowed=y. Geneva, Switzerland.

22. Obse N, Mossie A, Gobena T. Magnitude of anemia and associated risk factors among pregnant women attending antenatal care in Shalla Woreda, West Arsi Zone, Oromia Region, Ethiopia. *Ethiop J Health Sci*. 2013;23((2):):165–173.

23. Alem M, Enawgaw B, Gelaw A, et al., Prevalence of anemia and associated risk factors among pregnant women attending antenatal care in Azezo Health Center Gondar town, Northwest Ethiopia. *J Interdiscip Histopathol*.2013.

24. Walelign F, Gariba E, Roba K. Prevalence of Anemia and associated factors among pregnant women in Ethiopia. *Food Sci Nutri Res*. 2018;1(1).

25. Berhe B, Mardu F, Legese H, et al. Prevalence of anemia and associated factors among pregnant women in Adigrat General Hospital, Tigrai, northern Ethiopia, 2018. *BMC Res Notes*. 2019;12 (1):310. doi:10.1186/s13104-019-4347-4

26. Kitila KT, Tulu BL, Bedaso DG, Negwo DT, Gemeda MN. Prevalence of anemia and associated risk factors among pregnant women attending antenatal care in selected health centers in Addis Ababa, Ethiopia. *J Womens Health Care*. 2018;7(443):10.4172.

27. Geleta W, Babure Z. Anemia and its determinant factors among pregnant women in Ebantu District, East Wollega zone, Ethiopia. *J Womens Health Care*. 2020;9(490):2167–2420.

28. Abriha A, Yesuf ME, Wassie MM. Prevalence and associated factors of anemia among pregnant women of Mekelle town: a cross sectional study. *BMC Res Notes*. 2014;7(1):888. doi:10.1186/1756-0500-7-888

29. Bereka SG, Gudeta AN, Reta MA, Ayana LA. Prevalence and associated risk factors of anemia among pregnant women in rural part of JigJiga City, Eastern Ethiopia: a cross sectional study. *J Preg Child Health*. 2017;4((337):):2.

30. Gedefaw L, Ayele A, Asres Y, Mossie A. Anaemia and associated factors among pregnant women attending antenatal care clinic in Walayita Sodo town, Southern Ethiopia. *Ethiop J Health Sci*. 2015;25(2):155–164. doi:10.4314/ejhs.v25i2.8

31. Disler P, Lynch SR, Charlton RW, et al. The effect of tea on iron absorption. *Gut*. 1975;16(3):193–200. doi:10.1136/gut.16.3.193

32. Fan FS. Iron deficiency anemia due to excessive green tea drinking. *Clin Case Rep*. 2016;4(11):1053. doi:10.1002/ccr3.707

33. Nelson M, Poulter J. Impact of tea drinking on iron status in the UK: a review. *Journal of Hum Nut Dietetics*. 2004;17(1):43–54. doi:10.1046/j.1365-277X.2003.00497.x

34. Lönnerdal B. Calcium and iron absorption - mechanisms and public health relevance. *Int J Vitam Nutr Res*. 2010;80(45):293–299. doi:10.1024/0300-9831/a000036

Prevalence of Factor V Leiden G1691A and Prothrombin G20210A Gene Mutation Among Pregnant Women: Experience from a Multi-Center Study in Nigeria

Sarah O John-Olabode, Kehinde S Okunade, Ayorinde James, Gbenga Olorunfemi, Obiefuna I Ajie, Akinniyi A Osuntoki & Alani S Akanmu

ORIGINAL RESEARCH

Prevalence of Factor V Leiden G1691A and Prothrombin G20210A Gene Mutation Among Pregnant Women: Experience from a Multi-Center Study in Nigeria

Sarah O John-Olabode [1]
Kehinde S Okunade [2]
Ayorinde James [3]
Gbenga Olorunfemi [4]
Obiefuna I Ajie [5]
Akinniyi A Osuntoki [6]
Alani S Akanmu [1]

[1]Department of Hematology and Blood Transfusion, College of Medicine, University of Lagos, Lagos, Nigeria; [2]Department of Obstetrics and Gynecology, College of Medicine, University of Lagos, Lagos, Nigeria; [3]Department of Biochemistry and Nutrition, Nigerian Institute of Medical Research, Lagos, Nigeria; [4]Division of Epidemiology and Biostatistics, School of Public Health, University of Witwatersrand, Johannesburg, South Africa; [5]Department of Clinical Pathology, College of Medicine, University of Lagos, Lagos, Nigeria; [6]Department of Biochemistry, College of Medicine, University of Lagos, Lagos, Nigeria

Introduction: Inherited thrombophilia and venous thromboembolism (VTE) have been closely linked to adverse pregnancy outcomes such as preeclampsia/eclampsia contributing to increased maternal and perinatal morbidity and mortality. There is, however, little genetic data from Africa including Nigeria that explores the prevalence of common VTE genetic risk markers such as factor V Leiden mutation (FVL G1691A) and prothrombin gene mutation (F2 G20210A) among pregnant women in Nigeria.

Purpose: To determine the prevalence and distribution of FVL G1691A and F2 G20210A in pregnant women in Lagos, Nigeria.

Patients and Methods: This hospital-based cross-sectional pilot study was conducted among pregnant women between 1 July 2019 and 31 August 2020. The genotype of interest was determined through amplification by polymerase chain reaction using G1691A of FV and prothrombin A20210G specific primers. Descriptive data were presented using Stata version 15 (Stata Corp) statistical software.

Results: Of the 400 recruited participants, 397 and 389 samples were successfully processed for FVL G1691A and F2 G20210A mutations, respectively. Three participants had FVL heterozygous mutation; thus, the prevalence of heterozygous mutation of FVL among the study participants was 0.76%, 95% CI: 0.002–0.023%, n=3/397. There was no F2 G20210A mutation detected among the study participants.

Conclusion: This study indicates that screening for factor V Leiden mutation and prothrombin gene mutation in pregnancy might not be of any clinical significance among Nigerian women. However, carrying out a genome-wide associated study is recommended to determine the true impact of these two common inherited thrombophilias in this population.

Keywords: factor V Leiden G1691A, prothrombin G20210A gene mutation, factor 2, factor V, preeclampsia, venous thromboembolism

Introduction

Pregnant women with inherited thrombophilia have an over 50% increased risk of developing venous thromboembolism (VTE);[1] VTE has been attributed to 9–14% of maternal deaths.[2,3] Inherited thrombophilia and VTE have been closely linked to various adverse pregnancy outcomes contributing to increased maternal and perinatal morbidity and mortality.[4,5] One such adverse pregnancy outcome is preeclampsia/

Correspondence: Sarah O John-Olabode
Department of Hematology and Blood Transfusion, College of Medicine, University of Lagos, Lagos, Nigeria
Tel +234 8096608152
Email sarahajibola@yahoo.com

eclampsia (PET), which is responsible for 11% of maternal deaths in Nigeria.[6] Indeed, PET is one of the five leading causes of maternal mortality in Nigeria.[7] The most frequent inherited thrombophilias evaluated for VTE are notably factor V Leiden mutation (FVL G1691A) and prothrombin gene mutation (F2 G20210A). The prevalence of these two mutations is 3–15% in Caucasians but is assumed to be rare in other ethnic populations. There is, however, little genetic data from many parts of Africa, including Nigeria the country that contributes the largest proportion (19%) of the burden of maternal mortality (MM) worldwide and 29% of the total MM from Sub-Saharan African.[8,9] This study therefore evaluated the prevalence of these common VTE genetic risk markers among pregnant women in Lagos, Nigeria.

Patients and Methods
Study Setting
This cross-sectional multi-centre pilot study was conducted among 400 pregnant women receiving obstetric care at the antenatal clinics of the Lagos University Teaching Hospital (LUTH) and Randle General Hospital, Lagos over a period of 13 months (1 July 2019 to 31 August 2020).

This study was approved by the College of Medicine, University of Lagos Health Research Ethics Committee (Approval number: HREC/15/04/2015).

Study Population
Eligible participants were consecutively consenting pregnant women aged 15–49 years who registered for antenatal care in the two participating hospitals. Pregnant women below the age of 18 years were regarded as emancipated minors. "Emancipated minor" is a person who is not of legal age to give consent (below 18 years of age in Nigeria) for a research study but who by marriage, pregnancy, being the mother of a child whether married or not or has left home and is self-sufficient can be allowed to give consent legally. Written informed consent was obtained from each participant following an explanation of the nature and purpose of the study and before recruitment.

Data Collection
A structured interviewer-administered questionnaire was used to collect data on sociodemographic characteristics and obstetric history. Whole blood samples were collected from each study participant in ethylenediaminetetraacetic acid (EDTA) tubes. To improve the deoxyribonucleic acid (DNA) yield, white cells were separated from whole blood by centrifugation to produce buffy coats following the Qiagen protocol.[10]

Determination of Genotype
Genomic DNA Extraction
The extraction of DNA was performed at the Nigerian Institute of Medical Research, Lagos (NIMR) using a - Presto™ Well Blood Genomic DNA Extraction kit (Geneaid Biotech GB, Taiwan) following the vacuum protocol. Briefly, 20 µL of protease was added to the bottom of each microtube and 200 µL of buffy coats were carefully added without touching the rims of the microtubes. To each microtube 200 µL of lysis buffer (GB Buffer) was added, mixed thoroughly for 15 seconds, and incubated at 70°C for 10 minutes. Absolute ethanol (200 µL) was added to each sample and carefully mixed by pipetting up and down 5 times. Lysates were transferred to the Presto™ gDNA 96-well binding plate. A vacuum pressure of 15 inches Hg was applied until the lysates passed through the binding plate into the collection tube leaving nucleic acids abound to the plate. The binding plate was washed twice with 400 and 600 µL of wash buffer successively, with a vacuum pressure of 15 inches Hg applied at each wash step. An additional 10 minutes of the vacuum pressure was added to the dry binding plate membrane. The Presto™ gDNA 96-well binding plate was removed from the assembled manifold, the nozzles were cleaned, and it was re-assembled over DNase/RNase free microtubes to collect eluted DNA using 200 µL of preheated (60°C) elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0) from the binding plate. Eluted DNA were quantified using Nano Drop™ 2000 (Thermo Fisher Scientific, USA) and stored at −20°C prior to downstream applications.

rhAmp SNP Assay Design
Factor 2 (rs1799963) and factor V Leiden single nucleotide polymorphisms (SNPs) (rs6025) were selected from the dbSNP database and entered into the RNAase H amplification (rhAmp) Genotyping Design Tool (https://www.idtdna.com/site/order/designtool/index/) for biallelic blocked rhAmp primers conjugated with either FAM fluorophore (Allele Primer 1) or Yakima Yellow (Allele Primer 2), and a non-fluorogenic locus rhAmp primer (Table 1). Synthetic gBlocks® Gene Fragments

Table 1 rhAmp SNP Assay Design

Gene Name	SNP ID	Primer Name	Sequence
F5	rs6025	Allele Primer 1 Allele Primer 2 Locus Primer	/rhAmp-F/AAGGACAAAATACCTGTATTCCTCrGCCTG/GT1 /rhAmp-Y/AAGGACAAAATACCTGTATTCCTTrGCCTG/GT1/ GCCCAGTGCTTAACAAGACCATrACTAC/GT4/
F2	rs1799963	Allele Primer 1 Allele Primer 2 Locus Primer	/rhAmp-F/CCAATAAAAGTGACTCTCAGCGrAGCCT/GT3/ /rhAmp-Y/CCAATAAAAGTGACTCTCAGCArAGCCT/GT3/ GCAGCTGCCCATGAATAGCArCTGGG/GT4

(Integrated DNA Technologies, https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments) were used as known genotype controls (Supplementary Material). gBlocks Gene Fragments representing the wild type and mutant allele were mixed together in an equal molar ratio, representing the heterozygous genotype.

rhAmp SNP Assay

Purified genomic DNA samples were normalized with TE (Tris-EDTA) buffer (pH 7.5) to a concentration of 3 ng/μL. Synthetic gene templates for the reference (Allele 1) and alternate alleles (Allele 2) were diluted to 500 copies/μL, while the heterozygous control was a pool of equal volume of the controls for each allele. rhAmp Genotyping master-mix and reporter mix were combined at a volume ratio of 20:1 in a new tube. A 5 μL assay reaction was carried out in a tube containing 2.56 μL combined master mix and reporter mix, 0.25 μL rhAmp SNP assay, 0.1 μL Nuclease free water, and 2 μL (6 ng) of template DNA or 1000 copies of synthetic gene controls. Thermal cycling was done on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories 3, Marnes-la-Coquette, France) using the cycling profile enzyme activation at 95°C for 10 mins; and a 40X repeated cycle of denaturation at 95°C for 10 secs; annealing at 60°C for 30 secs, and extension at 68°C for 20 secs. Signal was captured at the extension step and the FAM channel was assigned to the reference allele while the VIC channel was assigned to the alternate allele. An allelic discrimination plate was set-up and identified using Bio-Rad analysis software. Automatic allele calls were reviewed and converted into genotypes.

Definition of Study Endpoints

The study endpoint was the prevalence of thrombophilic gene mutation among pregnant women within the age group, 15 to 49 years. Each single nucleotide polymorphism (SNP) result was classified as: homozygous A/A (mutant/mutant), heterozygous A/G (mutant/wild type) or normal G/G (wild type/wild type).[11]

Data Analysis

Stata version 15 (Stata Corp) statistical software was used for data analysis. The sociodemographic and clinical characteristics of the participants were described. Categorical variables were presented as frequencies, percentages and charts, while normally distributed continuous variables were presented as mean ± standard deviation and non-normally distributed continuous variables were presented as median (interquartile range). Prevalence of thrombophilic polymorphisms among the study participants was determined with a 95% confidence interval.

Results

The sociodemographic characteristics of the 400 pregnant women who participated in the study are presented in Table 2. The mean age and median parity were 31.9 ± 5.2 years and 1 (0–2), respectively.

Detailed information about the two polymorphisms of interest is presented in Table 3.[12] Of the 400 samples collected from the study participants, 14 (3F5, 11F2)

Table 2 Sociodemographic Characteristics of Study Participants

Characteristics	N (%)
Age (Year ±SD)	31.9 ± 5.2
Gestational age (median, IQR) weeks	20 (17–26)
Parity (median, IQR)	1 (0–2)
Primigravidae	110 (27.5)
Multigravida	290 (72.5)

Ethnicity
  Igbo
  Yoruba
  Hausa
	
	150 (39.6)
	225 (59.4)
	4 (1.1)

Table 3 Targeted Single Nucleotide Polymorphisms

Polymorphism	rsSNP	Gene Name	Symbol	Protein Change	Gene Consequence
20210G-A	rs1799963	Coagulation factor II, thrombin	F2	None	3 Prime UTR Variant
FV Leiden	rs6025	Coagulation factor V	F5	R (Arg) > Q (Gln)	Missense Variant

Abbreviations: rs, reference single nucleotide polymorphism; UTR, untranslated region; Arg, arginine; Gln, glutamine.

Table 4 Frequency of Normal and Mutant Genotypes in Study Population

Polymorphism	Normal (%)	Heterozygote (%)	Homozygote (%)
F2 (n=389)	389 (100)	0	0
FV (n=397)	394 (99.2)	3 (0.76)	0

Abbreviations: F2, factor II; FV, factor V.

Table 5 Characteristics of Participants with FVL Heterozygote Mutation

Characteristics	Participant A	Participant B	Participant C
Age (years)	35	27	31
Ethnicity	Yoruba	Yoruba	Igbo
Blood group	O Positive	AB positive	O Positive
Hemoglobin Genotype	AA	AA	AA
Parity	2	1	2
Birth weight (gm)	3420	3100	2200, 2500
Type of Pregnancy	Singleton	Singleton	Multiple
Mode of delivery	Normal delivery	Caesarean section	Caesarean section
Perinatal outcome	Alive	Alive	Alive

were not amplified successfully. The FVL heterozygous mutation was found in 3 of the 397 samples that amplified for FVL detection, thus the prevalence of heterozygous mutation of FV among the study participants was 0.76%, 95% CI: 0.002% - 0.023%, n=3/397 (Table 4).

In contrast, F2 G20210A mutation was not recorded in any of the 389 samples that amplified for F2 G20210A detection.

The presence of FVL mutation had no clinical significance in this population as the three participants who tested positive for FVL mutation had no prior history of adverse pregnancy outcomes and also recorded no complications in the index pregnancies (Table 5).

Discussion

Inherited thrombophilia polymorphisms have been linked with VTE and placenta mediated pregnancy complications that remain one of the major causes of perinatal and maternal morbidity and mortality in underdeveloped nations.[13–22]

There is limited information on the prevalence of inherited thrombophilias in Nigeria, and to the best of our knowledge this study is the first of its kind to determine the prevalence of rs6025 (FVL) and rs1799963 (F2) gene mutations in asymptomatic pregnant women whose thrombophilia status is unknown and the significance of screening for these inherited thrombophilias to aid clinical governance in our population.

Our study has revealed some key findings. First, we have shown that the prevalence of rs6025 (FVL) and rs1799963 (F2) mutation is very low in our study population. In this study, rs6025 (FVL) mutation was present in 0.76% of the study population, while rs1799963 (F2)

genetic mutation was not detected in any of the study participants. This finding is in agreement with previous reports that found a low incidence of FVL and F2 mutations in Africans.[23–26] Despite a paucity of data in the variants of rs6025 and rs1799963 in studies involving pregnant women in Nigeria, we used information deposited in the 1000 genome browser of the National Centre for Biotechnology Information as a comparative control in investigating allelic variants in this study (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes). The Yoruba and Esan ethnic groups were captured in the 1000 genomes project and the minor allele frequencies (MAF) for both rs6025 (FVL) and rs1799963 (F2) were reported to be 0%.[27] In this study, MAF for rs6025 (FVL) was found to be 0.76%, while that of rs1799963 remained at 0%.

Another important finding of this study is corroboration of the fact that women who have inherited thrombophilia can have a problem-free pregnancy, as women who had heterozygous FVL mutation in this study had uneventful pregnancies with no peripartum complications. Similarly, Said et al reported no adverse pregnancy outcomes in the majority of subjects among a cohort of

nulliparous women with inherited thrombophilia polymorphisms.[28]

The third key finding of this study was the ethnic variation in the prevalence of FVL mutation in participants who were heterozygotes for FVL mutation, as this mutation was found predominantly (66.7%, n=2/3) among women from the Yoruba ethnic group in the Southwestern part of Nigeria. This may however not be out of place as the study was conducted in the Southwestern part of Nigeria that is inhabited mainly by the Yoruba ethnic population.

Though our data suggest that FVL and F2 gene mutations have low prevalence and therefore no risk factors that should be considered in our population, it is still possible that associations with other specific pregnancy complications, such as fetal growth restriction, stillbirth, placental abruption and thrombophilias, may be seen if a genome-wide associated study (GWAS) is conducted in the future. Our study had a few limitations. First, it was a hospital-based study, which thus limits generalization of the findings to a larger population. Second, there was limited power to make any inference from the findings. However, this is the first known attempt in Nigeria to determine the prevalence of rs6025 (FVL) and rs1799963 (F2) polymorphisms in pregnant women and to examine the possible effects of these mutations on pregnancy outcomes.

Conclusion

Our data revealed a very low prevalence of FVL G1691A and FII G20210A mutations among pregnant women in Lagos, Nigeria and also suggests that these particular inherited thrombophilias may be of minimal clinical significance among heterozygotes in pregnancy. However, this study has provided valuable information for conducting large cohort studies on these polymorphisms among pregnant women in Nigeria. This could inform future policies that might be useful in the prediction and prevention of VTE and other related placenta-mediated pregnancy complications in Nigerian women.

Abbreviations

VTE, venous thromboembolism; PET, preeclampsia; FVL, factor V Leiden; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; rhAmp, RNAse H amplification; dbSNP, SNP database.

Data Sharing Statement

All data are available from the corresponding author on request. Supplementary data was submitted with this paper.

Ethics Approval and Consent to Participate

The study protocol was permitted by the Ethics Committee of the College of Medicine University of Lagos and adhered to the Declaration of Helsinki principles. Informed written consent was obtained from each subject.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Myers B, Pavord S. Heritable thrombophilias: implications for pregnancy and current evidence for treatment. *Obstetr Gynaecol*. 2011;13:225–230. doi:10.1576/toag.13.4.225.27687
2. Centers for Disease Control and Prevention. Pregnancy mortality surveillance system; 2016 Available from: http://www.cdc.gov/reproductivehealth/maternalinfanthealth/pmss.html. Accessed February 2, 2019.

3. Say L, Chou D, Gemmill A. Global causes of maternal deaths: a WHO systematic analysis. *Lancet Glob Health*. 2014;2(6):e329–e338. doi:10.1016/S2214-109X(14)70227-X

4. Kupferminc MJ. Thrombophilia and pregnancy. *Reprod Biol Endocrinol*. 2003;1:111. doi:10.1186/1477-7827-1-111

5. Robertson L, Wu O, Langhorne P, et al. Thrombophilia in pregnancy: a systematic review thrombosis: risk and economic assessment of thrombophilia screening (TREATS) study. *Br J Haematol*. 2006;132 (2):171–196. doi:10.1111/j.1365-2141.2005.05847.x

6. African Population and Health Research Center. Maternal health in Nigeria: facts and figures fact sheet June 2017. Adapted from the report: maternal health in Nigeria: a situation update; 2016. Available from: www.aphrc.org/publications/maternal-health-nigeria-situation-update/. Accesed April 30, 2021.

7. Agan TU, Monjok E, Akpan UB, Omoronyia OE, Ekabua JE. Trend and causes of maternal mortality in a Nigerian Tertiary Hospital: a 5-year retrospective study (2010–2014) at the University of Calabar Teaching Hospital, Calabar, Nigeria. *Open Access Maced J Med Sci*. 2018;6(6):1153–1158. doi:10.3889/oamjms.2018.220

8. Hulley SB, Cummings SR, Browner WS, Grady D, Newman TB. *Designing Clinical Research: An Epidemiologic Approach*. 4th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2013:Appendix 6B; 75.

9. Nelen WLDM, Steegers EAP, Eskes TKAB, Blom HJ. Genetic risk factor for unexplained recurrent early pregnancy loss. *Lancet Res Lett*. 1997;350(9081):861. doi:10.1016/S0140-6736(97)24038-9

10. QIAGEN. QIAsymphony SP protocol sheet: DNA_Buffy_Coat_400_V6_DSP by Qiagen; 2019. Available from: www.qiagen.com. Accessed June 2, 2019.

11. Ghermann RB, Goodwin TM. Obstetric implications of activated protein C resistance and factor V Leiden mutation. *Obstet Gynecol Surv*. 2000;55(2):117–122. doi:10.1097/00006254-200002000-00026

12. Traven E, Ogrinc A, Kunej T. Initiative for standardization of reporting genetics of male infertility. *Syst Biol Reprod Med*. 2017;63:58–66. doi:10.1080/19396368.2016.1250181

13. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet*. 2003;361:901–908. doi:10.1016/S0140-6736(03)12771-7

14. Howley HE, Walker M, Rodger MA. A systematic review of the association between factor V Leiden or prothrombin gene variant and intrauterine growth restriction. *Am J Obstet Gynecol*. 2005;192:694–708. doi:10.1016/j.ajog.2004.09.011

15. Agorastos T, Karavida A, Lambropoulos A, et al. Factor V Leiden and prothrombin G20210A mutations in pregnancies with adverse outcome. *J Matern Neonatal Med*. 2002;12:267–273. doi:10.1080/jmf.12.4.267.273

16. Benedetto C, Marozio L, Salton L, Maulà V, Chieppa G, Massobrio M. Factor V Leiden and factor II G20210A in preeclampsia and HELLP syndrome. *Acta Obstet Gynecol Scand*. 2002;81:1095–1100. doi:10.1034/j.1600-0412.2002.811201.x

17. Muetze S, Leeners B, Ortlepp JR, et al. Maternal factor V Leiden mutation is associated with HELLP syndrome in Caucasian women. *Acta Obstet Gynecol Scand*. 2008;87:635–642. doi:10.1080/00016340802112740

18. Nagy B, Tóth T, Rigó J, Karádi I, Romics L, Papp Z. Detection of factor V Leiden mutation in severe pre-eclamptic Hungarian women. *Clin Genet*. 1998;53:478–481. doi:10.1111/j.1399-0004.1998.tb02599.x

19. Mello G, Parretti E, Marozio L, et al. Thrombophilia is significantly associated with severe preeclampsia: results of a large-scale, case-controlled study. *Hypertens 1979*. 2005;46:1270–1274. doi:10.1161/01.HYP.0000188979.74172.4d.

20. Wang X, Bai T, Liu S, Pan H, Wang B. Association between thrombophilia gene polymorphisms and preeclampsia: a meta-analysis. *PLoS One*. 2014;9:e100789. doi:10.1371/journal.pone.0100789.

21. Dudding T, Heron J, Thakkinstian A, et al. Factor V Leiden is associated with pre-eclampsia but not with fetal growth restriction: a genetic association study and meta-analysis. *J Thromb Haemost*. 2008;6:1868–1875. doi:10.1111/j.1538-7836.2008.03134.x

22. Lin J, August P. Genetic thrombophilias and preeclampsia: a meta-analysis. *Obstet Gynecol*. 2005;105:182–192. doi:10.1097/01.AOG.0000146250.85561.e9

23. Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet*. 1995;346:1133–1134. doi:10.1016/S0140-6736(95)91803-5

24. Patel RK, Arya R. Venous thromboembolism: racial and ethnic influences. *Therapy*. 2008;5:169–175. doi:10.2217/14750708.5.2.169

25. Montagnana M, Favaloro EJ, Franchini M, Guidi GC, Lippi G. The role of ethnicity, age and gender in venous thromboembolism. *J Thromb Thrombolysis*. 2010;29(4):489–496. doi:10.1007/s11239-009-0365-8

26. Hira B, Pegoraro RJ, Rom L, Moodley J. Absence of factor V Leiden, thrombomodulin and prothrombin gene variants in black south African women with pre-eclampsia and eclampsia. *BJOG*. 2003;110:327–328. doi:10.1046/j.1471-0528.2003.01090.x

27. National Centre for Biotechnology Information. 100 genome project; 2021. Available from: https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes. Accessed April 4, 2021.

28. Said JM, Higgins JR, Moses EK, et al. Inherited thrombophilia polymorphisms and pregnancy outcomes in nulliparous women. *Obstet Gynecol*. 2010;115:5–13. doi:10.1097/AOG.0b013e3181c68907

Diagnostic Value of Neutrophil Lymphocyte Ratio and D-Dimer as Biological Markers of Deep Vein Thrombosis in Patients Presenting with Unilateral Limb Edema

Ikhwan Rinaldi, Rachmat Hamonangan, Mohamad Syahrir Azizi, Rahmat Cahyanur, Fadila Wirawan, Atikah Isna Fatya, Ageng Budiananti & Kevin Winston

ORIGINAL RESEARCH

Diagnostic Value of Neutrophil Lymphocyte Ratio and D-Dimer as Biological Markers of Deep Vein Thrombosis in Patients Presenting with Unilateral Limb Edema

Ikhwan Rinaldi [1]
Rachmat Hamonangan [2]
Mohamad Syahrir Azizi [3]
Rahmat Cahyanur [1]
Fadila Wirawan [4]
Atikah Isna Fatya [4]
Ageng Budiananti [4]
Kevin Winston [4]

[1]Division of Hematology and Medical Oncology, Department of Internal Medicine, Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia; [2]Department of Internal Medicine, PGI Cikini Hospital, Jakarta, Indonesia; [3]Division of Cardiology, Department of Internal Medicine, Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [4]Department of Internal Medicine, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Introduction: Patients with deep vein thrombosis (DVT) pose high morbidity and mortality risk thus needing fast and accurate diagnosis. Wells clinical prediction scores with D-dimer testing are traditionally used to rule out patients with low probability of DVT. However, D-dimer testing has a few limitations regarding its relatively low specificity. Neutrophil-lymphocyte ratio (NLR), a marker of inflammation, was found to increase in DVT. Hence, we aimed to evaluate the role of NLR for DVT diagnosis.

Methods: Data were collected from medical records of patients with suspected DVT at Cipto Mangunkusumo National General Hospital during January–December 2014. Diagnosis of DVT was conducted using lower limb ultrasonography. Diagnostic values for NLR, D-dimer, and NLR + D-dimer were determined by receiver operating characteristic (ROC) analysis to obtain area under the curve (AUC), sensitivity, specificity, negative predictive value, and positive predictive values. Sensitivity and specificity analyses of NLR and D-dimer were also conducted based on Wells score and divided into groups of low and high probability of DVT.

Results: The AUC values for NLR, D-dimer, and NLR + D-dimer were 72.6%, 70.4%, and 76.1%, respectively. The optimal cut-off value determined for NLR was 5.12 with sensitivity of 67.7%, specificity of 67.9%, PPV of 68.85%, and NPV of 64.91% in differentiating subjects with and without DVT. This study also found that D-dimer had sensitivity of 69.4%, specificity of 71.4%, PPV of 72.88%, and NPV of 67.8%. Meanwhile, the NLR + D-dimer combination had sensitivity of 66.1% and specificity of 72.6%. Multivariate analysis showed that NLR (OR: 2.636; 95% CI: 1.144–6.076; p: 0.023) and D-dimer (OR: 4.175; 95% CI: 1.810–9.633; p: 0.001) were associated with DVT.

Conclusion: NLR value has wider AUC than D-Dimer and is relatively easier to obtain and does not require specific assay, thus enabling rapid evaluation of symptomatic patients suspected of having DVT. Adding NLR to D-dimer increased AUC to detect DVT. Therefore, NLR could serve as a complementary diagnostic tool for D-dimer to exclude DVT, especially in low clinical probability patients.

Keywords: deep vein thrombosis, neutrophils lymphocyte ratio, NLR, inflammation, D-dimer

Introduction

Deep vein thrombosis (DVT) is a form of venous thromboembolism, which is the third most common cardiovascular disorder.[1] DVT is present in about two thirds in a group of venous thromboembolism that affects around 300,000–600,000

Correspondence: Ikhwan Rinaldi
Division of Hematology and Medical Oncology, Department of Internal Medicine, Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
Tel +62 811177997
Email ikhwanrinaldi@gmail.com

individuals in the US.[2] DVT poses a high risk of mortality and long-term complications if not treated properly.[3] Therefore, accurate diagnosis is imperative to initiate prompt treatment and prevent mortality. However, the diagnosis of DVT still remains a challenge for physicians due to lack of symptoms' accuracy.[1,4]

Venography as the gold standard for DVT diagnosis is rarely used nowadays due to its invasive property, high cost, and the requirement to use contrast agent.[2,5,6] Currently, venous ultrasonography is the first imaging modality choice for DVT diagnosis due to its high sensitivity and specificity, although the diagnostic value is limited for distal thrombosis.[5–9] On the other hand, performing ultrasonography for all patients suspected of having DVT is not a cost-effective approach as it is time-consuming and expensive.[10] Finally, not all physicians are trained to use venous ultrasonography. Hence, current practice implements the use of clinical prediction scores to estimate pretest probability of DVT. One of the most commonly used scores is Wells score for DVT which has been widely validated. Wells score considers clinical features and risk factors for DVT to categorize patients as high probability (Wells score $\geq$ 2) and low probability (Wells score < 2).[4,11]

While high probability Wells scores should be followed by diagnostic imaging to confirm the diagnosis, low probability scores are not enough to safely rule out suspected DVT inpatients. Therefore, Wells score should be used in conjunction with other diagnostic testing such as D-dimer.[11] D-dimer is a fibrin degradation product which may be elevated in DVT and other conditions such as pregnancy, infection, malignancy, and trauma. D-dimer test is highly sensitive but not specific in diagnosing DVT. The commonly used threshold for normal D-dimer levels is <500 ng/mL.[1,4,11] Combination of D-dimer and low probability Wells score yields high negative predictive value to rule out DVT in symptomatic patients.[2,12,13] Despite the important role of D-dimer testing in the diagnosis of DVT, it has some pitfalls such as high false positive rate.[14] D-dimer levels can be assessed using various assays with variable sensitivity and specificity. D-dimer levels can also be elevated in many conditions other than DVT.[15,16] Therefore, interpretation of D-dimer should be done carefully. D-dimer testing is also not available in all health care facilities, limiting its use to rule out suspected DVT in patients.[2,11]

Nowadays, the role of inflammation in the pathogenesis of DVT has been recognized.[17–20] Neutrophil-lymphocyte ratio (NLR) is a non-specific marker of inflammation which is related to many diseases, including venous thromboembolism. Several studies have shown the role of NLR as predictor and prognostic factor for mortality in pulmonary embolism and venous thromboembolism.[21–25] For example, a study by Farah et al showed that NLR was associated with acute pulmonary embolism (OR 1.2; 95% CI: 1.01-1.4; p: 0.041).[22]

NLR is obtained by dividing neutrophil count with lymphocyte count.[17,26,27] The rise of neutrophil count represents a systemic inflammatory process, while the decrease of lymphocyte shows an ongoing stress inflicted by the disease.[17] The role of neutrophils in thrombus formation has been elucidated. Neutrophils release neutrophil extracellular traps (NETs) that initiate and propagate thrombus formation.[28,29]

The role of neutrophils in thrombus formation presents a possibility to use NLR as a biological marker in DVT. Compared with D-dimer, NLR is easy to evaluate and widely available in many healthcare facilities since it does not require special assays. Therefore, this study aimed to evaluate the diagnostic value of NLR compared with D-dimer as a marker in DVT.

Methods
Study Design and Subjects
This was a cross-sectional study of secondary data obtained from medical records. All medical records were obtained from January 1st 2014 to December 31st 2014 and were looked through for adult patients suspected of having DVT presenting with unilateral lower limb edema who underwent ultrasonography for diagnosis at Cipto Mangunkusumo National General Hospital, Jakarta, Indonesia. This study identified a total of 160 subjects suspected of having DVT. However, 42 subjects were excluded due to lack of USG Doppler examination and/or incomplete laboratory data, resulting in the study size of 118 patients.

Clinical characteristics of excluded patients were presented in Supplementary File. We excluded patients without USG Doppler examination to prevent bias in this study and incorrect diagnosis. Another exclusion criterion was pregnancy which was not found in any subjects. As this was a cross-sectional study from secondary data of medical records, the authors chose total sampling method to achieve the minimal sample size.

Data Collection

Data were collected from medical records which included subjects' baseline characteristics, the presence of comorbidities, clinical findings, Wells score, complete blood count, differential count, D-dimer, and results of ultrasonography (USG) of the lower limbs.

The Wells score of patients was previously determined, evaluated, and recorded by internal medicine specialist in Cipto Mangunkusumo National General Hospital. Additionally, USG Doppler examination for DVT diagnosis was also conducted by internal medicine specialist. Both total Wells score and USG Doppler examination status were obtained directly from medical records.

Wells score of <2 is classified as low probability for DVT while score ≥2 is classified as high probability. NLR was calculated by dividing absolute neutrophil count with absolute lymphocyte count. The presence of uncompressible vein on USG confirmed the diagnosis of DVT.

The presence of comorbidities including diabetes mellitus, chronic kidney disease, heart disease, infection, cerebrovascular disease, hypertension, hematological disorders, and malignancy was documented in medical records and obtained for this study.

Ethics Approval

Informed consent of the subjects in this study was represented by the ethical approval from Ethics Committee of the Faculty of Medicine, Universitas Indonesia number 0558/UN2.F1/ETIK/2018 with protocol number 18–06-0646 due to the study having a cross-sectional design using secondary data from medical records. Hence, no informed consent from the patients was required. Finally, this study complied with Declaration of Helsinki and confidentiality of patients' data was maintained.

Statistical Methods

Baseline characteristics of samples were described as mean or median for numerical data and percentage for categorical data. Age was categorized as <45 years and ≥45 years since rate of DVT was found to increase significantly after the age of 45 years.[30] Study subjects were classified into two groups based on the presence of DVT confirmed by lower limb USG.

Statistical analysis was performed using IBM SPSS Statistics 20.0. In all analysis, P value ≤0.05 for the two-tailed test was considered significant. The relationship between NLR and DVT was analyzed with unpaired

t-test. Distribution of NLR was skewed, so the data were log transformed before being analyzed. Back-transformation was then performed to obtain the geometric mean which was presented in the results.

Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic value of NLR and identify its optimal cut-off value. The area under the ROC curve (AUC), sensitivity, specificity, negative predictive value, positive predictive value, and the likelihood ratio were presented to describe the diagnostic value of NLR.

D-dimer was categorized as positive if the D-dimer level was ≥ 500 ng/dL and negative if <500 ng/dL, as this is a universal cutoff used in clinical setting. The area under the ROC curve (AUC), sensitivity, specificity, negative predictive value, and positive predictive value were presented to describe the diagnostic value of NLR.

The diagnostic value of NLR was then compared with D-dimer. A model incorporating both NLR and D-dimer was analyzed to see if NLR could add diagnostic value to D-dimer in diagnosing DVT. The diagnostic value of both NLR and D-dimer were also analyzed while subjects were divided into low and high probability groups based on Wells score. Statistical comparison between AUC of each variable was also conducted. Logistic regression was then performed to determine the independent association between NLR and D-dimer with DVT.

Results

A total of 160 subjects were gathered but 42 subjects were excluded from the study. Hence, a total of 118 subjects were included in this study. Clinical characteristics of excluded patients can be seen in Supplementary Table 1.

The mean age was 54.78 (SD: 13.87) years with 22.1% of them younger than 45 years. Half (50%) of the subjects were male. The majority of subjects (87.3%) had comorbidities. Subject baseline characteristics can be viewed in Table 1.

Out of 118 subjects, 62 (52.5%) had DVT. Baseline characteristic of hematological parameters showed indication of higher leukocyte levels in the DVT-positive group when compared with non-DVT group (13,174.52 versus 10,015.36). Additionally, the comparison revealed higher percentage of neutrophil in DVT-positive group (80.2% versus 68.7%) while the percentage for lymphocyte was lower in DVT-positive group (10.6% versus 20%). The median NLR was higher in DVT-positive group (7.45 versus 3.35).

Table 1 Subject Characteristics

Variables	All Subjects	Presence of DVT	
		Positive	Negative
	n = 118	n = 62 (52.5%)	n = 56 (47.5%)
Age (years), [mean ± SD]	54.78 ± 13.87	51.85 ± 14.94	58.02 ± 11.89
Age categories, [n (%)]			
<45	26 (22.1)	19 (30.6)	7 (12.5)
≥45	92 (77.9)	43 (69.4)	49 (87.5)
Gender, [n (%)]			
Male	59 (50)	29 (46.8)	31 (55.5)
Female	59 (50)	33 (53.2)	25 (44.6)
Wells score, [n (%)]			
Low Probability	21 (17.8)	3 (4.8)	18 (32.1)
High Probability	97 (82.2)	59 (95.2)	38 (67.9)
D-Dimer, [n (%)]			
≥500 ng/dl (positive)	55 (46.6)	43 (69.4)	16 (28.6)
<500 ng/dl (negative)	63 (53.4)	19 (30.6)	40 (71.4)
Complete Blood Count			
Hb (mg/dL), mean ± SD	10.53 ± 2.19	10.14 ± 1.94	10.96 ± 2.38
Hematocrit (%), mean ± SD	31.57 ± 6.22	30.501 ± 5.56	32.62 ± 6.71
Leukocyte (cells/μL), median (min-max)	9,330 (1,100–72,100)	13,174.52 ± 9,143.48	10,015.36 ± 9,290.04
Thrombocyte (cells/μL), median (min-max)	267,000 (20,000–121,800)	261,500 (20,000–996,000)	282,000 (51,800–1,218,000)
Basophil (%), median (min-max)	0.20 (0–3)	0.10 (0–3)	0.30 (0–1)
Eosinophil (%), median (min-max)	1.85 (0–16.80)	0.4 (0–11.40)	2.30 (0–16.80)
Neutrophil (%), median (min-max)	76.0 (8.20–96)	80.2 (8.20–96)	68.7 (42.1–95)
Lymphocyte (%), median (min-max)	14.3 (3–184)	10.6 (3–184)	20 (3–47.20)
Monocyte (%), median (min-max)	6.11 ± 2.95	5.81 ± 3.27	6.45 ± 2.53
NLR, median (min-max)	5.20 (0.41–30.67)	7.45 (0.41–30.67)	3.35 (0.89–30.67)
Total Comorbidities, [n (%)]	103 (87.30)	54 (52.40)	49 (47.60)
Malignancy, [n (%)]	30 (29.12)	24 (44.44)	6 (12.24)
Diabetes, [n (%)]	26 (25,24)	11 (20.37)	15 (30.61)
Chronic Kidney Disease, [n (%)]	17 (16.50)	8 (14.81)	9 (18.36)

The proportion of subjects with high probability of DVT (Wells score ≥ 2) were significantly higher in the group with DVT. The presence of comorbidities was equal between subjects who had and did not have DVT. However, subjects who had DVT had higher number of malignancies. The main comorbidities in our subjects were presence of malignancies with 24 subjects in DVT group having malignancies and only 6 subjects in non-DVT group having malignancies.

ROC Analysis of NLR

ROC analysis was performed to find out the optimal cut-off value for NLR. ROC analysis was conducted using NLR as continuous data. The area under ROC curve (AUC) for NLR was 72.6% (63.4%–81.8%) with $p < 0.001$ (Figure 1) (Table 2). The optimal cut-off value for NLR based on sensitivity and specificity curve analysis was determined to be 5.12 (Figure 2). This cutoff had sensitivity of 67.7%, specificity of 67.9%, negative predictive value (NPV) of 64.91%, and positive predictive value (PPV) of 68.85% (Table 2). Coordinates of the curve for NLR can be seen in Supplementary Table 2.

ROC Analysis of D-Dimer

ROC analysis for D-dimer with a cut-off value of 500 showed the area under curve 70.4% (60.8%-80.0%)

ROC Curve
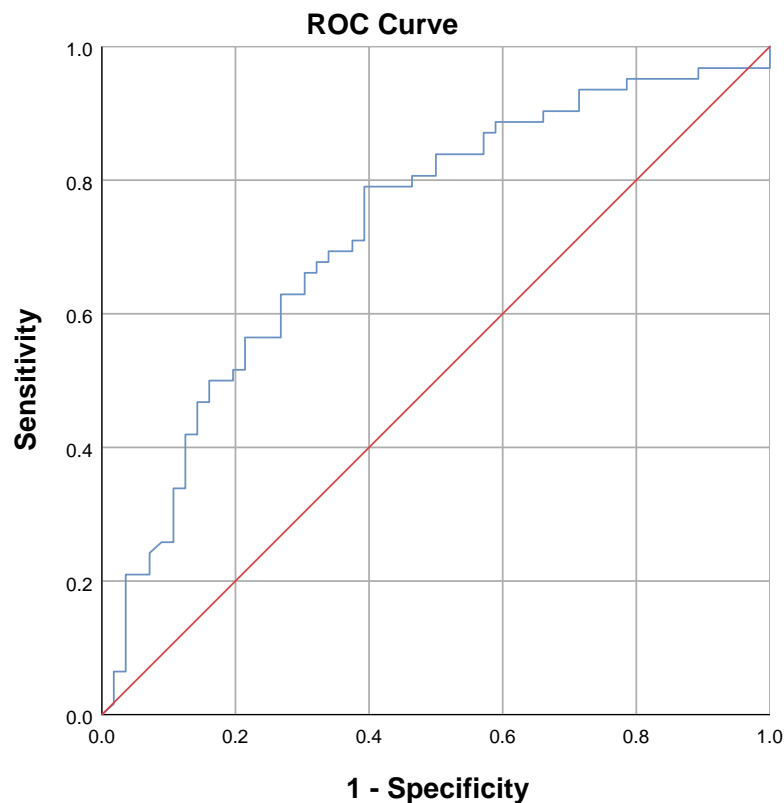
Figure 1 Receiver operating characteristic (ROC) analysis of NLR. AUC was 72.6% (63.4%-81.8%). NLR optimal cut-off value was 5.12 (n=118).

(Figure 3). Analysis showed that D-Dimer with a cut-off value of 500 had sensitivity of 69.4%, specificity of 71.4%, NPV of 67.8%, and PPV of 72.88% (Table 3).

ROC Analysis of NLR + D-Dimer
When NLR and D-dimer were used together, ROC analysis showed AUC of 76.1% with a sensitivity of 66.1% and specificity of 72.6% (Table 4) (Figure 4). Coordinates of the curve for NLR + D-Dimer can be seen in Supplementary Table 3.

AUC Difference Analysis
Comparisons of the 3 AUCs were conducted. Lowest AUC difference was 2.2% between NLR and D-Dimer. Highest AUC difference was 5.7% between D-Dimer and NLR +

D-Dimer combination. The difference of 5.7% was statistically significant with p value of 0.047. Based on this, addition of NLR to D-Dimer improved AUC significantly.

We also assessed the overall model quality of the variables using SPSS Software. The model quality for NLR, D-Dimer, and NLR + D-Dimer were 0.63, 0.62, and 0.67 respectively (Figure 5). A good model has a value of more than 0.5.

Sensitivity and Specificity of NLR + D-Dimer Based on Wells Score Probability
When the subjects were divided based on DVT probability, NLR had higher sensitivity compared to D-dimer in

Table 2 ROC Analysis of NLR

	AUC (%)	CI 95%	Standard Error	Sensitivity (%)	Specificity (%)	Positive Predictive Value	Negative Predictive Value
NLR (≥5.12)	72.6	0.634–0.818	0.047	67.7	67.9	68.85	64.91
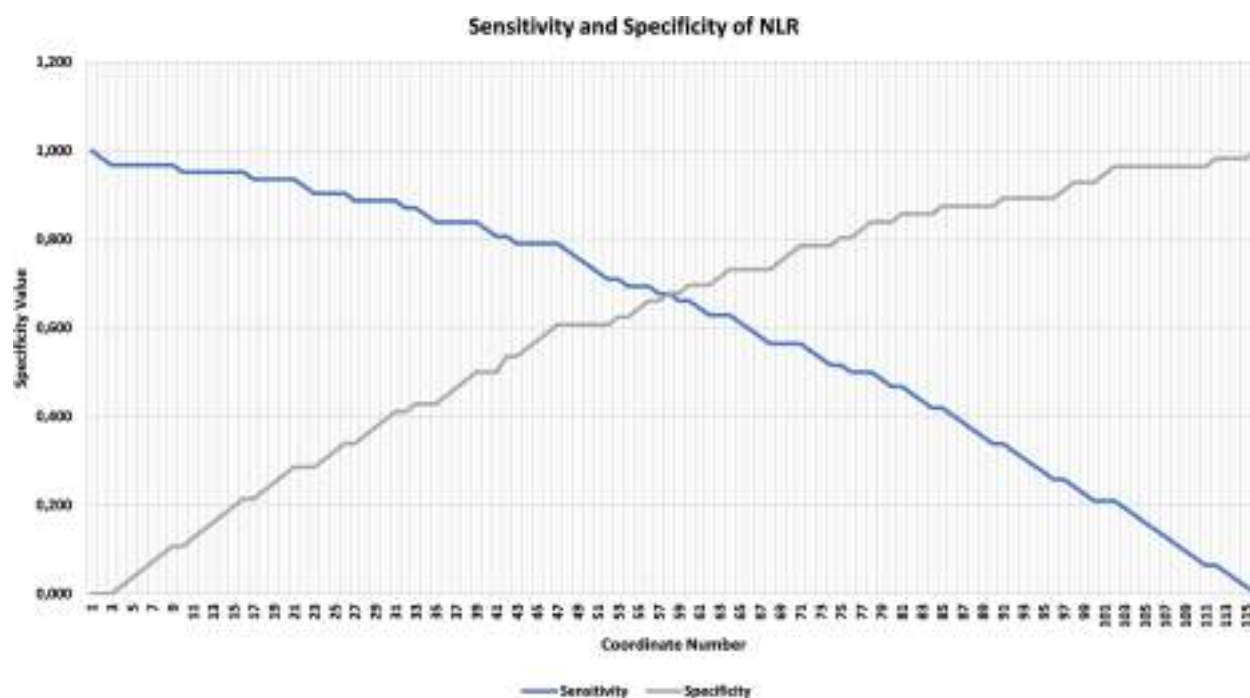
Figure 2 Sensitivity and specificity analysis of NLR. NLR optimal cut-off value was determined to be 5.12 (n=118).
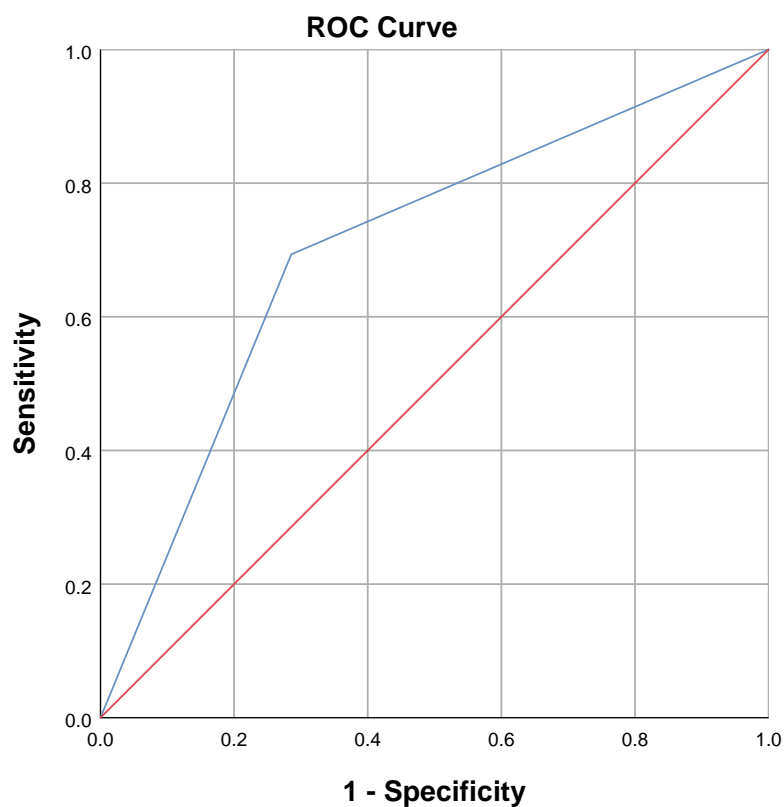
Diagonal segments are produced by ties.

Figure 3 Receiver operating characteristic (ROC) analysis of D-dimer. AUC was 70.4% (60.8%–80.0%).

318

Journal of Blood Medicine 2021:12

Table 3 ROC Analysis of D-Dimer

	AUC (%)	CI 95%	Standard Error	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
Positive D-dimer (>500 ng/dL)	70.4	63.4%–81.8%	0.049	69.4	71.4	72.88	67.8

Table 4 ROC Analysis of NLR and D-Dimer

	AUC (%)	CI 95%	Standard Error	Sensitivity (%)	Specificity (%)
NLR + D-dimer	76.1	67.3%–84.8%	0.045	66.1	72.6

subjects with low probability of DVT. However, D-dimer was shown to be more specific than NLR to differentiate DVT in subjects with low probability (Table 6).

Univariate Analysis

We then conducted univariate analysis of NLR with the cutoff value obtained from ROC curve. Result of univariate analysis for NLR using cutoff value of 5.12 showed that subjects with NLR $\geq$ 5.12 had higher likelihood of having DVT (OR: 4.089;

95% CI:1.898–8.813; p: <0.01). Other variables were also analyzed for univariate analysis (Table 7). Result of the univariate analysis showed that D-Dimer was also significantly associated with DVT.

Multivariate Analysis

Variables which had p value of <0.25 were selected for multivariate analysis. Those variables were NLR, D-Dimer, and age. In the multivariate analysis, interaction

ROC Curve
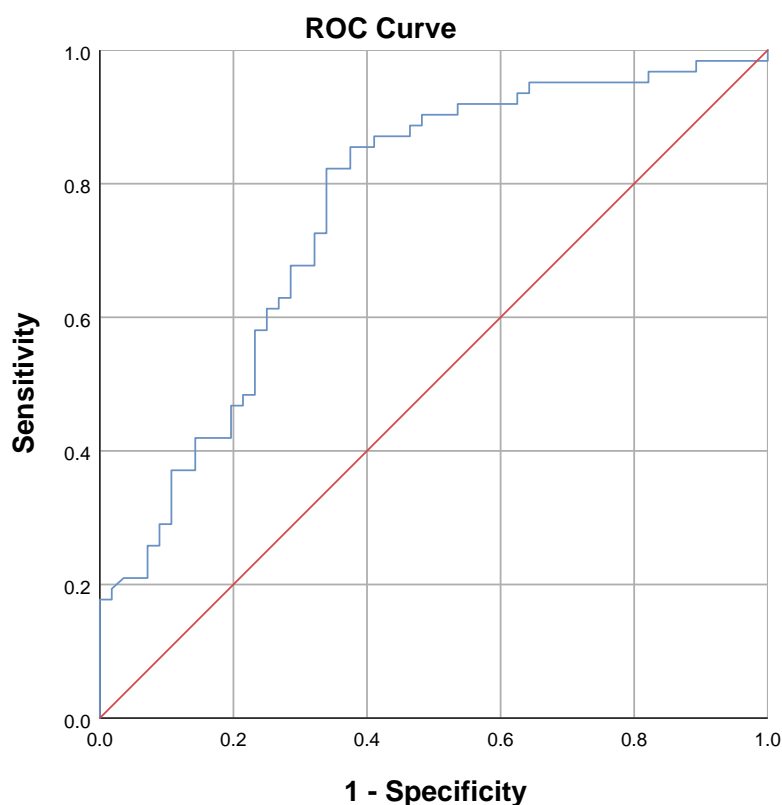
Diagonal segments are produced by ties.

Figure 4 Receiver operating characteristic (ROC) analysis of NLR and D-dimer. AUC is 76.1% (67.3%–84.8%).

Overall Model Quality

A good model has a value above 0.5
A value less than 0.5 indicates the model is no better than
random prediction
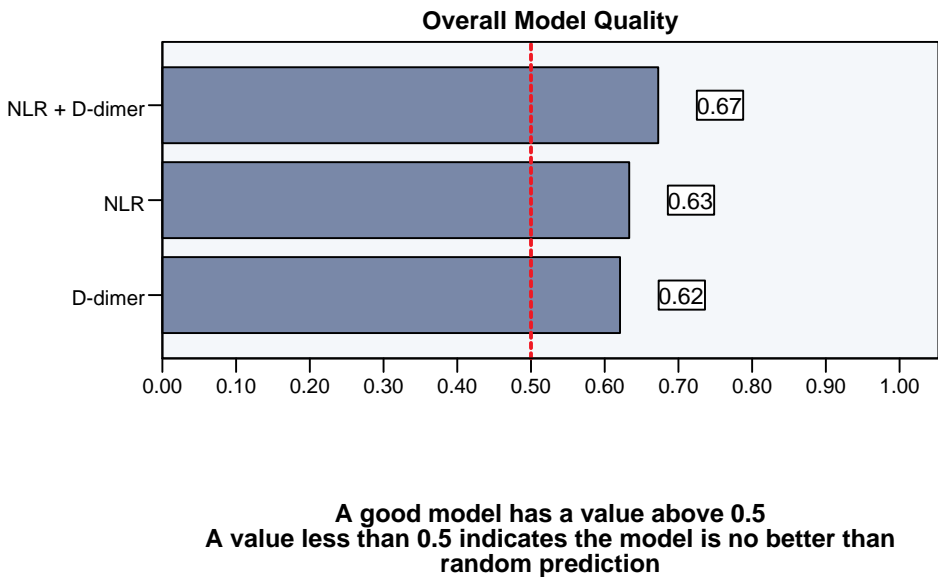
Figure 5 Overall model quality.

with each variable was also included. Result of step 1 multivariate analysis is presented in Table 8.

Step 4 of multivariate analysis showed that NLR, D-Dimer, and age had statistically significant association with DVT (Table 9). However, the variable interaction between D-Dimer and NLR did not achieve statistical significance (p: 0.080). Hence, we conducted another multivariate analysis without D-Dimer by NLR interaction to obtain the best model.

Final Model of NLR Diagnostic Study for DVT

Result of multivariate analysis without D-Dimer by NLR interaction showed that NLR (OR: 2.636;95% CI: 1.144–6.076); p: 0.023) and D-Dimer (OR: 4.175; 95% CI: 1.810–9.633; p: 0.001) were associated with DVT (Table 10). Hosmer and Lemeshow test for goodness of fit showed p value of 0.210 which indicated that the model was well calibrated (Table 11). Finally, the formulae from logistic regression were made (Figure 6).

Table 5 Statistical Comparison of Obtained AUCs

Test Result Pairs	AUC Difference	P-value
NLR and D-Dimer	2.2%	0.684
NLR and NLR + D-Dimer Combination	3.5%	0.262
D-Dimer and NLR + D-Dimer Combination	5.7%	0.047

Discussion

The role of inflammation in DVT has been demonstrated in many studies. Neutrophils are thought to mediate the pathogenesis of DVT.[30,31] The initiation of thrombus formation involves an inflammatory process which induces activation of endothelial cells, platelets, and leukocytes.[18,31] Activated endothelial cells express P-selectin, an adhesion molecule that mediates the attachment of leukocytes and platelets. Additionally, pro-inflammatory cytokines are secreted by the endothelium to recruit innate immune cells, particularly neutrophils and monocytes. Those cells, especially neutrophils, are abundantly found in early thrombi, forming clusters or layers adjacent to the endothelium.[23,25,26] Attachment of neutrophils to the endothelium is then followed by platelet adhesion. Finally, neutrophils may propagate thrombus formation through neutrophil extracellular traps (NETs).[26,28,29] NETs are released as a response to cellular damage and inflammatory stimuli.[29] Other function of NETs is to enhance coagulation by recruiting factor XIIa and cleave tissue factor pathway inhibitor, an inhibitor of coagulation. Additionally, binding of NETs to fibrin and von Willebrand Factor (VWF) leads to recruitment of platelets and red blood cells to the site of thrombus formation.[28,29]

Neutrophil-lymphocyte ratio (NLR), a non-specific inflammatory marker represents the relationship between neutrophils and lymphocytes in inflammation.[14,28,29] Higher NLR indicates high neutrophil count and low

Table 6 Sensitivity and Specificity of NLR and D-Dimer in Low Probability and High Probability Groups

	Low Probability		High Probability	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
D-Dimer	60.0	76.7	73.8	53.8
NLR	65.0	67.4	69.0	61.5%

Table 7 Univariate Analysis of Variables

Variables	Odds Ratio	P value
NLR ≥ 5.12 NLR <5.12	4.089 (1.898–8.813) Reference	0.000
D-Dimer (>500 ng/dL) D-Dimer (<500 ng/dL	5.658 (2.562–12.495) Reference	0.000
Age > 60 years Age ≤ 60 years	0.542 (0.251–1.172) Reference	0.120
Male Female	0.709 (0.343–1.463) Reference	0.352
Malignancies Comorbidity No Malignancies Comorbidity	1.500 (0.647–3.480) Reference	0.345

Table 8 Step 1 of Multivariate Analysis with Interaction Variable

Variables	Odds Ratio	Coefficient	P value
NLR ≥ 5.12 NLR <5.12	5.506 (1.367–22.185) Reference	1.706	0.016
D-Dimer (>500 ng/dL) D-Dimer (<500 ng/dL	9.434 (2.240–39.729) Reference	2.244	0.002
Age > 60 years Age ≤ 60 years	0.483 (0.103–2.255) Reference	−0.728	0.355
D-Dimer by NLR Interaction D-Dimer by Age Interaction	0.200 (0.035–1.162) 1.049 (0.171–6.425)	−1.608 0.048	0.073 0.959
NLR by Age Interaction Constant	1.194 (0.190–7.516) 0.317	0.177 −1.150	0.850 0.011

Table 9 Step 4 of Multivariate Analysis with Interaction Variable

Variables	Odds Ratio	Coefficient	P value
NLR ≥ 5.12 NLR <5.12	5.500 (1.664–18.182) Reference	1.705	0.001
D-Dimer (>500 ng/dL) D-Dimer (<500 ng/dL	9.600 (2.681–35.207) Reference	2.262	0.001
D-Dimer by NLR interaction Constant	0.213 (0.023–1.204) 0.250	−1.544 −1.386	0.080 0.000

Table 10 Best Model of Multivariate Analysis

Variables	Odds Ratio	Coefficient	P value
NLR ≥ 5.12 NLR <5.12	2.636 (1.144–6.076) Reference	0.969	0.023
D-Dimer (>500 ng/dL) D-Dimer (<500 ng/dL	4.175 (1.810–9.633) Reference	1.429	0.001
Constant	0.336	−1.089	0.001

Table 11 Hosmer and Lemeshow Test

Chi-Squared	Df	P-value
3.120	2	0.210

lymphocyte count. The rise of neutrophil count represents a systemic inflammatory process while the decrease of lymphocytes shows ongoing stress inflicted by the disease.[17] Hence, we aimed to evaluate the role of NLR to diagnose DVT.

Baseline Characteristics

Baseline characteristics showed that the median of NLR is higher in DVT-positive group than in non-DVT group (7.45 versus 3.35). Thrombus development is associated with inflammation and recruitment of leukocytes.[32,33] Hence, the difference of median NLR in this study is in concordance with the pathophysiology described previously, where there is an increase of neutrophils due to inflammation. NLR levels therefore correlate with higher level of inflammation. The mean age of the patients was above 50 years old which is in the age range when DVT occurs.

$$Y = \alpha + \beta_1 X_1 + \ldots + \beta_i X_i$$

$$Y = -1.089 + (0.969 \times NLR) + (1.429 \times D\text{-}Dimer)$$

Figure 6 Logistic regression formulae obtained from best model of multivariate analysis for odds ratios of having DVT.

ROC Curve Analysis

We conducted ROC analysis to obtain the AUC value for NLR, D-Dimer, and NLR + D-Dimer with the results of 72.6%, 70.4%, and 76.1% respectively. AUC value of above 70% is considered moderately good for diagnosis. As a result, NLR, D-Dimer, and NLR + D-Dimer combination are good modalities to help diagnose DVT. Of interest is a similar AUC value of NLR with D-Dimer in this study.

D-Dimer is already a well-established marker for thrombosis. D-dimer is a fibrin degradation product which acts as a marker of endogenous thrombus fibrinolysis.[16] Therefore, elevated D-dimer levels can indicate the presence of thrombus.[34] Meanwhile, NLR represents the inflammatory process that initiates and propagates thrombus formation.[26] Our study showed that the addition of NLR to D-Dimer increased the AUC by 5.7% when compared to D-Dimer only (76.1% versus 70.4%). This difference is statistically significant as shown by AUC difference analysis (Table 5). Hence, the addition of NLR increased the diagnostic value of D-Dimer, and NLR therefore may be used as a marker to supplement D-Dimer in diagnosing DVT. NLR is also a laboratory parameter which is easy to obtain in a clinical setting. However, despite the promising result, this needs to be confirmed and evaluated in a clinical setting as the values may be different clinically.

Sensitivity and Specificity Analysis

In our study, the optimal cut-off value for NLR was 5.12. This cutoff had sensitivity of 67.7% and specificity of 67.9% in differentiating subjects with and without DVT. Meanwhile, the NPV and PPV for NLR were 64.91% and 68.85%, respectively. This study also found that D-dimer had sensitivity of 69.4%, specificity of 71.4%, PPV of 72.88%, and NPV of 67.79%. Meanwhile, the NLR + D-dimer combination had sensitivity of 66.1% and specificity of 72.6%.

The cutoff of 5.12 for NLR in this study is different to other studies. For example, in a study by Ferroni et al, which consisted of 810 cancer patients undergoing chemotherapy and aimed to analyze the prognostic value of NLR to predict VTE events yielded a cut-off value of >3 with AUC 55%, sensitivity 59%, and specificity 57%.[35] Using that cut-off value, NLR could predict the occurrence of symptomatic VTE with HR 2.5 (95% CI 1.0–6.4, p=0,06). The study by Ferroni et al showed lower

sensitivity and specificity of NLR with lower cut-off value compared with the findings in our study.[35] Another example was shown in a case-control study by Bakirci et al, which evaluated the relationship between NLR and venous thromboembolism (VTE) consisting of DVT and pulmonary embolism (PE).[13] The study showed NLR with a cut-off value of 1.84 with sensitivity of 88.2% and specificity of 67.6%.

These different cutoffs may be explained by several factors. The first example of the study by Ferroni et al was a prognostic study to predict VTE occurrence which differs from this study where the aim was to diagnose VTE.[35] The other example is the study by Bakirci et al which included both DVT and PE, in which PE had a more acute setting than DVT which may have produced lower NLR ratio and influenced the results.[13] Additionally, the study excluded subjects with comorbidities such as malignancies, inflammatory diseases, rheumatic, antiphospholipid syndrome, chronic kidney disease, and liver disease while most of the subjects in our study had those conditions.[13] The inclusion of comorbidities could increase inflammatory conditions, thus increasing the all-subjects median of NLR value in our study.[25,34,36–38]

Sensitivity and Specificity of NLR Based on DVT Probability

In subjects with low probability of DVT based on Wells score, NLR had higher sensitivity than D-Dimer (65% versus 60%). In contrast, for subjects with high probability of DVT, NLR had higher specificity than D-Dimer (69.2% versus 53.8%). Due to better sensitivity of NLR in low probability group, NLR may be more effective as additional screening in low probability group. Additionally, NLR is easily accessible and does not require specific assay, thus enabling rapid evaluation of symptomatic patients suspected of having DVT and reducing the need to perform USG in low probability patients.[37] Therefore, NLR might be better than D-dimer to correctly diagnose DVT in patients with low probability. Finally, in patients with high probability of DVT, the higher specificity of NLR can be useful to exclude patients without DVT.

Multivariate Analysis

Various comorbidities presented in this study were associated with an increase in NLR, resulting in the possibilities of comorbidities as a confounding factor.[25,34,36–38] We first conducted univariate logistic regression of

malignancies, age, and gender, however, malignancies and gender had p value above 0.25 (Table 8). Hence, they were not statistically significant and were not selected for multi-variate analysis. Additionally, this also meant that malignancies did not affect the relationship of DVT and NLR in this study. Other comorbidities beside malignancies were not analyzed due to lack of sufficient high number of subjects with "non-malignancies" comorbidities.

The lack of correlation between malignancies and DVT is in contrast with Wells score where malignancies have positive correlation with DVT. The difference can be explained due to this study being designed as a diagnostic study with the aim to analyze value of NLR and D-Dimer for DVT diagnosis instead of finding variables associated with DVT. Furthermore, sample size calculations for variables such as malignancies were not conducted.

In multivariate analysis, we also included interaction between the variables. In the first model, only NLR, D-dimer, and D-dimer by NLR interaction were left as variables. However, D-dimer by NLR interaction had p value above 0.05 and was eliminated from the model due to being statistically insignificant. Final model showed that only NLR and D-dimer were the significant variables in this study (Table 10). Hosmer and Lemeshow test of the final model showed p value of 0.210, which concluded that the final model had good calibration.

Study Limitations

This study used a relatively small sample size of 118 patients. Hence, the result of this study requires further confirmation from prospective studies with higher sample size to evaluate and confirm the diagnostic value of NLR in DVT. In addition, the effect of additional comorbidities other than malignancies on the results could not be studied here. Finally, this study did not specify whether the DVT was proximal or distal, while the location of DVT may be associated with higher NLR.[14,37] Therefore, other factors that may influence NLR such as the location of DVT should also be considered and analyzed in future studies.

Conclusion

The diagnostic value of NLR was comparable with D-dimer in DVT subjects with unilateral limb edema. In subjects with low probability of DVT, NLR had higher sensitivity compared to D-dimer. NLR value is much easier to obtain and does not require specific assay. Therefore, NLR was shown to be a useful complementary diagnostic tool for D-dimer to exclude DVT, especially in low clinical probability patients.

Abbreviations

DVT, deep vein thrombosis; NLR, neutrophil-lymphocyte ratio; ROC, receiver operating characteristic; AUC, area under the curve; NETs, neutrophil extracellular traps; VTE, venous thromboembolism; PE, pulmonary embolism.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgment

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

Disclosure

An abstract entitled "The Relationship between Neutrophil Lymphocyte Ratio and Deep Vein Thrombosis in Unilateral Limb Edema in Suspected Deep Vein Thrombosis Patients" has been presented as a poster at The 64th Annual Scientific and Standardization Committee (SSC) Meeting of The International Society on Thrombosis and Haemostasis (ISTH) in Dublin, Ireland on July 18–12, 2018 and has been published as an abstract in Research and Practice in Thrombosis and Haemostasis, 2018, Volume 2, page 1–368, doi: 10.1002/rth2.12125, available from: https://onlinelibrary.wiley.com/doi/epdf/10.1002/rth2.12125. Significant new content, analysis, and discussion of the diagnostic values of

D-dimer, combination of NLR and D-dimer, and comparison of diagnostic value between NLR and D-dimer in high and low clinical probability DVT have been added to this present manuscript. The authors declare that they have no competing interests.

References

1. Wells P, Anderson D. The diagnosis and treatment of venous thromboembolism. *Hematology Am Soc Hematol Educ Program*. 2013;2013:457–463.

2. Beckman MG, Hooper WC, Critchley SE, Ortel TL. Venous thromboembolism: a public health concern. *Am J Prev Med*. 2010;38 (4suppl):S495–501. doi:10.1016/j.amepre.2009.12.017

3. Beyth J, Cohen AM, Landefeld CS. Long-term outcomes of deep-vein thrombosis. *Arch Intern Med*. 1995;155:1031–1037. doi:10.1001/archinte.1995.00430100053006

4. Le Gal G, Righini M. Controversies in the diagnosis of venous thromboembolism. *J Thromb Haemost*. 2015;13(S1):S259–65. doi:10.1111/jth.12937

5. Lesmana A, Pratama D, Wangge G. Comparison of wells score, D–dimer and combination of wells score and D–dimer with venous duplex ultrasonography in diagnosis of acute deep vein thrombosis in lower extremity andy. *New Ropanasuri J Surg*. 2017;2(1):e103–e109. doi:10.7454/nrjs.v2i1.17

6. Tan S, Chong B, Thoo F, Ho J, Boey H. Diagnosis of deep vein thrombosis: accuracy of colour Doppler ultrasound compared with venography. *Singapore Med J*. 1995;36:362–366.

7. Lewiss RE, Kaban NL, Saul T. Point-of-care ultrasound for a deep venous thrombosis. *Glob Heart*. 2013;8(4):329–333. doi:10.1016/j.gheart.2013.11.002

8. Mazzolai L, Aboyans V, Ageno W, et al. Diagnosis and management of acute deep vein thrombosis: a joint consensus document from the European Society of Cardiology working groups of aorta and peripheral vascular diseases and pulmonary circulation and right ventricular function. *Eur Heart J*. 2018;39(47):4208–4218. doi:10.1093/eurheartj/ehx003

9. Bogavac-Stanojević N, Dopsaj V, Jelić-Ivanović Z, Lakić D, Vasić D, Petrova G. Economic evaluation of different screening alternatives for patients with clinically suspected acute deep vein thrombosis. *Biochem Medica*. 2013;23(1):96–106. doi:10.11613/BM.2013.012

10. Huisman MV, Klok FA. Diagnostic management of acute deep vein thrombosis and pulmonary embolism. *J Thromb Haemost*. 2013;11 (3):412–422. doi:10.1111/jth.12124

11. Geersing GJ, Zuithoff NPA, Kearon C, et al. Exclusion of deep vein thrombosis using the Wells rule in clinically important subgroups: individual patient data meta-analysis. *BMJ*. 2014;348(g1340):1–13. doi:10.1136/bmj.g1340

12. Ahearn GS, Bounameaux H. The role of the D-dimer in the diagnosis of venous thromboembolism. *Semin Respir Crit Care Med*. 2000;21 (6):521–536. doi:10.1055/s-2000-13186

13. Bakirci EM, Topcu S, Kalkan K, et al. The role of the nonspecific inflammatory markers in determining the anatomic extent of venous thromboembolism. *Clin Appl Thromb*. 2015;21(2):181–185. doi:10.1177/1076029613494469

14. Chopra N, Doddamreddy P, Grewal H, Kumar PC. An elevated D-dimer value: a burden on our patients and hospitals. *Int J Gen Med*. 2012;25(5):87–92. doi:10.2147/IJGM.S25027

15. Bounds EJ, Kok SJ, Dimer D. *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2020. Available from http://www.ncbi.nlm.nih.gov/books/NBK431064/. Accessed April 7, 2021.

16. Linkins L-A, Takach Lapner S. Review of D-dimer testing: good, bad, and ugly. *Int J Lab Hematol*. 2017;39(Suppl 1):98–103. doi:10.1111/ijlh.12665

17. Branchford BR, Carpenter SL. The role of inflammation in venous thromboembolism. *Front Pediatr*. 2018;6(May):142. doi:10.3389/fped.2018.00142

18. Christiansen SC, Nass IA, Cannegieter SC, Hammerstrom J, Rosendaal FR, Reitsma PH. Inflammatory cytokines as risk factors for a first venous thrombosis: a prospective population-based study. *PLoS Med*. 2006;3(8):1414–1419. doi:10.1371/journal.pmed.0030334

19. Dowling NF, Hooper WC, Austin H. Understanding and predicting venous thromboembolism: the role of coagulation factors and inflammatory markers. *Am J Med*. 2002;113(8):689–690. doi:10.1016/S0002-9343(02)01395-5

20. Sharma K, Patel AK, Shah KH, Konat A. Is neutrophil-to-lymphocyte ratio a predictor of coronary artery disease in Western Indians? *Int J Inflamm*. 2017;2017:1–8. doi:10.1155/2017/4136126

21. Köse N, Yıldırım T, Akın F, Yıldırım SE, Altun İ. Prognostic role of NLR, PLR, and LMR in patients with pulmonary embolism. *Bosn J Basic Med Sci*. 2020;20(2):248–253. doi:10.17305/bjbms.2019.4445

22. Farah R, Nseir W, Kagansky D, et al. The role of neutrophil-lymphocyte ratio, and mean platelet volume in detecting patients with acute venous thromboembolism. *J Clin Lab Anal*. 2019;34(1):e23010. doi:10.1002/jcla.23010

23. Phan T, Brailovsky Y, Fareed J, Hoppensteadt D, Iqbal O, Darki A. Neutrophil-to-lymphocyte and platelet-to-lymphocyte ratios predict all-cause mortality in acute pulmonary embolism. *Clin Appl Thromb*. 2020;Jan(26):1076029619900549.

24. Du P. Neutrophil-lymphocyte ratio is a predictor of venous thromboembolism in gastric cancer patients. *Clin Lab*. 2019;65(5). doi:10.7754/Clin.Lab.2018.181005

25. Go SI, Lee A, Lee US, et al. Clinical significance of the neutrophil–lymphocyte ratio in venous thromboembolism patients with lung cancer. *Lung Cancer*. 2014;84(1):79–85. doi:10.1016/j.lungcan.2014.01.014

26. Borazan E, Balik AA, Bozdağ Z, et al. Assessment of the relationship between neutrophil lymphocyte ratio and prognostic factors in non-metastatic colorectal cancer. *Turk J Surg*. 2017;33(3):185–189. doi:10.5152/turkjsurg.2017.3528

27. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. 2018;18(2):134–147. doi:10.1038/nri.2017.105

28. Pfeiler S, Stark K, Massberg S, Engelmann B. Propagation of thrombosis by neutrophils and extracellular nucleosome networks. *Haematologica*. 2017;102(2):206–213. doi:10.3324/haematol.2016.142471

29. Cushman M. Epidemiology and risk factors for venous thrombosis. *Semin Hematol*. 2007;44(2):62–69. doi:10.1053/j.seminhematol.2007.02.004

30. von Brühl M-L, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*. 2012;209(4):819–835. doi:10.1084/jem.20112322

31. Michel J, Ho-tin-noé B. Thrombi and neutrophils. *Circ Res*. 2015;116:1107–1109. doi:10.1161/CIRCRESAHA.115.306050

32. Cavuş UY, Yildirim S, Sönmez E, Ertan C, Ozeke O. Prognostic value of neutrophil/lymphocyte ratio in patients with pulmonary embolism. *Turk J Med Sci*. 2014;44(1):50–55. doi:10.3906/sag-1302-47

33. Aue G, Lozier JN, Tian X, et al. Inflammation, TNFα, and endothelial dysfunction link lenalidomide to venous thrombosis in chronic lymphocytic leukemia. *Am J Hematol*. 2011;86(10):835–840. doi:10.1002/ajh.22114

34. Papa A, Emdin M, Passino C, Michelassi C, Battaglia D, Cocci F. Predictive value of elevated neutrophil-lymphocyte ratio on cardiac mortality in patients with stable coronary artery disease. *Clin Chim Acta*. 2008;395:27–31. doi:10.1016/j.cca.2008.04.019

35. Ferroni P, Riondino S, Formica V, et al. Venous thromboembolism risk prediction in ambulatory cancer patients: clinical significance of neutrophil/lymphocyte ratio and platelet/lymphocyte ratio. *Int J Cancer*. 2015;136(5):1234–1240. doi:10.1002/ijc.29076

36. Imtiaz F, Shafique K, Mirza S, Ayoob Z, Vart P, Rao S. Neutrophil lymphocyte ratio as a measure of systemic inflammation in prevalent chronic diseases in Asian population. *Int Arch Med*. 2012;5:2. doi:10.1186/1755-7682-5-2

37. Ertaş G, Sönmez O, Turfan M, et al. Neutrophil/lymphocyte ratio is associated with thromboembolic stroke in patients with non-valvular atrial fibrillation. *J Neurol Sci*. 2013;324(1–2):49–52. doi:10.1016/j. jns.2012.09.032

38. Binnetoğlu E, Şengül E, Halhallı G, Dindar S, Şen H. Is neutrophil lymphocyte ratio an indicator for proteinuria in chronic kidney disease? *J Clin Lab Anal*. 2014;28(6):487–492. doi:10.1002/jcla.21715

Overcoming Drug Interference in Transfusion Testing: A Spotlight on Daratumumab

Marilyn T Nedumcheril, Robert A DeSimone, Sabrina E Racine-Brzostek, Ok Kyong Chaekal & Ljiljana V Vasovic

REVIEW

Overcoming Drug Interference in Transfusion Testing: A Spotlight on Daratumumab

Marilyn T Nedumcheril[1,2]
Robert A DeSimone [1]
Sabrina E Racine-Brzostek[1]
Ok Kyong Chaekal[1,3]
Ljiljana V Vasovic [1]

[1]Department of Pathology and Laboratory Medicine, New York-Presbyterian Hospital, Weill Cornell Medicine, New York, NY, USA; [2]New York Blood Center Enterprises, New York, NY, USA; [3]Department of Medicine/Division of Hematology-Oncology New York-Presbyterian Hospital, Weill Cornell Medicine, New York, NY, USA

Abstract: Daratumumab, a monoclonal antibody therapeutic, is highly efficacious and widely used in all stages of multiple myeloma and amyloidosis and has promising activity in other hematologic disorders. Daratumumab interacts with red blood cells, interfering with pre-transfusion testing. This interference can lead to compromising transfusion safety, extensive blood bank work ups and delays in provision of compatible units. Several methods have been developed to negate daratumumab interference with indirect antiglobulin testing. They are based on i) standard blood bank techniques including dithiothreitol and enzymatic treatment of reagent cells, using reagent red blood cells negative for CD38, ii) blocking CD38 antigens on reagent or donor cells, iii) neutralization of anti-CD38 antibody in patient plasma prior to testing, and iv) extended antigen typing of patient red blood cells in conjunction with provision of phenotypically matched units for transfusion. Implementation of those methods by the blood bank should be a planned effort coordinated with the patient's clinical team. Timely involvement of blood bank and transfusion services and educational efforts by both blood banks and clinical providers can improve the overall daratumumab safety profile in regard to blood transfusion.

Keywords: immunohematology, CD38, drug neutralization, dithiothreitol, incompatible crossmatch, indirect antiglobulin testing

Introduction

Pre-transfusion testing is a cornerstone of safe blood transfusion practices.[1] Principal components of pre-transfusion testing consist of recipient's and donor's blood typing, detection of red blood cell (RBC) antibodies in recipient's plasma, selection of compatible blood units including extended typing beyond ABO/Rh when clinically significant alloantibodies are identified, and finally confirming compatibility by serologic crossmatching. Interference with pre-transfusion testing can lead to compromised transfusion safety, extensive blood bank work-ups and delays in the provision of compatible RBC units. Drug interference can be observed as a drug-induced immune hemolytic anemia, in either a drug-dependent fashion or through drug-independent antibodies that are serologically indistinguishable from idiopathic warm autoantibodies that can persist after the offending agent is removed or metabolized. A new challenge is presented by a new generation of monoclonal antibody therapeutics such as anti-cluster differentiation (CD)-38 and Anti-CD47 that target antigens expressed by hematological malignancies that are also expressed by RBCs.[2,3] Most notable is the Anti-CD38 monoclonal antibody daratumumab, targeting multiple myeloma, which has gained wide attention for its clinical utility and will be further discussed.[4,5]

Correspondence: Ljiljana V Vasovic
Department of Pathology and Laboratory Medicine, New York-Presbyterian Hospital, Weill Cornell Medicine, 525 East 68th Street, Box 251, New York, NY, 10065, USA
Tel +1 212 746 6106
Fax +1 212 746 8435
Email ljv9004@med.cornell.edu

Daratumumab in the Treatment of Plasma Cell Neoplasms

Plasma cell neoplasms, mostly myeloma but also closely related disorders such as amyloidosis, account for approximately 10% of all hematologic malignancies and remain largely incurable. In 2021, the American Cancer Society estimates 34,920 newly diagnosed cases and about 12,410 deaths attributed to MM.[6] Daratumumab is a humanized IgG1κ monoclonal antibody targeting CD38, a surface protein highly expressed in multiple myeloma (MM) cells. Human CD38 antigen is a 46 kDa, type II transmembrane glycoprotein which functions as an ADP-ribosyl cyclase. In addition to its expression on plasma cells and malignant myeloma cells, CD38 is also expressed at low levels on other hematopoietic cells, including RBCs and epithelial cells.[7] Its expression may be stimulated by proinflammatory cytokines in patients with cancer.[8]

Daratumumab effectively induces antibody dependent cellular toxicity (ADCC), complement dependent cytotoxicity, antibody dependent cellular phagocytosis (ADCP), and apoptosis in MM cells[9] and has become one of the most effective drugs for treatment of myeloma.[10] It was first approved by the United States Food and Drug Administration (FDA) for use in adult patients in late 2015. Initially approved as monotherapy for multiple myeloma patients with double-refractory disease who failed at least three prior lines of therapy, it has since been incorporated in front-line treatment and is mostly used in combination with other agents such as proteasome inhibitors, imids and corticosteroids.[11,12] Such triplet or quadruplet therapies have dramatically improved response, response duration, and life-expectancy of patients with multiple myeloma. Daratumumab is also one of the most active agents approved for treatment of amyloidosis. It has occasionally been used for treatment of delayed RBC engraftment occurring after stem cell transplant, and it has shown promise in treatment of T-ALL.[13,14] Initially daratumumab was given intravenously. It frequently causes infusion-related toxicity such as fever, rigors, flushing, and in extreme cases anaphylactic shock. This is prevented by premedication with steroids, antihistamines, and acetaminophen. A more recent formulation combines daratumumab with hyaluronidase (Darzalex Faspro™, Janssen Biotech, Horsham, PA) and is administered subcutaneously with better tolerance. Premedication is still required. The half-life of daratumumab, like that of other antibody treatments, is measured in weeks. It is dosed weekly or every other week during initial treatment. In many patients this is followed by monthly maintenance treatments so that detectable blood levels are present throughout treatment. Several other CD38 antibody therapies are in development, including isatuximab and MOR202.[15]

No significant clinically detectable hemolysis has been observed to preclude daratumumab therapy in MM patients. However, therapy-related anemia may be observed requiring RBC transfusion.[16,17] Interestingly, Schuetz et al. found that daratumumab may be an effective therapeutic agent against autoimmune hemolytic anemia (AIHA) post-hematopoietic stem cell transplantation (HSCT) refractory to established first and second-line therapies. The mechanism of action to induce rapid remission of AIHA post-HSCT is now known, and use should be confined to those with life-threatening disease unresponsive to established therapies.[18]

Daratumumab interference with serological testing in transfusion medicine and other laboratory tests including serum protein and immunofixation electrophoresis and flow cytometry have been readily observed. Despite promising benefits, concerns have been raised regarding daratumumab interference with serological testing and transfusion safety, particularly in urgent situations.

Daratumumab Interferes with Serological Testing in Transfusion Medicine

Daratumumab does not interfere with ABO/RhD typing or with immediate-spin crossmatches. Daratumumab is known to cause interference with serological testing, including red cell antibody screening, antibody identification and cross-matching based on indirect antiglobulin testing (IATs).[19] Routine pre-transfusion testing algorithm consists of ABO/Rh typing, antibody detection and identification, direct antiglobulin test (DAT), and crossmatching whether a patient is on daratumumab or not. ABO typing consists of a forward type that identifies antigens present on the RBC surface by mixing patient's RBCs and antiserum (anti-A and anti-B) and a reverse type to identify what antibodies are present in a patient's plasma/serum by mixing patient plasma with reagent RBCs (A and B). Rh typing is a forward type only. Antibody screens detect the presence of alloantibodies in a patient's plasma by mixing patient plasma with group O red cells of known phenotype and observing for agglutination. The DAT identifies

antibodies attached to red blood cells. The patient's red cells are screened by mixed with polyspecific DAT reagent and confirmed by mixing with monospecific anti-IgG and anti-C3b/d reagents. When the DAT is positive for agglutination with anti-IgG reagent. Elutions may be performed to identify the antibody of interest. Prior to issue of a RBC unit, RBC crossmatching is performed to check for blood compatibility. When no RBC antibody is detected, pre-transfusion testing involves either an electronic crossmatch (computer matches compatible units) or immediate spin to confirm ABO compatibility. Immediate spin crossmatching mixes patient plasma with RBCs from a selected unit at room temperature. However, when an antibody has been detected, an antiglobulin (full or Coomb's) crossmatch is performed by incubating patient plasma, donor RBCs, and AHG reagent at 37C. CD38 expression on reagent red cells will be recognized by daratumumab and will result in a panreactivity in vitro. Initially, it was often confused with an antibody to a high prevalence antigen. Ficin-treated red cells, dilution and adsorption techniques failed to eliminate the panreactivity. The direct antiglobulin test (DAT) can be positive following daratumumab therapy. However, studies of patients on daratumumab, where antibody screening yielded positive reactions by IATs, had negative DATs.[20] Acid eluates failed to show agglutination with patients' own RBCs as well as donor RBCs. These results were observed for two to six months after the last daratumumab infusion. The positivity in IAT testing was not unique to daratumumab but was also seen with other anti-CD38 clones.

In patients taking daratumumab, the DAT is frequently negative and reactivity with the patient's own RBCs is inconsistent, and the auto-control is frequently negative. There is some evidence to suggest that daratumumab induces loss of CD38 on RBCs over time.[16] This confusion led to unnecessary testing and significant delays in blood provision. In the package insert, the manufacturer recommends that patients should be typed and screened prior to administration of daratumumab. However, another salient point is that blood banks should be consistently notified by providers or pharmacy when a patient is planned to or has received daratumumab. This is also important if daratumumab is used in outpatient setting and for off label use in other lymphoid malignancies.[21,22] Unrecognized daratumumab interference with blood bank serologic testing can lead not only to unnecessary delays in pre-transfusion testing but also in issuing crossmatch incompatible RBC units as an emergency release

procedure. This raises concerns regarding transfusion safety due to interference with alloantibody identification that can result in an acute or delayed hemolytic transfusion reaction, particularly in urgent situations. Thus, methods to mitigate daratumumab interference were widely investigated and implemented in clinical practice (see Table 1 and Figure 1). Clinicians taking care of patients receiving daratumumab should contact the blood bank to inform them of daratumumab use when ordering a pre-daratumumab type and screen. Algorithm for clinicians managing patients treated with daratumumab is provided (see Figure 2.)

Methodologies to Negate Daratumumab Interference During Pre-Transfusion Testing

RBCs with Destroyed or Lacking CD38 Antigens Used for Pre-Transfusion Testing

Prior studies investigating the structure and function of CD38 revealed that the reducing agent dithiothreitol (DTT) can denature CD38, and enzymatic digestion with trypsin can cleave CD38 from the cell surface.[23] Interestingly, it has also been reported that samples from patients on anti-CD38 therapy can cause false positivity on subsequent samples via carryover on solid phase instruments such as the TANGO optimo (Bio-Rad Laboratories, Hercules, CA).[24]

To resolve the issue of daratumumab interfering with serological testing, Chapuy et al. investigated methods to prevent binding of daratumumab to CD38 on reagent RBCs.[25] As a model they used HL60 cells transduced with either CD38 or green fluorescent protein (CD38 negative control). When exposed to daratumumab spiked plasma, there was a dose dependent binding to CD38+ HL60 cells but not CD38- and controls. When CD38 +HL60 cells are incubated with 10 mmol/L DTT and 2% trypsin, daratumumab binding decreases by 92% and 40% respectively.

The same researchers compared the eluate reactivity of untreated and DTT-treated RBCs incubated with daratumumab. The eluates of untreated RBCs contained specific binding to CD38+HL60 cells and a mini-5 RBC panel. Eluates of DTT-treated RBCs had no detectable binding to CD38+HL60 cells or the mini-panel of 5 RBCs. DTT-sensitive blood groups include Kell, Cartwright, Dombrock, Indian, John Milton Hagen, Knops, Landsteiner-Weiner, Lutheran, and Raph.[26] However,

Table 1 Key Features of Methodologies Utilized to Abrogate Daratumumab Interference in Pretransfusion Testing

I. RBCs with lacking, diminished or destroyed CD38 antigens used in pre-transfusion testing
Long standing technique
Highly reliable as a blood bank procedure
Training and competency of technologists well established
  • **Dithiothreitol (DTT)**
    Denatures CD38 antigen
    DTT treated donor cells can be used for a crossmatch
    Caution needed as DTT denatures Kell and other clinically significant antigens
  • **Trypsin/Papain**
    Cleaves CD38 antigen
    Destroys many other significant RBC antigens
    Less reliable than DTT
  • **Cord blood cells**
    Lacking CD38 antigen
    Cord blood cell antigen panels reagent are not routinely available
  • **In (Lu) RBCs**
    Lacking CD38 antigen
    Rare and not routinely available reagent cells
  • **Panels from RBC of Daratumumab treated Patients**
    Not standardized

II. Blocking CD38 antigens on reagent and donor cells
Not recognized by antihuman globulin (AHG)
  • **Anti-CD38 F(ab')2 fragments**
    Bind and mask CD38 on reagent cells
    Not commercially available.
  • **Anti-CD38 + monospecific anti-human IgG**
    Pre-Adsorbed RBCs with Daratumumab blocked with mono-specific anti-human IgG competing out AHG
  • **Non-human anti-CD38**
    Not recognized by AHG

III. Neutralization of anti-CD38 antibody in patient plasma prior to IAT testing
Routine blood bank testing providing adequate anti-CD38 antibody neutralization with:
  • **Anti-idiotype antibody**
    Commercially available
    Not typically available in standard blood bank inventory
  • **Soluble CD38 antigen**
    Requires large amounts of soluble CD38 required for neutralization of therapeutic daratumumab levels
    Less efficacious than anti-idiotype
    Method not standardized
  • **CD38 antigen from myeloma lines lysate/stroma**
    Difficult to standardize methodology
  • **Anti-CD38 aptamers**

(Continued)

Table 1 (Continued).

IV. Extended patient antigen typing with phenotypical matching of RBC units
Delays in transfusion if extended-match units difficult to acquire
Historically a method of choice for patients with known auto- and allo-antibodies
Antibody identification testing not required when matched units available
Serologic Crossmatch will be incompatible due to presence of Anti-CD38
  • **Extended RBC phenotype of recipients**
    To be performed prior initiating daratumumab therapy
  • **RBC genotyping**
    Can be performed at any time
    Universal genotyping is expensive
    Extended matching not necessary if:
    negative antibody screen and no prior history of auto- and allo-antibodies

Abbreviations: AHG, antihuman globulin; DTT, dithiothreitol; IAT, indirect anti-globulin test; RBC, red blood cells.

aside from Kell, the majority of clinically significant alloantibodies can be ruled out. Thus, Kell negative units are administered unless that patient is known to be K positive. ABO and Rh typing are not affected.

Chapuy et al. published a 0.2 M DTT validation study that investigated the applicability of the DTT method to negate daratumumab interference through the Biomedical Excellence for Safer Transfusion (BEST) Collaborative.[27] The study included 25 worldwide blood bank laboratories in North America, South America, Europe, Australia and the Asia Pacific region. The objective was to determine if the DTT method could reliably permit alloantibody identification in the presence of daratumumab. Regardless of the method of testing, all sites observed daratumumab interference using patients sample with the initial antibody screen with untreated cells. All sites correctly identified an unknown alloantibody in samples spiked with daratumumab. In a follow up survey, 84% of the respondents agreed that the DTT method fulfills the standard of care when evaluating patients on daratumumab and 89% of participants said they plan to use the DTT method to test samples of patients on daratumumab.

In order to mitigate destructive effects of 0.2 M DTT on the red cell membrane, particularly the destruction of the Kell antigen, Hosokawa et al. created the Osaka method.[28] Their method uses 0.01 M DTT in pH 7.3
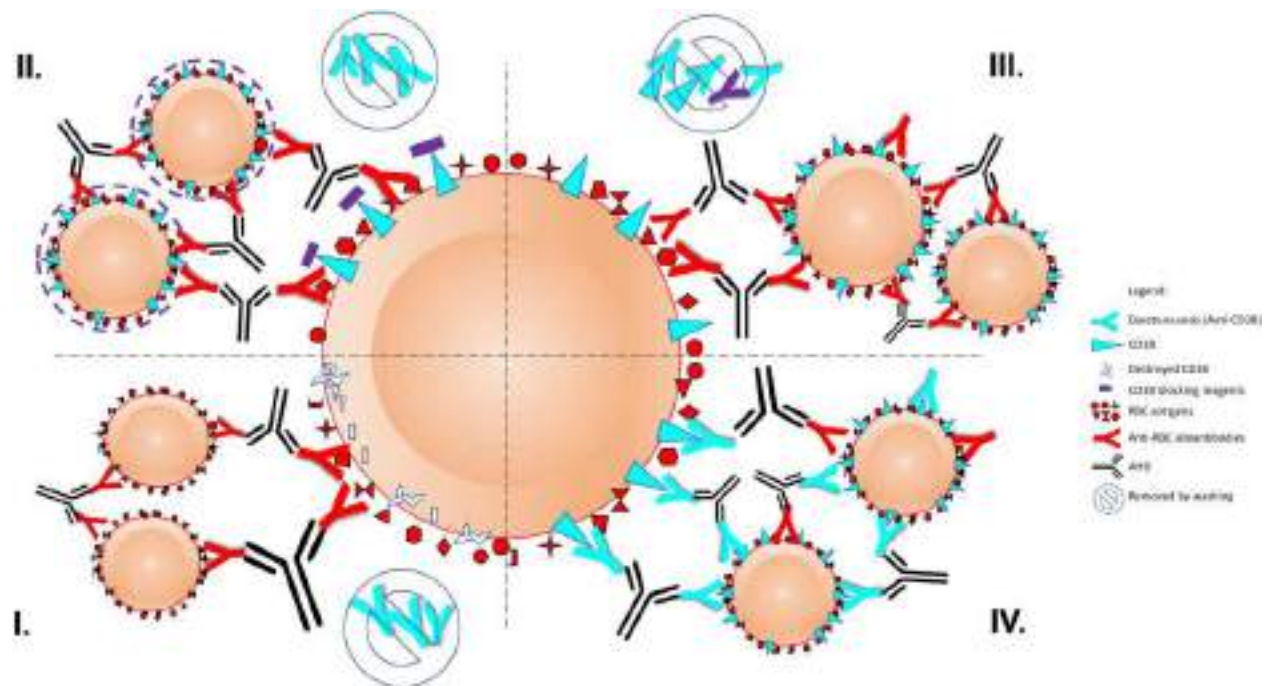
Figure 1 Methodologies utilized to abrogate daratumumab interference in pre-transfusion testing.
Notes: RBC agglutination due to presence of RBC specific alloantibodies or autoantibodies is not interfered by anti-CD38 after interventions I–III. I. RBCs lacking CD38 antigens used in pre-transfusion testing; II. Blocking CD38 antigens on reagent and donor cells; III. Neutralization of anti-CD38 antibody in patient plasma prior to IAT testing. IV. Extended patient antigen typing with transfusion of phenotypically matched RBC units does not require for RBC specific alloantibodies or autoantibodies to be identified (It is not necessary to distinguish if RBC agglutination is due to anti-CD38 or alloantibody or autoantibody presence).
Abbreviations: AHG, antihuman globulin; IAT, indirect antiglobulin test; RBC, red blood cells.

phosphate-buffered saline (PBS) and an automatic blood cell washing centrifuge to negate daratumumab interference while preserving K antigenicity. In a follow up article, the authors validated the Osaka method by using commercially available IgM anti-B (or anti-A) antibody as a quality control indicator instead of loss of Kell.[29] They also investigated the ability to detect anti-K and anti-Ku in daratumumab treated individuals. Anti-K reactivity was detected and daratumumab interference was negated. The authors do note that the Osaka method failed to detect low-titer (<4) anti-K. Large volumes of antigen typed RBCs can be treated with DTT and stored in Alsever's solution for convenience.[30]

Reagent red cells treated with trypsin or ficin enzyme are frequently used for ancillary testing. Trypsin does not degrade Kell antigens, but does destroy a number of other clinically significant antigens including M, N, EnaTS, Lutheran, Ge2, Ge3, Ge4 and Ch/Rg antigens.[26,31] Papain degrades antigens from Duffy and MNS blood group systems, Ge2, Ge4 and Ch/Rg.[32]

There is a report that use of polybrene, a quaternary ammonium polymer, could negate the positive results for

the IAT using standard PEG-antihuman globulin (PEG-AHG) in patients on daratumumab therapy.[33] Polybrene is used in many laboratories in Taiwan for antibody detection. The main limitation of the manual polybrene (MP) test is the lack of adequate sensitivity in the Kell system.

Reagent Cells Lacking CD38 Antigen

Schmidt et al. described testing patients' plasma against cord blood cells.[34] No reactivity was observed with all cord cells. The group tested the patients' plasma against adult cells with only the "i" antigen and no "I" on the surface which showed weak to 1+ reactivity. They concluded the cord cells express extremely low to no CD38 on their surface. They created a cord RBC panel consisting of 6–7 antigen typed cord cells and made 3% suspensions using Alsever's solution. Tube testing with low ionic strength saline (LISS) was performed, and they report they were successful at screening and transfusing 2 daratumumab patients 17 times. The method may not be feasible in routine laboratories that do not have cord blood cells readily available.[35]
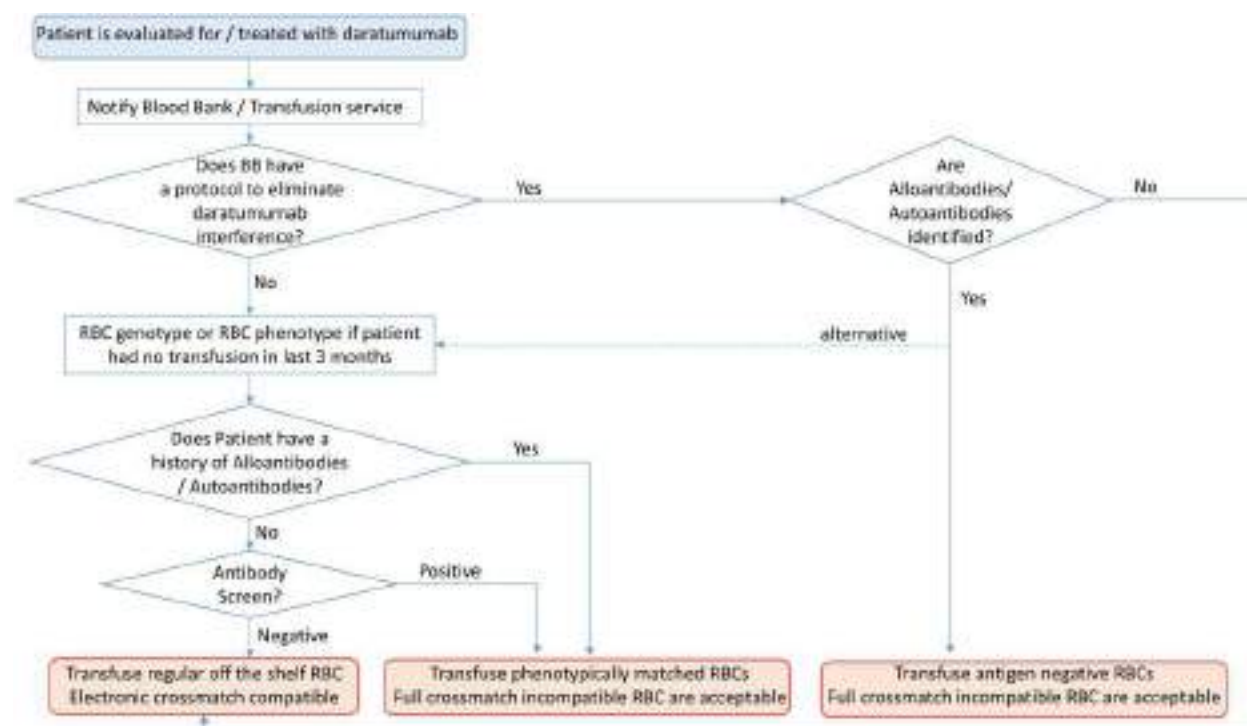
Figure 2 Algorithm for clinicians managing patients treated with daratumumab.
Abbreviations: AHG, antihuman globulin; RBC, red blood cells.

Alternatively, red cells that are negative for CD38 (red cells from daratumumab patients) or In (Lu) red cells can also be used for antibody investigations.[36]

Blocking CD38 Antigens on Reagent or Donor Cells

Another novel solution to daratumumab interference is pre-incubation of RBC with F(ab′)2 fragments of daratumumab.[37] F(ab′)2 fragments of daratumumab are created by digestion with pepsin. F(ab′)2 fragments bind the same epitope on CD38 and prevent binding of RBCs by free daratumumab. Known antibodies in patients' plasma and serum (anti-D, anti-C, anti-E, anti-e, anti-K, anti-Fya, anti-Lua, anti-S, or anti-M) were detected with reproducible results. According to authors, this is a relatively easy to use method but requires commercial availability and further validations.

In another attempt to alleviate daratumumab interference but maintain the integrity of the red cell structure, Chinoca Ziza et al. proposed the blockage monoclonal antibody protocol (BMAP).[38] Donor RBCs of known phenotype are treated with anti-CD38 (titer of anti-CD38 was previously determined by dilution resulting in 1+ agglutination). These anti-CD38 treated RBCs were then blocked with monospecific anti-

human IgG (Fc specific). Their experiments showed that daratumumab interference was eliminated in 20 of 20 patients. This method would ideally be performed in parallel to DTT treatment to allow detection of alloantibodies to high prevalence antigens eliminated by DTT. The method has limitations which include technical difficulty, unavailability of anti-CD38, and need to titrate anti-CD38 prior to testing (to reduce false positives associated with heavily coated RBCs). Of note, the protocol was validated for noncommercial RBCs collected within 7 days. The method has advantages of being inexpensive, accurate, and possibly applicable in the future to help negate interference by other monoclonal drugs targeting RBC antigens. Mouse anti-CD38 neutralized the detection of human IgG observed in daratumumab treated patient serum without affecting controls. A similar approach can be explored during pre-transfusion testing.[39]

Neutralization of Anti-CD38 Antibody in Patient Plasma Sample Prior to IAT Testing

Oostendorp et al. successfully blocked interference by daratumumab and other similar anti-CD38 monoclonal antibodies using an anti-idiotype antibody and recombinant soluble CD38

extracellular domain protein (sCD38).[20] These two solutions permitted correct irregular antibody identification. Work by Oostendorp et al. is consistent with the work of Chapuy et al. documenting inhibited binding of daratumumab to CD38 +HL60 cells with the addition of sCD38 which reduced daratumumab binding in a dose dependent manner.[25] Neutralization with anti-idiotype antibody reduced binding by 95%. The neutralization methods are highly effective and simpler to perform than DTT treatment of RBCs. Although costs are higher and large amounts of sCD38 would be required to treat samples from patients on daratumumab, the primary obstacle is limited availability of reagents. In contrast, DTT is relatively inexpensive and already standardized by blood banks.

Another method for overcoming daratumumab interference is the use of Daudi cell stroma generated by sonication.[40] Daudi B-cell stroma expresses high levels of CD38. Adsorbing a sample of plasma with Daudi cell stroma led to removal of daratumumab interference while allowing detection of alloantibodies such as anti-K, anti-Yta, and anti-Gya. According to authors, Daudi cell stroma is relatively inexpensive and large quantities can be stored and frozen for convenience. Interestingly, Tremblay et al. compared the AABB standard 0.2 M DTT method with the Osaka method, and their own Daudi cell stroma method. Treatment with Daudi cell stroma did not affect anti-Jsb detection and displayed reduced reactivity of anti-K. Although testing with the Osaka method reduced daratumumab interference, there was still K antigen loss, denaturing of Jsb, and less strong reaction with anti-K than without 0.01 M DTT treatment. Their findings suggest adsorption with Daudi cell stroma cells removes anti-CD38 without compromising antibody detection and identification. The main limitation is the standardization of the method and the requirement of specialized equipment such as an ultrasonic processor and centrifuge with performance up to 20,000g.

A high affinity single-stranded DNA oligonucleotides, aptamers, specific to daratumumab can also be used to neutralize binding to CD38.[41]

Establishing Baseline Phenotype or Genotype Prior to Starting Treatment with Daratumumab

Additional blood bank methods include RBC phenotyping and genotyping. Antigen phenotyping is a routine serologic procedure and aids in selection of phenotype similar

RBC units for transfusion.[35,42] Extended RBC phenotyping should be performed prior to treatment with daratumumab in patients who have not been transfused in the prior 3 months.

Genotyping can be performed after initiation of anti-CD38 treatment but is expensive and can be associated with increased turnaround times for results.[36] One of the benefits to always providing phenotypically similar RBCs is the reduced risk of sensitization and future alloantibody formation for the red cell antigens that are matched. Anti-RBC alloantibodies that may be present will not react against matched RBC units. AHG crossmatches with phenotypically or genotypically matched units will still be crossmatch incompatible due to the presence of Anti-CD38 in the patient sample.

Transfusion of RBC Units with Incompatible Crossmatches

To aid transfusion services, the AABB published a bulletin with recommendations for handling daratumumab interference.[43] Prior to daratumumab therapy, a baseline type and screen should be performed; a baseline genotype or phenotype is also recommended. After therapy, antibody screening and identification should be performed using DTT treated cells, and K-negative units should be issued unless the patient is known to be K-positive. Crossmatch should be performed as per standard requirements. Alternatively, an AHG crossmatch may be performed using DTT-treated donor cells. For patients with no history of antibodies and a negative antibody screen using DTT-treated cells, an electronic or immediate-spin crossmatch with ABO/RhD-compatible, K-matched units may be performed. If an emergency transfusion is required, uncrossmatched ABO/RhD-compatible RBCs may be given per local blood bank practices. Current evidence shows no increased risk of hemolysis after transfusion in patients treated with daratumumab.[44] Further, a recent article by Ye et al. found no significant daratumumab associated difference in alloimmunization risk between 45 MM patients receiving transfusions on daratumumab and 46 MM patient receiving transfusions but not on daratumumab as the control group. The study is limited due to the number of patients enrolled. It may be useful to study larger cohorts of patients receiving daratumumab to investigate if there is a clinically significant increase in risk of alloimmunization.[45]

Impact of Daratumumab on Transfusion Service Costs

Anani et al retrospectively reviewed 62 patients on daratumumab and compared the costs of: DTT method with K-negative RBC transfusion, patient phenotyping or genotyping with antigen matched transfusion, and combination of genotyping and DTT treated RBC investigation with genotype predicted antigen-matched RBC transfusion.[46] They found that genotyping was most cost effective in patients requiring long-term transfusion when matching less than 4 antigens. Cushing et al. published a detailed cost analysis comparing theoretical universal genotyping with a provision of phenotypically similar RBC transfusions versus implementation of a standardized notification and testing/transfusion algorithm, an approach combining DTT-based testing with selective genotyping and the provision of phenotypically similar RBCs only for patients with clinically significant antibodies.[47] The targeted, standardized approach was found to be more cost effective in a cohort of patients followed over a year. Cost-related considerations are especially important for mitigating daratumumab interference in national general blood bank services and for blood banks in developing countries where specialized reagent are not readily available.[48]

Conclusion

The prevalence of monoclonal antibody therapies will only continue to rise. Daratumumab epitomizes the need for the recognition of potential interferences of therapeutic agents with pre-transfusion testing, the development of appropriate methodologies to overcome these interferences as well as good communication between the blood bank and the treating physician in order to secure safe blood products for the patient. Established blood banking pre-transfusion testing standards and practices for the clinical management of patients undergoing blood transfusions serve as building blocks for creating pre-transfusion testing protocols for those patients receiving monoclonal antibody therapies. As the clinical use of daratumumab increased, several methodologies have been utilized to resolve daratumumab interference during pre-transfusion testing, which have been discussed in this article. Equally as important is the timely communication between the transfusion service and clinical teams. Clinical providers should be aware of daratumumab interference and should contact the blood bank prior to start of Daratumumab therapy to ensure the patient has a baseline type and screen, and that blood bank has a protocol to negate Daratumumab interference. Emphasis

on educational efforts by blood banking and clinical professional societies are also key for continued blood transfusion safety.

Abbreviations

DTT, dithiothreitol; AHG, antihuman globulin; IAT, indirect antiglobulin test; MM, multiple myeloma; RBC, red blood cells.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Fung MG, Hillyer BJ. *AABB Technical Manual*. 18th ed. AABB; 2014.
2. Pietsch EC, Dong J, Cardoso R, et al. Anti-leukemic activity and tolerability of anti-human CD47 monoclonal antibodies. *Blood Cancer J*. 2017;7(2):e536. doi:10.1038/bcj.2017.7
3. Tong B, Wang M. CD47 is a novel potent immunotherapy target in human malignancies: current studies and future promises. *Future Oncol*. 2018;14(21):2179–2188. doi:10.2217/fon-2018-0035
4. Palumbo A, Chanan-Khan A, Weisel K, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;375(8):754–766. doi:10.1056/NEJMoa1606038
5. Kaufman GP, Schrier SL, Lafayette RA, Arai S, Witteles RM, Liedtke M. Daratumumab yields rapid and deep hematologic responses in patients with heavily pretreated AL amyloidosis. *Blood*. 2017;130(7):900–902. doi:10.1182/blood-2017-01-763599
6. Cancer Facts & Figures 2021. American Cancer Society journal, CA: a cancer journal for clinicians. Available from: https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2021.html. Accessed May 3, 2021.
7. Plesner T, Arkenau HT, Gimsing P, et al. Phase 1/2 study of daratumumab, lenalidomide, and dexamethasone for relapsed multiple myeloma. *Blood*. 2016;128(14):1821–1828. doi:10.1182/blood-2016-07-726729
8. Albeniz I, Demir Ö, Turker-Sener L, Yalcintepe L, Nurten R, Bermek E. Erythrocyte CD38 as a prognostic marker in cancer. *Hematology*. 2007;12(5):409–414. doi:10.1080/10245330701383841
9. de Weers M, Tai YT, van der Veer MS, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol*. 2011;186(3):1840–1848. doi:10.4049/jimmunol.1003032
10. McKeage K. Daratumumab: first global approval. *Drugs*. 2016;76 (2):275–281. doi:10.1007/s40265-015-0536-1
11. Clark CA, Cornell RF, Scott EC, Chung J, Costa LJ. Management of relapsed and refractory multiple myeloma in modern times: incorporating new agents into decision-making. *Am J Hematol*. 2016;91 (10):1044–1051. doi:10.1002/ajh.24478
12. Darzalex package insert. Horsham, PA: Janssen Biotech; 2015. Available from: http://wwwdarzalexcom/shared/product/darzalex/darzalex-prescribing-informationpdf. Accessed March 3, 2021.
13. Chapuy CI, Kaufman RM, Alyea EP, Connors JM. Daratumumab for delayed red-cell engraftment after allogeneic transplantation. *N Engl J Med*. 2018;379(19):1846–1850. doi:10.1056/NEJMoa1807438
14. Mirgh S, Ahmed R, Agrawal N, et al. Will daratumumab be the next game changer in early thymic precursor-acute lymphoblastic leukaemia? *Br J Haematol*. 2019;187(2):e33–e35. doi:10.1111/bjh.16154

15. van de Donk N, Richardson PG, Malavasi F. CD38 antibodies in multiple myeloma: back to the future. *Blood*. 2017;131(1):13–29. doi:10.1182/blood-2017-06-740944

16. Sullivan HC, Gerner-Smidt C, Nooka AK, et al. Daratumumab (anti-CD38) induces loss of CD38 on red blood cells. *Blood*. 2017;129(22):3033–3037. doi:10.1182/blood-2016-11-749432

17. Chari A, Arinsburg S, Jagannath S, et al. Blood transfusion management and transfusion-related outcomes in daratumumab-treated patients with relapsed or refractory multiple myeloma. *Clin Lymphoma Myeloma Leuk*; 2017.

18. Schuetz C, Hoenig M, Moshous D, et al. Daratumumab in life-threatening autoimmune hemolytic anemia following hematopoietic stem cell transplantation. *Blood Adv*. 2018;2(19):2550–2553. doi:10.1182/bloodadvances.2018020883

19. Murphy MF, Dumont LJ, Greinacher A. Interference of new drugs with compatibility testing for blood transfusion. *N Engl J Med*. 2016;375(3):295–296. doi:10.1056/NEJMc1515969

20. Oostendorp M, Lammerts van Bueren JJ, Doshi P, et al. When blood transfusion medicine becomes complicated due to interference by monoclonal antibody therapy. *Transfusion*. 2015;55(6 Pt 2):1555–1562. doi:10.1111/trf.13150

21. Vidal-Crespo A, Matas-Céspedes A, Rodriguez V, et al. Daratumumab displays in vitro and in vivo anti-tumor activity in models of B-cell non-Hodgkin lymphoma and improves responses to standard chemo-immunotherapy regimens. *Haematologica*. 2020;105(4):1032–1041. doi:10.3324/haematol.2018.211904

22. Salles G, Gopal AK, Minnema MC, et al. Phase 2 study of daratumumab in relapsed/refractory mantle-cell lymphoma, diffuse large B-cell lymphoma, and follicular lymphoma. *Clin Lymphoma Myeloma Leuk*. 2019;19(5):275–284. doi:10.1016/j.clml.2018.12.013

23. Berthelier V, Laboureau J, Boulla G, Schuber F, Deterre P. Probing ligand-induced conformational changes of human CD38. *Eur J Biochem*. 2000;267(10):3056–3064. doi:10.1046/j.1432-1033.2000.01329.x

24. Anani WQ, Duffer K, Kaufman RM, Denomme GA. How do I work up pretransfusion samples containing anti-CD38? *Transfusion*. 2017;57(6):1337–1342. doi:10.1111/trf.14144

25. Chapuy CI, Nicholson RT, Aguad MD, et al. Resolving the daratumumab interference with blood compatibility testing. *Transfusion*. 2015;55(6 Pt 2):1545–1554. doi:10.1111/trf.13069

26. M L-fc R, Olsson ML. *The Blood Group Antigen Factsbook*. 3rd ed. MA: Academic Press; 2012.

27. Chapuy CI, Aguad MD, Nicholson RT, et al. International validation of a dithiothreitol (DTT)-based method to resolve the daratumumab interference with blood compatibility testing. *Transfusion*. 2016;56 (12):2964–2972. doi:10.1111/trf.13789

28. Hosokawa M, Kashiwagi H, Nakayama K, et al. Distinct effects of daratumumab on indirect and direct antiglobulin tests: a new method employing 0.01 mol/L dithiothreitol for negating the daratumumab interference with preserving K antigenicity (Osaka method). *Transfusion*. 2018;58(12):3003–3013. doi:10.1111/trf.14900

29. Hosokawa M, Kashiwagi H, Nakayama K, et al. Additional validation of Osaka method (0.01 mol/L dithiothreitol) for negating the daratumumab interference. *Transfusion*. 2019;59(7):2479–2480. doi:10.1111/trf.15305

30. Disbro WL. Stability guidelines for dithiothreitol-treated red blood cell reagents used for antibody detection methods in patients treated with daratumumab. *Immunohematology*. 2017;33(3):105–109. doi:10.21307/immunohematology-2019-015

31. Lancman G, Arinsburg S, Jhang J, et al. Blood transfusion management for patients treated with anti-cd38 monoclonal antibodies. *Front Immunol*. 2018;9:2616. doi:10.3389/fimmu.2018.02616

32. Bub CB, Reis IND, Aravechia MG, et al. Transfusion management for patients taking an anti-CD38 monoclonal antibody. *Rev Bras Hematol Hemoter*. 2018;40(1):25–29. doi:10.1016/j.bjhh.2017.09.003

33. Yeh TJ, Yeh CJ, Liu YC, Hsiao HH. Manual polybrene method for pretransfusion test could overcome the interference of daratumumab therapy in myeloma. *Transfusion*. 2019;59(8):2751–2752. doi:10.1111/trf.15341

34. Schmidt AE, Kirkley S, Patel N, et al. An alternative method to dithiothreitol treatment for antibody screening in patients receiving daratumumab. *Transfusion*. 2015;55(9):2292–2293. doi:10.1111/trf.13174

35. De Vooght KM, Oostendorp M, van Solinge WW. Dealing with anti-CD38 (daratumumab) interference in blood compatibility testing. *Transfusion*. 2016;56(3):778–779. doi:10.1111/trf.13474

36. Quach H, Benson S, Haysom H, et al. Considerations for pre-transfusion immunohaematology testing in patients receiving the anti-CD38 monoclonal antibody daratumumab for the treatment of multiple myeloma. *Intern Med J*. 2018;48(2):210–220. doi:10.1111/imj.13707

37. Selleng K, Gebicka PD, Thiele TF. (ab')2 fragments to overcome daratumumab interference in transfusion tests. *N Engl J Med*. 2018;379(1):90–91. doi:10.1056/NEJMc1804751

38. Chinoca Ziza KN, Paiva TA, Mota SR, et al. A blockage monoclonal antibody protocol as an alternative strategy to avoid anti-CD38 interference in immunohematological testing. *Transfusion*. 2019;59 (5):1827–1835. doi:10.1111/trf.15202

39. Baig NA, Dukek BA, Falbo DK, et al. Daratumumab interference in flow cytometric anti-granulocyte antibody testing can be overcome using non-human blocking antibodies. *Vox Sang*. 2021;116 (1):116–122. doi:10.1111/vox.12989

40. Tremblay T, Branch DR, Loubaki L. Daudi cell stroma: an alternative to dithiothreitol to resolve daratumumab interference in pretransfusion testing. *Transfusion*. 2020;60(9):2090–2096. doi:10.1111/trf.15932

41. Tapia-Alveal C, Olsen TR, Worgall TS. Personalized immunoglobulin aptamers for detection of multiple myeloma minimal residual disease in serum. *Commun Biol*. 2020;3(1):781. doi:10.1038/s42003-020-01515-x

42. Hannon JL, Clarke G. Transfusion management of patients receiving daratumumab therapy for advanced plasma cell myeloma. *Transfusion*. 2015;55(11):2770. doi:10.1111/trf.13267

43. Mitigating the anti-CD38 interference with serologic testing AABB association bulletin #16-02; 2016. Available from: http://www.aabb.org/programs/publications/bulletins/Documents/ab16-02.pdf. Accessed May 3, 2021.

44. Chari A, Arinsburg S, Jagannath S, et al. Blood transfusion management and transfusion-related outcomes in daratumumab-treated patients with relapsed or refractory multiple myeloma. *Clin Lymphoma Myeloma Leuk*. 2018;18(1):44–51. doi:10.1016/j.clml.2017.09.002

45. Ye Z, Wolf LA, Mettman D, Plapp FV. Risk of RBC alloimmunization in multiple myeloma patients treated by daratumumab. *Vox Sang*. 2020;115(2):207–212. doi:10.1111/vox.12864

46. Anani WQ, Marchan MG, Bensing KM, et al. Practical approaches and costs for provisioning safe transfusions during anti-CD38 therapy. *Transfusion*. 2017;57(6):1470–1479. doi:10.1111/trf.14021

47. Cushing MM, DeSimone RA, Goel R, et al. The impact of daratumumab on transfusion service costs. *Transfusion*. 2019;59 (4):1252–1258. doi:10.1111/trf.15134

48. Setia R, Dogra M, Sachdeva P, Handoo A, Choudhary D, Agarwal A. Daratumumab (Anti-CD38) interference with serological testing: an emerging challenge for blood banks in developing countries. *Glob J Transfus Med*. 2017;2(2):163. doi:10.4103/GJTM.GJTM_42_17

Ferric Carboxymaltose for Anemic Perioperative Populations: A Systematic Literature Review of Randomized Controlled Trials

John Jeffrey Jones, Linda M Mundy, Nicole Blackman & Michelle Shwarz

Published online: 26 May 2021.

Submit your article to this journal

Article views: 241

View related articles

View Crossmark data

Citing articles: 5 View citing articles

R E V I E W

Ferric Carboxymaltose for Anemic Perioperative Populations: A Systematic Literature Review of Randomized Controlled Trials

John Jeffrey Jones [1]
Linda M Mundy [2]
Nicole Blackman [2]
Michelle Shwarz [2]

[1]College of Pharmacy and Health Sciences, St. John's University, Queens, NY, USA; [2]American Regent, Inc, Norristown, PA, USA

Importance: Perioperative anemia is a common comorbid condition associated with increased risk of morbidity and mortality in patients undergoing elective surgical procedures.

Objective: We conducted a systematic literature review (SLR) to determine the efficacy and safety of the use of intravenous ferric carboxymaltose (FCM) for the treatment of perioperative anemia in preoperative, intraoperative, and postoperative elective surgical care.

Evidence Review: Studies meeting inclusion criteria for the SLR reported on treatment efficacy in an adult study population randomly allocated to FCM for the treatment of perioperative anemia during the perioperative period. After screening, 10 of 181 identified studies from searches in MEDLINE and EMBASE databases were identified for inclusion in this review.

Findings: Preoperative treatment was reported in six studies, intraoperative treatment in one study, postoperative treatment in two studies, and both pre- and postoperative treatment in one study. Together, 1975 patients were studied, of whom 943 were randomized to FCM, of whom 914 received FCM treatment. The 10 studies reported elective surgical populations for colorectal, gastric, orthopedic, abdominal, urologic, plastic, neck, gynecologic, and otolaryngologic procedures. Given the clinical and methodological heterogeneity of the studies, the analyses were limited to qualitative assessments without meta-analyses. All 10 studies reported statistically greater changes in hemoglobin concentration, serum ferritin, and/or transferrin saturation with FCM treatment compared with comparators (placebo, oral iron, standard care, or a combination of these). Two studies reported statistically significant differences in transfusion rate and 2 studies reported significant differences in length of hospital stay between FCM and its comparator(s).

Conclusions and Relevance: This SLR adds to existing data that administration of FCM in preoperative and postoperative settings improves hematologic parameters. Several studies in the review supported the beneficial effects of FCM in reducing transfusion rate and length of stay. Larger, well-designed, longer-term studies may be needed to further establish the efficacy and safety of FCM in elective surgery patients with perioperative anemia.

Keywords: intravenous iron, hemoglobin, elective surgery, patient blood management, iron-deficiency anemia

Plain Language Summary

Preoperative and postoperative anemia occur frequently in patients undergoing elective surgery.[1]

Perioperative anemia is associated with an increased risk of morbidity, mortality, and other adverse postoperative outcomes.[1–4]

Correspondence: Nicole Blackman
American Regent, Inc, 800 Adams Avenue, Suite 200, Norristown, PA, 19403, USA
Tel +1 631 772 3524
Email nblackman@americanregent.com

Iron deficiency is a common cause of anemia in surgical patients, affecting at least 10% of surgical patients during the preoperative period and at least 20% during the postoperative period.[5,6]

Blood transfusion is frequently administered as part of the standard of care for perioperative anemia[7] and may produce a temporary increase in hemoglobin levels[8] but is associated with poor outcomes.[9]

This systematic review evaluated the effectiveness and safety of ferric carboxymaltose in patients with perioperative anemia, with a focus on patients' health states during the preoperative and postoperative period.

The 10 identified studies included patient populations undergoing elective colorectal, gastric, orthopedic, abdominal, urologic, plastic, neck, gynecologic, and otolaryngologic procedures.

Comparators were placebo, oral iron, standard care, or a combination of these interventions.

Overall, this review found that ferric carboxymaltose significantly increased hemoglobin level, serum ferritin concentrations, and transferrin saturation in patients with perioperative anemia who were undergoing elective surgery.

Introduction

Perioperative anemia is a common comorbid condition associated with increased risk of morbidity and mortality in patients undergoing elective surgical procedures. The most common etiology of perioperative anemia in adults is iron deficiency. Clinical signs and symptoms of iron-deficiency anemia (IDA) are protean and vary widely, and perioperative health outcomes associated with IDA include reduced physical function, prolonged postoperative recovery, increased length of hospital stay, and mortality.[1–4,10] Among orthopedic patients, the preoperative prevalence of anemia is estimated at 24% to 44%, and postoperative estimates are significantly higher.[1] Among patients with advanced colon cancer undergoing colectomy, the estimated prevalence of anemia is reported to be as high as 75.8%.[11]

In preoperative evaluation for IDA, diagnostic assessment and pre-surgical treatment varies. One recognized intervention is blood transfusion, with an estimated 6.6 million red blood cell (RBC) units administered annually to elective surgery patients in the United States.[4] To assess the use of an intravenous iron in anemic perioperative patient populations, a systematic literature review (SLR) was conducted and focused on the safety and efficacy of ferric carboxymaltose.

Methods
Study Design

The study protocol was designed and executed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).[12] All review methods were conducted a priori according to the established protocol. Publication screening, selection, and assessments of bias were carried out by the authors, with consensus for reconciliation. Included studies were conducted in adults (≥18 years of age) with anemia (if not IDA) who had been identified for elective surgery and randomly allocated to receive intravenous (IV) FCM or comparator-(s). Inclusion also required study publication in peer-reviewed journals. Studies were excluded if the design was nonexperimental, if efficacy was not reported, or if the investigative treatment consisted of only IV FCM in combination with either erythropoietin or an erythropoi-esis-stimulating agent; animal and pediatric studies were also excluded. Conference abstracts and grey literature were excluded.

Literature Search

Comprehensive, computer-based literature searches for records were performed systematically using the MEDLINE (PubMed, US National Library of Medicine, National Institutes of Health) and EMBASE databases. The electronic search strategy was peer reviewed and developed with guidance from a university librarian at St. John's University College of Pharmacy and Health Sciences. Databases were searched from their inception to January 2021 for abbreviations and full-phrase versions of keywords and Medical Subject Headings combined with Boolean operators as outlined in the Supplementary Data, Appendix 1. Two reviewers independently and in duplicate assessed titles and abstracts of identified records for trial eligibility, and a third reviewer resolved any discrepancies by discussion or adjudication until consensus was reached. Relevant records were further assessed by examining the full-text articles. A snowball method (using a key manuscript as a starting point and consulting the bibliography to find other relevant titles on the subject) was used to search the identified literature to additional studies. Data were extracted onto a standardized extraction form.

Risk of Bias and Quality Assessment

Internal validity and risk of bias for each study meeting final inclusion criteria were assessed in duplicate by 2 independent reviewers using the Cochrane Risk of Bias Tool.[13] The key types of bias that were examined were selection, performance, detection, attrition, and reporting. The overall quality of evidence was assessed using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE)[14] approach to determine the strength of the conclusions/recommendations from this review.

Outcome Measures

The primary outcomes were the absolute or relative change from baseline in hemoglobin (Hb) concentration (g/dL), serum ferritin (ng/mL), and% transferrin saturation (TSAT). Primary outcomes that were reported in alternative units were converted for consistency across studies for ease of interpretability; conversions were quality controlled for accuracy. Secondary outcome measures were the proportion of patients who received a perioperative blood transfusion, the occurrence of adverse events, treatment-related adverse events, postoperative mortality, length of hospital stay, and any validated measure of quality of life (QOL).

Results

Study Selection

We identified 244 citations, 208 through database searching and 36 through other sources. The number was decreased to 181 after removing duplicates (Figure 1). The 181 citations were screened based on title and abstract, and 139 were excluded. After screening the full-text articles of the remaining 42 citations, 32 were excluded. Ultimately, 10 eligible studies were included in this systematic review.

Study Characteristics

All 10 studies included in the systematic review were randomized clinical trials and were published between 2014 and 2020.[7,15–23] Five studies were conducted in Europe (United Kingdom,[15,21,23] Spain[15,16]), 2 studies in Oceania (Australia[7,17]), and 3 studies in Asia (South Korea[19,20,22]).

The design and patient flow of the included studies are summarized in Table 1. Studies investigated the use of FCM in the following surgical populations: cancer (colorectal,[18] gastric[19]), cardiac,[21] menorrhagia,[20] orthopedic (hip arthroplasty,[16,22] total knee arthroplasty[15,22]), abdominal,[17,23] and a combination of orthopedic, abdominal, neck, gynecologic, urologic, plastic, and ear, nose, and throat (ENT).[7] Ferric carboxymaltose was administered preoperatively in 5 studies,[16,18,20,21,23] intraoperatively in 1 study,[22] postoperatively in 3 studies,[7,15,19] and perioperatively (both preoperatively and postoperatively) in 1 study.[17] With respect to blinding, 2 studies used a double-blind design,[16,23] 3 were single-blind,[15,19,22] and 5 were open-label.[7,17,18,20,21] Follow-up periods for patients varied, spanning from 2 weeks post-infusion,[20] 4 weeks postoperatively[15,17,22] to 2 to 6 months after hospital discharge.[7,15,16,19,21,23]

In total, 1975 study patients were randomized across the 10 studies, 943 (48%) of whom were randomized to receive IV FCM, and of these, 914 (97%) received their assigned treatment. Three studies compared IV FCM with oral ferrous sulfate (dose: 100 mg per day[15] to 200 mg twice daily[15,21]), 1 study compared IV FCM with IV iron sucrose (dose: 200 mg iron x 3/week),[20] and 2 studies compared IV FCM with standard of care (no intervention[7] or no treatment, continued observations, oral iron recommendations, and allogeneic blood transfusion [ABT]).[17] The comparator for 3 of the remaining 4 studies was IV saline placebo,[19,22,23] and the comparators for the other remaining study (triple-arm, parallel design) were 1 mL subcutaneous (SC) saline placebo with 250 mL of IV saline placebo and 40,000 IU/1 mL SC erythropoietin (EPO) with 1000 mg IV FCM diluted in 250 mL saline.[16] The dosages of IV FCM across studies varied in terms of total dose and dosage calculation as well as administration time, which spanned 6 to 30 minutes. In 3 studies, IV FCM was given in a single dose of 1000 mg;[7,22,23] in the 7 others, the dose was determined by the Ganzoni formula to correct the total iron deficit,[15] manufacturer recommendation (15 mg/kg),[7,17] weight cutoffs (<50 or ≥50 kg),[19,20] weight cutoffs in combination with inclusion Hb values,[18,21] or blood loss (5 x 10^{-7} mcg/L) if blood loss was ≥100 mL.[17] For 8 of the 10 studies, the maximum single dose of IV FCM was 1000 mg;[7,15–17,19,22,23] Keeler et al and Padmanabhan et al allowed a maximum total dose of up to 2000 mg (2 doses of 1000 mg administered 7 days apart and when required).[15,21]

The hematologic inclusion criteria varied among the studies, with 5 requiring IDA with Hb limits (9–12 g/dL,[16] <11 g/dL [women] or <12 g/dL [men],[18] <12 g/dL [women] or <13 g/dL [men][21,23] and ≥7–<10 g/dL[19]); 4 requiring IDA with specific hematologic profiles (serum
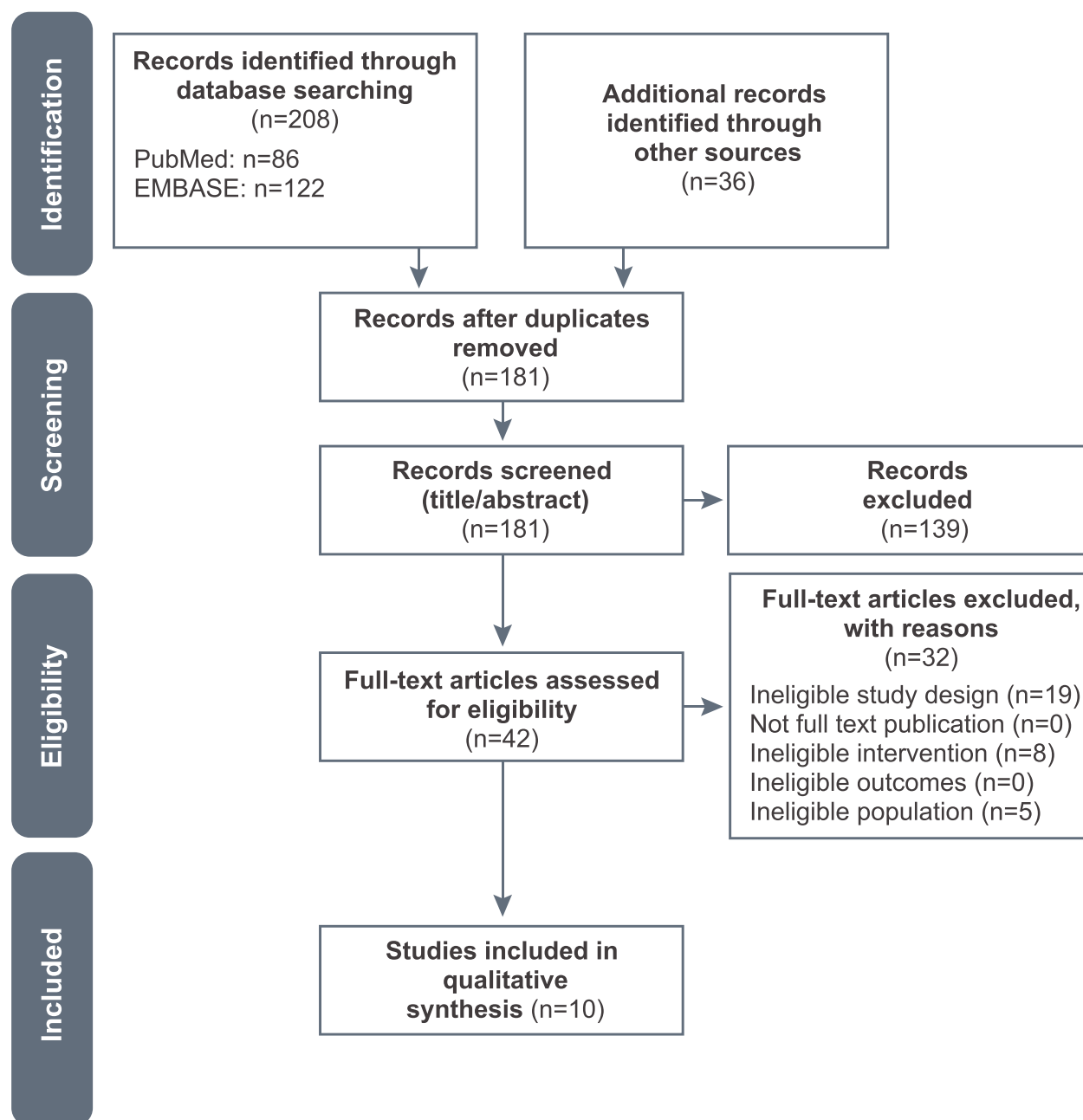
Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flowchart of the study selection process.
Note: Adapted from Page MJ, Moher D, Bossuyt PM, et al. PRISMA 2020 explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. *BMJ*. 2021;372:n160.[37]

ferritin <300 μg/L, transferrin saturation <25%, and Hb <12 g/dL [women] or <13 g/dL [men];[17] Hb 7–12 g/dL with serum ferritin ≤100 μg/L or TSAT ≤20%;[7] serum ferritin <300 μg/L and Hb <10 g/dL);[20,22] and 1 requiring anemia and/or IDA with a specific hematologic profile (Hb 8.5–12 g/dL, TSAT <20%, or both[15]).

Institution-specific medical care standards specifically relating to perioperative blood management (PBM) were explicitly stated in 3 of the 10 studies.[15–17] The first study used thromboprophylaxis with low-molecular-weight heparin and prophylaxis with proton pump inhibitors for upper gastrointestinal bleeding.[16] The second used

Table I Design and Patient Flow of Included Studies

	Study Description							
Study	**Perioperative State (Surgery)**	**Study Group Assignment**	**Design**	**Prespecified Blood Transfusion Criteria**	**Randomized N**	**Treated N**	**Withdrawn N**
Bisbe et al (2014)[15]	Postoperative (orthopedic)	**G1:** IV FCM 700–1000 mg single dose on postop day 1 **G2:** Oral ferrous sulfate 100 mg/d from postop day 7 to 30	Single-blinded	3. Hb <70 g/L=3 RBC transfusions independent of symptoms 4. Hb 71–89 g/L=2 RBC transfusions in presence of severe symptoms (HF with NYHA Class ≥III dyspnea, ACS, hemodynamic instability [hypotension, orthostatism], and/or acute or exacerbated chronic respiratory failure)	122 **G1:** 60 **G2:** 62	121 **G1:** 59 **G2:** 62	**G1:** 7 (LTFU) **G2:** 11 (LTFU)
Bernabeu-Wittel et al (2016)[16]	Preoperative (orthopedic hip)	**G1:** EPO 40,000 IU/1 mL EPO SC + IV FCM 1000 mg (2500 mg vials diluted in 250 mL of saline) infused over 20 min **G2:** 1 mL placebo (saline) SC + IV FCM 1000 mg (2500 mg vials diluted in 250 mL of saline) infused over 20 min **G3:** 1 mL placebo (saline) SC + IV placebo (250 mL of saline) infused over 20 min	Double-blind	1. Hb <70 g/L=3 RBC transfusions independent of symptoms 2. Hb 71–89 g/L=2 RBC transfusions in presence of severe symptoms (HF with NYHA Class ≥III dyspnea, ACS, hemodynamic instability [hypotension, orthostatism], and/or acute or exacerbated chronic respiratory failure)	303 **G1:** 100 **G2:** 103 **G3:** 100	293 **G1:** 97 (hip surgery—osteosynthesis, n=66 [68%]; partial arthroplasty, n=27 [28%]; total arthroplasty, n=4 [4%]) **G2:** 101 (hip surgery—osteosynthesis, n=70 [69%]; partial arthroplasty, n=28 [28%]; total arthroplasty, n=3 [3%]) **G3:** 97 (hip surgery—osteosynthesis, n=60 [62%]; partial arthroplasty, n=33 [34%]; total arthroplasty, n=4 [4%])	**G1:** 14 (discontinued intervention, n=6 [not operated, n=3, iron sucrose administration in follow-up period, n=3]; excluded from per protocol analysis,[a] n=8) **G2:** 17 (discontinued intervention, n=6 [not operated, n=2; iron sucrose administration in follow-up period, n=4]; excluded from per protocol analysis,[a] n=11) **G3:** 11 (discontinued intervention, n=4 [not operated, n=3, iron sucrose administration in follow-up period, n=1]; excluded from per protocol analysis, n=7)[a]
Froessler et al (2016)[17]	Perioperative (abdominal)	**G1:** Preop—IV FCM single 15 mg/kg injection (maximum: 1000 mg) administered over 15 min Postop—IV FCM 0.5 mg per recorded 1 mL blood loss if blood loss ≥100 mL, given within 2 days of surgery **G2:** Usual care consisting of no treatment, observation, oral iron recommendations, and ABT; prescription and administration of IV iron allowed	Open (masking not used)	Not reported	72 **G1:** 40 **G2:** 32	72 **G1:** 40 **G2:** 32	Not reported

(Continued)

Table 1 (Continued).

Study	Study Description — Perioperative State (Surgery)	Study Group Assignment	Design	Prespecified Blood Transfusion Criteria	Randomized N	Treated N	Withdrawn N
Khalafallah et al (2016)[7]	Postoperative (orthopedic, abdominal, ENT, plastic, gynecologic, urologic)	**G1:** IV FCM single 15 mg/kg injection (maximum: 1000 mg) over 15 min. **G2:** No intervention (standard care)	Open-label	Not reported	201; **G1:** 103; **G2:** 98	201; **G1:** 97; **G2:** 94	**G1:** 6 (withdrawn from study, n=5; LTFU, n=1); **G2:** 4 (withdrawn from study, n=2; LTFU, n=2)
Keeler et al (2017)[18]	Preoperative (oncological)	**G1:** IV FCM diluted in 250 mL 0.9% NS over 15 min; dose calculated using body weight and inclusion hemoglobin value. Maximum dose 1000 mg/wk and 2000 mg during trial; second dose ≥7 days after first, if needed. **G2:** Oral ferrous sulphate 200 mg BID continued until surgery (patients advised to take without food and with liquid high in ascorbic acid to maximize enteric absorption)	Open-label	Not reported	116; **G1:** 55; **G2:** 61	116; **G1:** 55; **G2:** 61	**G1:** 4 (did not undergo surgical resection); **G2:** 2 (did not undergo surgical resection)
Kim et al (2017)[19]	Preoperative (oncological)	**G1:** IV FCM 1–2 1000 mg injections for patients <50 kg or 500 mg injections for ≥50 kg over 6–15 min. **G2:** IV saline placebo	Single-blinded	Per guidelines, Hb <7 g/dL	454; **G1:** 228; **G2:** 226	445; **G1:** 222; **G2:** 223	**G1:** 5 (did not complete study [LTFU, n=3; withdrew consent after treatment, n=1; physician withdrew patient for surgical complication, n=1]) **G2:** 10 (did not complete study [withdrew consent after treatment, n=6; LTFU, n=2; eligibility criteria violation recognized after treatment/received blood transfusion before treatment, n=2])
Lee et al (2019)[20]	Preoperative (benign uterine diseases)	**G1:** IV FCM single dose administered over 15 min (maximum: 1000 mg); amount determined based on patient's body weight (<50 kg, 500 mg iron; ≥50 kg, 1000 mg iron). **G2:** IV IS single administration over 3 dosing visits per week (maximum: 600 mg iron/week in 200 mg iron doses. Dosage based on calculated iron deficit using the Ganzoni formula	Open-label	Not reported	101; **G1:** 52; **G2:** 49	101; **G1:** 52; **G2:** 49	**G1:** 0 **G2:** 0

Study	Setting	Intervention	Blinding	Transfusion threshold	N	Randomized	Dropouts
Padmanabhan et al (2019)[21]	Preoperative (cardiac)	**G1:** IV FCM diluted in 250 mL of NS administered over 30 min (maximum: 1000 mg dose). A second dose was offered when required. Calculation of dosing regimen as follows: Hb > 100 and weight < 70 kg = 1000 mg; Hb > 100 and weight > 70 kg = 1500 mg; Hb 70–100 and weight < 70 kg = 1500 mg; Hb 70–100 and weight > 70 kg = 2000 mg **G2:** 200 mg ferrous sulphate PO BID	Open-label	Not reported	50 **G1:** 26 **G2:** 24	44 **G1:** 22 **G2:** 22	**G1:** 4 (LTFU) **G2:** 1 (LTFU)
Park et al (2019)[22]	Intraoperative (Unilateral TKA or THA)	**G1:** IV FCM 1000 mg diluted in 100 mL NS administered over 15 min. **G2:** IV 100 mL NS placebo	Single-blinded	Hb < 8 g/dL throughout entire perioperative period. If significant hemodynamic instability was observed despite adequate fluid administration or a requirement of an increasing amount of vasopressor was essential, allogenic transfusion of packed RBC was permitted with the Hb ≥ 8 g/dL. In case of transfusion, the subjects received one unit of packed RBC and the Hb level was followed to assess the further administration of additional packed RBC.	69 **G1:** 32 **G2:** 34	58 **G1:** 29 **G2:** 29	**G1:** 3 (withdrew consent, n=1, LTFU, n=2) **G2:** 5 (unnoticed cancer diagnosis, n=1; withdrew consent, n=1; LTFU, n=3)
Richards et al (2020)[23]	Preoperative (abdominal)	**G1:** IV FCM single dose 1000 mg in 100 mL NS administered over 15 min **G2:** IV 100 mL NS placebo	Double-blinded	Not reported	487 **G1:** 244 **G2:** 243	481 **G1:** 240 **G2:** 241	**G1:** 8 (not treated, n=4; withdrew consent, n=4) **G2:** 6 (not treated, n=2; withdrew consent, n=4)

Note: [a]Patients did not receive intervention, were not operated, or received iron sucrose administration in the follow-up period.
Abbreviations: ABT, allogeneic blood transfusion; ACS, acute coronary syndrome; BID, twice daily; EPO, erythropoietin; FCM, ferric carboxymaltose; G, treatment group; Hb, hemoglobin; HF, heart failure; IV, intravenous; IS, iron sucrose; LTFU, lost to follow-up; NS, normal saline; NYHA, New York Heart Association; PO, by mouth; RBC, red blood cell; SC, subcutaneous; TDD, total daily dose; TID, three times daily; THA, total hip arthroplasty; TKA, total knee arthroplasty.

a robust PBM protocol including preoperative treatment with iron, SC EPO, or both 1 month before surgery, discretionary tranexamic acid during and after surgery, and antithrombotic treatment with SC bemiparin (a low-molecular-weight heparin not approved in the United States); additionally, shed blood exceeding 400 mL was automatically reinfused via an autotransfusion device.[15] In the third study, patients in the usual care group received perioperative anemia management, which did not disallow prescription or administration of IV iron.[17] Five of the 10 studies prespecified blood transfusion thresholds; 3 specified Hb <7 g/dL,[7,15–17,19] and 1 of these set the number of RBC transfusions at 3.[16] Three of the 5 studies specified Hb <8 g/dL[15,22] or 7.1–8.9 g/dL in the presence of severe symptoms.[16]

Study Populations

Baseline characteristics of patients are provided in Table 2. Most of the study populations (9/10) had an average age of 60 years or older, and 9 of the 10 were generally evenly distributed with respect to age across treatment groups. The difference in age distribution between the FCM group and the comparator arm in the sixth study occurred by chance and was not explained by the authors. Eight studies had a greater percentage of women.[7,15,16,19–22] Mean weight was lowest among patients undergoing surgery for menorrhagia[20] and highest among those undergoing abdominal surgery.[17] The American Society of Anesthesiologists (ASA) physical status (grades ranged from I [defined as normal and healthy] to VI [declared brain-dead, organs to be donated]) varied by surgical population in the intervention group; 27–58% of patients undergoing abdominal surgery,[17,23] 45% undergoing colorectal cancer surgery,[18] and 20% undergoing orthopedic surgery had ASA Grade III (presence of severe systemic disease) or greater.[15,24] In each of the studies reporting baseline hematopoietic parameters, the patients met criteria for IDA (mean baseline Hb <11 g/dL, serum ferritin <300 ng/mL, and TSAT <25%).

Risk of Bias Assessment

Overall, the studies were considered to have a high risk of bias, largely with regard to the blinding of patients and personnel and blinding of outcome assessments (Figures 2 and 3).

Random Sequence Generation

Eight out of 10 studies were considered to have a low risk of bias for random sequence generation, as they clearly reported

appropriate randomization Che techniques.[15,17–21,23,25] However, the random sequence method using Excel in Khalafallah et al[7] was questionable and not described in Park et al,[22] making the risk of bias unclear.

Allocation Concealment

Eight out of 10 studies were rated as having low risk of bias for allocation concealment.[7,15,17,19,22,23,25] The studies by Keeler et al[18] and Lee et al[20] did not adequately describe the concealment methods of treatment allocation by an independent unit; selection bias was thus deemed unclear in the risk of bias for allocation concealment.

Blinding

Five of the 10 studies had an open-label design,[7,17,18,20,21] and 3 were patient-blinded only,[15,19,22] leading to a high risk of performance bias; the remaining studies had a double-blind design, which were assigned a low risk of bias.[16,23] Three studies had an unclear risk of detection bias; neither Keeler et al[18] nor Kim et al[19] clearly stated who performed the outcome assessments, and Bernabeu-Wittel et al[16] did not describe the study's blinding measures. Detection bias was low in Bisbe et al[15] and Richards et al[23] but was high in Froessler et al, Lee, Padmanabhan, and Park owing to a lack of description of outcome blinding measures in these open-label studies[17,20–22] and in Khalafallah et al owing to the recording of adverse events (AEs) by personnel who administered the study drug.[7]

Incomplete Outcome Data

Risk of attrition bias was considered low in 3 studies, in Bernabeu-Wittel et al because losses to follow-up were disclosed and the attrition was reasonable and not expected to affect results,[16] in Froessler et al because the rationale was given for study termination[17] and Richards et al because attrition was described and accounted for in statistical analysis.[23] Risk of attrition bias was deemed unclear for 6 studies because no explanations were offered for withdrawal for either treatment group,[7] for the relevance of incomplete data sets available at the end of the study period,[15,21,22] for the impact of incomplete adherence—which was assessed in 1 study—to allocated treatment,[18] and for interpolation of missing data using linear regression analysis when the total sample size is very small.[20] The risk of attrition bias was high for Kim et al, in which a modified intention-to-treat analysis was used.[19]

Table 2 Baseline Patient Characteristics of Included Studies

Author	Age, y, Mean ± SD	Sex, n (%)	Weight, kg, Mean ± SD	ASA Physical Status Classification, n (%)	Preoperative Hb, g/dL, Mean ± SD	Serum Iron, μg/dL, Mean ± SD	Preoperative Serum Ferritin, ng/mL, Mean ± SD	Preoperative TSAT, %, Mean ± SD
Bernabeu-Wittel (2016)[16]	G1: 84.6 ± 6.2 G2: 83.4 ± 6.4 G3: 82.3 ± 6.9	G1: 84 (81.5%) (F) G2: 87 (87.0%) (F) G3: 87 (87.0%) (F)	Not reported	G1: III (II–III)a G2: III (II–III)a G3: III (II–III)a	G1: 11.0 ± 0.8 G2: 11.0 ± 0.7 G3: 11.0 ± 0.7	Not reported	G1: 155 ± 170 G2: 137 ± 150 G3: 141 ± 130	Not reported
Keeler (2017)[18]	G1: Median (IQR): 73.8 (67.4–78.6) G2: Median (IQR): 74.7 (67.9–80.8)	G1: 35:20 (M:F) G2: 37:24 (M:F)	G1: Mean (95% CI): 79.0 (74.9, 83.2) G2: Mean (95% CI): 72.8 (68.7, 76.9)	G1: I–II, n=30; III–IV, n=25 G2: I–II, n=43; III–IV, n=18	G1: Recruitment: ~10.1; day of surgery: ~11.9 G2: Recruitment: ~10.4; day of surgery: ~11.0	Not reported	Not reported	Not reported
Kim (2017)[19]	G1: 60.9 ± 13.7 G2: 61.2 ± 12.6	G1:125 (54.8%) (F) G2:124 (54.9%) (F)	G1: 57.6 ± 9.7 G2: 58.1 ± 9.9	Not reported	G1: 11.6 ± 1.6 G2: 11.8 ± 1.6	G1: 24.6 ± 15.2 G2: 24.0 ± 11.1	G1: 115.9 ± 104.8 G2: 137.1 ± 123.9	G1: 10.8 ± 7.2 G2: 10.5 ± 5.4
Froessler (2016)[17]	G1: 64 ± 15 G2: 68 ± 15	G1: 19:21 (M:F) G2: 17:15 (M:F)	G1: 86 ± 27 G2: 88 ± 20	G1: II: 17 (42.5); III: 22 (55); IV: 1 (2.5) G2: II: 17 (53.1); III: 15 (46.9); IV: 0	G1: Randomization: 10.7 ± 1.3 (n=40); admission: 11.5 ± 1.3 (n=36) G2: Randomization: 10.6 ± 1.4 (n=32; P=0.76); admission: 10.7 ± 1.7 (n=29; P=0.12) Difference between randomization and admission: G1: 0.8 ± 0.8 (n=36) G2: 0.1 ± 1.3 (n=29; P=0.010)	Not reported	G1: Randomization, median (IQR): 19 (6–48) G2: Randomization, median (IQR): 37 (11–82)	G1: Randomization, median (IQR): 6 (3–10) G2: Randomization, median (IQR): 9 (7–15)
Bisbe (2014)[15]	G1: Mean (range): 72.7 (56–86) G2: Mean (range): 72.8 (40–86)	G1: 47 (78.3%) (F) G2: 50 (80.6%) (F)	G1: 79.6 ± 14.9 G2: 76.2 ± 11.9	G1: ≥III: 12 (20.0) G2: ≥III: 12 (19.4)	G1: 13.6 ± 0.9 G2: 13.6 ± 0.9	G1: 77 ± 22 G2: 74 ± 21	G1: 120 ± 111 G2: 164 ± 95	G1: 20.1 ± 6.6 G2: 20.4 ± 6.7

(*Continued*)

Table 2 (Continued).

Author	Age, y, Mean ± SD	Sex, n (%)	Weight, kg, Mean ± SD	ASA Physical Status Classification, n (%)	Preoperative Hb, g/dL, Mean ± SD	Serum Iron, µg/dL, Mean ± SD	Preoperative Serum Ferritin, ng/mL, Mean ± SD	Preoperative TSAT, %, Mean ± SD
Khalafallah (2016)[7]	**G1:** Median (IQR): 65 (55–73) **G2:** Median (IQR): 67 (60–76)	**G1:** 65 (63%) (F) **G2:** 50 (51%) (F)	**G1:** Median (IQR): 82 (70–91) **G2:** Median (IQR): 81 (69–88)	Not reported	**G1:** Median (IQR), postop day 1: 10.9 (9.9–12.0) **G2:** Median (IQR), postop day 1: 10.6 (9.6–11.7)	**G1:** Preop: 81.6 ± 15.6; postop, day 1: 33.5 ± 21.2; period effect,[†] mean difference (95% CI): −48.0 (−54.8 to −41.9) [*P*<0.0001] **G2:** Preop: 81.0 ± 21.2; postop, day 1: 33.5 ± 25.1; period effect,[†] mean difference (95% CI): −47.4 (−53.9 to −41.0) [*P*<0.0001]	**G1:** Postop day 1, median (IQR): 170 (108–284) **G2:** Postop day 1, median (IQR): 243 (134–471)	**G1:** Postop day 1, median (IQR): 10 (7–15) **G2:** Postop day 1, median (IQR): 11 (9–15)
Lee (2019)[20]	**G1:** 44.0 ± 5.7 **G2:** 43.4 ± 5.0	**G1:** 52 (100%) (F) **G2:** 49 (100%) (F)	**G1:** 56.2 ± 6.7 **G2:** 57.6 ± 9.1	Not reported	**G1:** 8.4 ± 1.4 **G2:** 8.4 ± 1.1	Not reported	**G1:** 58 ± 57 **G2:** 57 ± 39	**G1:** 6.1 ± 7.3 **G2:** 4.8 ± 5.9
Padmanabhan (2019)[21]	**G1:** 73 ± 12 **G2:** 75 ± 10	**G1:** 9 (41%) (F) **G2:** 8 (36%) (F)	**G1:** 78.9 ± 18.2 **G2:** 80.5 ± 13.1	Not reported	**G1:** 11.9 ± 0.89 **G2:** 113.9 ± 1.11	Not reported	**G1:** median (IQR) 62.8 (26.2–86.4) **G2:** median (IQR) 49.7 (19.7–88.8)	Not reported
Parl[22] (2019)	**G1:** 65.1 ±10.2 **G2:** 62.0 ± 9.5	**G1:** 19 (65.5%) (F) **G2:** 21 (72.4%) (F)	**G1:** 64.2 ± 9.8 **G2:** 67.1 ± 11.8	**G1:** ≥III: 0 **G2:** ≥III: 0	**G1:** 12.5 ± 1.3 **G2:** 13.4 ± 1.1	**G1:** 92.4 ± 37.0 **G2:** 101.0 ± 46.5	**G1:** 90.6 ± 82.5 **G2:** 87.0 ± 105.2	**G1:** 29.4 ± 16.3 **G2:** 33.2 ± 17.2
Richards (2020)[23]	**G1:** Mean (range): 66 (57–72) **G2:** Mean (range): 65 (50–72)	**G1:** 125 (51%) (F) **G2:** 142 (58%) (F)	Not reported	**G1:** ≥III: 57 (23.4) **G2:** ≥III: 66 (27.2)	**G1:** 11.1 ± 1.2 **G2:** 11.1 ± 1.2	Not reported	**All Groups:** <100 – 57%	**All Groups:** <20 – 76%

Notes: [a]N's were not specified; [†]Estimated in the standard care group (G2) using repeated measures mixed effects linear regression with time from operation as a random factor, adjusted for sex. Outcomes were compared with preoperative sample concentrations: postoperative samples were taken on the first day after the operation.
Abbreviations: ASA, American Society of Anesthesiologists; CI,confidence interval; G, treatment group (as defined in Table 1 in Intervention Groups and Regimen row); IQR, interquartile range; SD, standard deviation; TSAT, transferrin saturation.

	Bernabeu-Wittel et al (2016)	Keeler et al (2017)	Kim et al (2017)	Froessler et al (2016)	Bisbe et al (2014)	Khalafallah et al (2016)	Lee et al (2019)	Padmanhaban et al (2019)	Park et al (2019)	Richards et al (2020)
Random sequence generation	⊕	⊕	⊕	⊖	⊕	⊖	⊕	⊕	?	⊕
Allocation concealment	⊕	?	?	⊕	⊕	⊕	?	⊕	⊕	⊕
Blinding of patients and personnel	⊕	⊖	⊖	⊖	⊖	?	⊖	⊖	⊖	⊕
Blinding of outcome assessment	?	?	⊖	⊖	⊕	⊖	⊖	⊖	⊖	⊕
Incomplete outcome data	⊕	⊖	⊕	⊕	⊖	?	?	?	?	⊕
Selective reporting	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
Other Bias	⊕	?	?	?	?	⊕	⊖	⊖	⊖	⊕

Figure 2 Risk of bias summary: authors' judgment about risk of bias for each item for each of the 6 included studies. The symbol "+" indicates low risk of bias, "?" indicates unclear risk of bias, and "–" indicates high risk of bias.
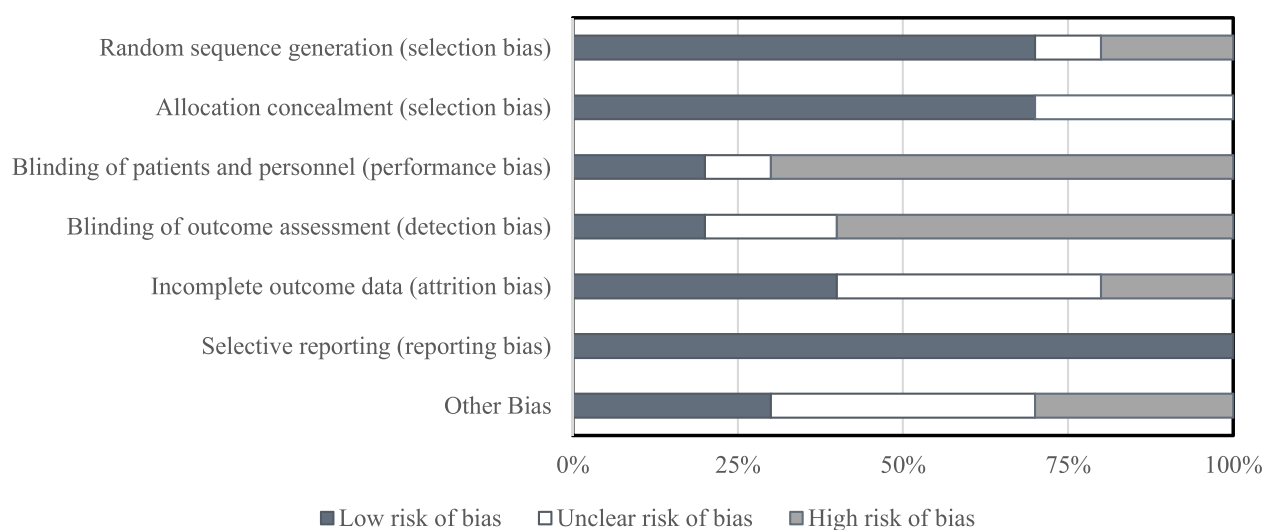
Figure 3 Risk of bias: authors' judgment about risk of bias presented as percentages across all 6 included studies.

Selective Reporting

In Keeler et al, the treatment effects for secondary endpoints of serum ferritin and TSAT were not reported and reported outcomes, including postoperative length of hospital stay and 90-day mortality rates, were not prespecified,[18] suggesting a high risk of reporting bias. For the other 9 included studies, all planned outcomes were reported.

Other Potential Sources of Bias

One of the 6 studies had a robust institution-specific patient blood management program in place that may have influenced outcome assessments.[15] In 1 study, IV iron was not considered part of usual care at the time of study commencement, but was nonetheless not prohibited.[17] A differential number of patients experiencing protocol deviations was noted in the study groups of

1 study.[18] Kim et al elected to examine a binary primary outcome measure instead of a continuous outcome measure, which may have skewed response.[19] Subjects in the Lee et al study who were assigned to iron sucrose returned to the study site for three dosing visits, whereas those assigned to FCM only had one visit, which may have skewed the results in favor of IS.[20] A large portion of patients in the Padmanabhan et al study did not return to receive their second dose of FCM, which may have skewed the results against IV iron.[21] Lastly, the Park et al study was significantly underpowered for the primary outcomes selected resulting in potential high risk of bias.[22]

Outcomes

All studies included reported absolute or relative change from baseline Hb concentrations. Change from baseline Hb concentration ranged from 1.3 g/dL[21] to 4.7 g/dL[23] among patients receiving preoperative FCM (including in the perioperative study) and 1.7 g/dL[15] to 3.2 g/dL[7] among those receiving postoperative FCM (Table 3). Six of the 10 studies[15,20,21,23] reported this outcome to be statistically significant versus comparators.[7,16–19,22] A key outcome measure was change from baseline in iron stores; findings showed that preoperative FCM administration resulted in an increase in serum ferritin concentrations of 229 μg/L (vs 19 μg/L at baseline)[17] to 558 μg/L (levels at randomization not reported)[18] and a 15%[17] to 35%[19] increase in TSAT. Postoperative administration resulted in a 114[7] to 571 μg/L[15] increase in serum ferritin concentration and 7.2%[15] to 20%[17] increase in TSAT. Both Bernabeu-Wittel et al[16] and Lee et al[20] did not report change from baseline TSAT.

A secondary efficacy outcome measure was proportion of patients who received perioperative ABT (Table 4). Two[7,17] of the 10 studies found statistically significantly lower rates of ABT in patients receiving FCM versus comparators in the perioperative surgical settings (absolute difference: 18.75%)[17] and postoperative (absolute difference: 4%).[7] The highest proportion of FCM–treated patients who received an ABT was 51.5% (53/103) in Bernabeu-Wittel et al, compared with 52% (52/100) in the FCM plus EPO group and 54% (54/100) in the placebo group;[16] in Khalafallah et al, 1% (1/103) of patients receiving FCM versus 5% (5/98) receiving standard of care received an ABT.[7]

Outcome measures included the proportion of patients who experienced AEs, postoperative mortality rate, and hospital length of stay (Table 4). The proportion of patients with AEs was 6.8% (15/222) in the FCM group and 0.4% (1/223) in the placebo group in the study by Kim et al.[19] Bisbe et al reported AEs in 33% (20/60) of patients in the FCM group and 32% (20/62) in the ferrous glycine sulfate comparator group (P=NS);[15] this included all AEs reported during hospitalization (ie, before initiation of iron replacement therapy) and AEs related to surgical complications from initiation to 30 days postoperatively.[15] Five percent of patients experienced AEs in the FCM group compared to 2% in the placebo group (risk ratio 2.20, 95% CI 0.78–6.24).[23] Zero AEs among treatment groups were reported in both Lee et al[20] and Park et al.[22] Postoperative mortality was reported in 6 of the 10 included studies and ranged from 1% (2/237)[23] to 11.7% (12/103) among patients receiving FCM[16] compared with 0[17,20,21] to 10% (10/100) receiving placebo.[16] The deaths were not attributed to study drug in the 5 studies; 3 studies did not discuss the possibility of a relationship between the deaths and study drug,[17,18,23] 1 study stated that the single death was due to unrelated causes,[21] and 1 study stated that the causes of death were similar among the 3 treatment groups (IV FCM, IV FCM in combination with EPO, and placebo).[16]

The length of hospital stay ranged from 6[17,18] to 10.7 days[19] with FCM versus6[18] to 11.6 days[7] with comparators (Table 5). Two of the 8 studies reported statistically significant differences in transfusion rate[7,17] and 2 studies reported significant differences in length of hospital stay between FCM and its comparators; the absolute difference in hospital stay was 3 days in Froessler et al[17] and 2.8 days in Khalafallah et al.[7] The variations in discharge criteria at the different institutions may have contributed to the differences in lengths of stay.

Validated measures of QOL included the Short Form Health Survey (SF-36 and SF-12) in 5 studies,[7,16,17,20,21] the EuroQol 5-dimension quality of life scale (EQ-5D) in 3 studies,[15,21,23] the Barthel Index questionnaires in 1 study,[15] the Multidimensional Fatigue Inventory (MFI),[23] and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30 (EORTC QLQ-C30) questionnaire and the QLQ Gastric Cancer Module (QLQ-STO22) in 1 study.[19] There were no statistically significant differences between IV FCM and comparators in QOL (overall score, change in score, or treatment effect) in any of these 7 studies.[7,15–17,19,21,23] Bisbe et al reported no significant differences between IV FCM and the comparator (ferrous

Table 3 Primary Outcome Measures: Change from Baseline in Hb, Serum Ferritin, and TSAT

Study	Surgery	Surgical Setting	Change from Baseline Hb (g/dL)			Change from Baseline Serum Ferritin (μg/L), Mean (SD)			Change from Baseline TSAT (%), Mean (SD)		
			Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Bisbe et al (2014)[15]	Orthopedic	Postop	1.7 (1.2)[a]	1.3 (1.0)[a]	—	571 (229)[a]	60 (88)[a]	—	7.2 (−5.2)[a]	0.7 (−5)[a]	—
Bernabeu–Wittel et al (2016)[16]	Hip fracture	Preop	1.38 (0.72)[b]	1.53 (0.56)[b]	0.88 (0.43)[b]	317 (140)[b]	288 (110)[b]	−4 (−10)[b]	—	—	—
Froessler et al (2016)[17]	Abdominal	Periop	1.9 (1.4)[c]	0.9 (1.4)[c]	—	229 (131–498)[d]	62 (24–146)[d]	—	15 (13–16)[d]	5 (0–3)[d]	—
Khalafallah (2016)[7]	Orthopedic, abdominal, ENT, plastic, gynecologic, urologic	Postop	3.2 (0.16)[e]	2.81 (0.18)[e]	—	114 (73.2)[e]	−133 (−41.1)[e]	—	20 (1.1)[e]	13.3 (1.4)[e]	—
Keeler et al (2017)[18]	Colorectal cancer	Preop	1.55 (0.93–2.58)[f]	0.50 (−0.13–1.33)[f]	—	558 (330–1085)[f]	27.5 (17–51.5)[f]	—	19 (16–29)[f]	9 (5–14)[f]	—
Kim et al (2017)[19]	Gastrectomy (cancer)	Preop	3.3[g]	1.6[g]	—	233.3[h]	53.4[h]	—	35.0[h]	19.3[h]	—
Lee et al (2019)[20]	Benign uterine diseases	Preop	2.2 (0.3)	1.9 (0.2)	—	—	—	—	—	—	—
Padmanabhan et al (2019)[21]	Cardiac	Preop	1.3 (0.9)[i]	4.4 (0.9)[i]	—	313 [228, 496][j]	5.5 [−1.4, 19.4][j]	—	—[k]	—[k]	—
Park et al (2019)[22]	Unilateral TKA or THA	Intraop	0.3 (1.00)[l]	−0.8 (0.78)[l]	—	1157.5 (412.3)[m]	83.8 (52.5)[m]	—	8.7 (10.4)[m]	−19.5 (−5.2)[m]	—
Richards et al (2020)[23]	Open abdominal surgery	Preop	4.7 (2.7–6.8)[n]; 10.7 (8–13.8)[o]; 7.3 (3.6–11.1)[p]	—	—	—	—	—	—	—	—

Notes: Treatment groups are defined in Table 1 in Intervention Groups and Regimen row; [a]Values are increase in mean (SD) from baseline to 60 days postdischarge (change in SD from baseline to 60 days postdischarge); [b]Values are increase in mean (SD) from discharge to postoperative week 4; [c]Values are increase in median (first quartile–third quartile) from randomization to postoperative week 4. [e]Values are increase in median (first quartile–third quartile) from baseline to day of surgery; [g]Values are increase in median from baseline to postoperative week 12; [h]Values are increase in mean from baseline to postoperative week 12; [i]Values are increase in median (interquartile range) between enrolment and surgical admission; [k]Statistical difference reported in favor of FCM (P=0.001); no data provided; [l]Values are increase in mean (SD) from baseline to postoperative day 30; [m]Values are increase in median (interquartile range) from baseline to postoperative day 5; [n]Values are increase in mean (95% CI) from baseline to 8 weeks following intervention; [o]Values are increase in mean (95% CI) from baseline to time of surgery; [o]Values are increase in mean (95% CI) from baseline to postoperative day 5; [n]Values are increase in mean (SD) from postoperative day 4 to postoperative day 30; [b]Values are increase in mean (SD) from baseline to 60 days postdischarge; [d]Values are increase in median (first quartile–third quartile) from baseline to day of surgery; [f]Values are increase in median (interquartile range) from baseline to postoperative day 30; [m]Values are increase in mean (95% CI) from baseline to 6 months following intervention.

Abbreviations: ENT, ear, nose, and throat; FCM, ferric carboxymaltose; Hb, hemoglobin; THA, total hip arthroplasty; TKA, total knee arthroplasty; TSAT, transferrin saturation.

Table 4 Secondary Outcome Measures: ABT, AEs, and Mortality

Study	Surgery	Surgical Setting	Proportion of Patients Receiving an ABT, n/N (%)			Proportion of Patients Experiencing AEs, n/N (%)			Postoperative Mortality, n/N (%)		
			Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Bisbe (2014)[15]	Orthopedic	Postop	3/59 (5.1)	2/62 (3.2)	—	20/59 (33.9)	20/62 (32.3)	—	—	—	—
Bernabeu-Wittel (2016)[16]	Hip fracture	Preop	53/103 (51.5)	52/100 (52.0)	54/100 (54)	—	—	—	12/103 (11.7)	12/100 (12.0)	10/100 (10.0)
Froessler (2016)[17]	Abdominal	Periop	5/40 (12.5)	10/32 (31.3)	—	—	—	—	1/40 (2.5)	0/32 (0)	—
Khalafallah (2016)[7]	Orthopedic, abdominal, ENT, plastic, gynecologic, urologic	Postop	1/103 (0.1)	5/98 (5.1)	—	—	—	—	—	—	—
Keeler (2017)[18]	Colorectal cancer	Preop	6/55 (10.9)	6/61 (9.8)	—	—	—	—	5/55 (9.1)	4/61 (6.6)	—
Kim (2017)[19]	Gastrectomy (cancer)	Preop	3/218 (1.4)	4/219 (1.8)	—	15/222 (6.8)	1/223 (0.4)	—	—	—	—
Lee et al (2019)[20]	Benign uterine diseases	Preop	0/52	0/49	—	0/52	0/49	—	0/52	0/49	—
Padmanabhan et al (2019)[21]	Cardiac	Preop	16/20 (80)	12/20 (60)	—	0/20	3/20	—	1/20 (5) (unrelated causes)	0/29	—
Park et al (2019)[22]	Unilateral TKA or THA	Intraop	2/29 (6.9)	4/29 (12.8)	—	0/29	—	—	—	—	—
Richards et al (2020)[23]	Open abdominal surgery	Preop	68/237 (29)	67/237 (28)	—	11/237 (5)	5/237 (2)	—	12/238 (5)	10/236 (4)	—

Note: Treatment groups are defined in Table 1.
Abbreviations: ABT, allogenic blood transfusion; AE, adverse event; ENT, ear, nose, and throat; FCM, ferric carboxymaltose; IV, intravenous; THA, total hip arthroplasty; TKA, total knee arthroplasty.

Table 5 Secondary Endpoint Outcomes: Hospital Length of Stay and QOL

Study	Surgery	Surgical Setting	Length of Hospital Stay, d			Instrument	Validated QOL Measure		
			Group 1	Group 2	Group 3		Group 1	Group 2	Group 3
Bisbe (2014)[15]	Orthopedic	Postop	7.9 (1.7)[a]	7.6 (0.9)[a]	—	EQ-5D Barthel questionnaire	Total EQ-5D increase: 0.6 (0.9)[b] Barthel increase: 92.6 (6.2)[b]	Total EQ-5D increase: 0.6 (0.17)[b] Barthel increase: 93.0 (8)[b]	—
Bernabeu-Wittel (2016)[16]	Hip fracture	Preop	7 (5-10)[c]	8 (6-11)[c]	8 (6-10)[c]	SF-36v2	Physical decrease: 4.4 (14)[d] Mental decrease: 2.7 (20)[d]	Physical decrease: 7.4 (11)[d] Mental decrease: 1.2 (13)[d]	Physical: 4.5 (15)[d] Mental: -2 (20)[d]
Froessler (2016)[17]	Abdominal surgery	Periop	6 (1, 19)[e]	9 (1, 23)[e]	—	SF-36	SF-26 decrease (pre-post): 8 (18)[f]	Decrease (preop to postop): 6 (17)[f]	—
Khalafallah (2016)[7]	Orthopedic, abdominal, ENT, plastic, gynecologic, urologic	Postop	7.8 (10.3)[a]	11.6 (15.6)[a]	—	SF-36	SF-36 increase: 13.4[g] Physical increase: 13.4[g] Mental increase: 13.3[g]	SF-36 increase: 9.1[g] Physical increase: 7.9[g] Mental increase: 9.9[g]	—
Keeler (2017)[18]	Colorectal cancer	Preop	6 (5-10)[c]	6 (4-9)[c]	—	—	—	—	—
Kim (2017)[19]	Gastrectomy (cancer)	Preop	10.7 (7.9)[a]	10.9 (13.8)[a]	—	EORTC QLQ-C30v3 QLQ-STO22	Fatigue (week 3): 30.0 (26.8, 33.1)[h] Dyspnea (week 12): 9.5 (7.2, 11.8)[h]	Fatigue (week 3): 34.6 (31.3, 37.9)[h] Dyspnea (week 12): 14.2 (11.3, 17.1)[h]	—
Lee et al (2019)[20]	Benign uterine diseases	Preop	—	—	—	SF-12	PCS (visit 3 vs visit 1): 3.7 ± 10.0 MCS (visit 3 vs visit 1): 4.5 ± 8.0	PCS (visit 3 vs visit 1): 2.3 ± 8.0 MCS (visit 3 vs visit 1): 1.6 ± 6.5	—
Padmanabhan et al (2019)[21]	Cardiac	Preop	7 (6-12)[c]	9 (6-14)[c]	—	EQ-5D SF-36	—	—	—
Park et al (2019)[22]	Unilateral TKA or THA	Intraop	—	—	—	—	—	—	—
Richards et al (2020)[23]	Open abdominal surgery	Preop	9 (7-14)[c]	9 (5-14)[c]	—	EQ-5D-5L MFI	EQ-5D-5L Utility score: 10-day: 0.80 (0.20); 8-week: 0.79 (0.20); 6-month: 0.82 (0.22) EQ-5D-5L Health score: 10-day: 70.6 (20.5); 8-week: 70.7 (19.4); 6-month: 75.0 (18.4) MFI: 10-day: 53.2 (18.4); 8-week: 52.9 (17.1); 6-month: 48.8 (18.9)	EQ-5D-5L Utility score: 10-day: 0.81 (0.21); 8-week: 0.77 (0.21); 6-month: 0.82 (0.21) EQ-5D-5L Health score: 10-day: 73.8 (19.6); 8-week: 71.1 (19.5); 6-month: 76.2 (19.2) MFI: 10-day: 50.5 (18.9); 8-week: 53.9 (17.7); 6-month: 47.4 (19.1)	—

Notes: Treatment groups are defined in Table 1; [a]Values are the mean (standard deviation); [b]Values are median (first quartile–third quartile); [c]Values are median (minimum, maximum); [d]Values are mean (standard deviation) decrease from baseline to 60 days postdischarge; [e]Values are median (minimum, maximum); [f]Values are mean (standard deviation) increase from presurgery/intervention to postoperative week 4; [g]Values are the increase from postoperative day 1 to postoperative week 12; [h]Values are mean (95% confidence interval).

Abbreviations: ED-5Q, EuroQol 5-dimension quality of life scale; EORTC, European Organization for Research and Treatment of Cancer; FCM, ferric carboxymaltose; IV, intravenous; MCS, mental health composite scores; MFI, Multidimensional Fatigue Inventory; PCS, physical health composite scores; QLQ-C30v3, Quality of Life Questionnaire-Core 30 version 3; QLQ-STO22, Quality of Life Questionnaire-Stomach; QOL, quality of life; SF-12, Short Form-12 Health Survey; SF-36, 36-Item Short Form Survey; THA, total hip arthroplasty; TKA, total knee arthroplasty.

glycine sulfate) in total EQ-5D and Barthel scores.[15] However, among patients with preoperative iron deficiency (serum ferritin <100 ng/mL), the IV FCM group had significantly better (ie, lower) mean ± SD EQ-5D subscores than the comparator group for "usual activities" (1.9 ± 0.3 vs 2.1 ± 0.3; *P*=0.026) and "anxiety or depression" (1.3 [0.6] vs 1.6 [0.7]; *P*=0.074) at the end of the study.[15] In addition, among patients with severe postoperative anemia (Hb <10.0 g/dL), the FCM group had significantly better scores for "usual activities" than the comparator group at the end of the study (1.9 vs 2.3 [SD not reported]; *P*=0.04).[15]

Overall Quality of Evidence

The GRADE criteria were applied to rate the quality of evidence for all specified outcomes in this review (Supplementary Data, Appendix 2). Evidence quality was downgraded for risk of bias, inconsistency, indirectness, and imprecision. Across both primary and secondary endpoints, the overall quality of evidence was determined to be very low (Table 6). Publication bias was not detected. The rationale for these judgments are outlined in the Supplementary Data, Appendix 3.

Discussion

This SLR supports the use of FCM as treatment for adult patients with IDA in the preoperative and postoperative health states. In both the preoperative and postoperative elective surgery settings, IDA is a modifiable risk factor for elective surgical management.[26,27] The 10 studies identified for inclusion in this SLR consistently reported change from baseline improvement in the three primary outcome measures: hemoglobin, ferritin, and TSAT.[7,15–19] For these studies, there was less consistency for the association of FCM as treatment for perioperative IDA with each of the five secondary outcomes: ABT, adverse events, postoperative mortality, hospital length of stay, and QoL measures (see Tables 4 and 5).

Current guidelines from the Australian National Health and Medical Research Council[29] and the United Kingdom National Institute for Health and Care Excellence[30] recommend consideration of alternatives to blood transfusions for patients undergoing surgery and advise restrictive hemoglobin thresholds (<7.0 g/dL) for blood transfusions. In this SLR, three of the postoperative surgery studies reported either significantly fewer total ABT events[17] or a significantly smaller proportion of patients receiving ABT[7,19] with FCM

than with the comparator. In the six preoperative surgery studies, the percentage of patients receiving ABT was not significantly different between assigned groups.[16,18–21,23] In one preoperative surgery study, FCM was associated with reduced hospital readmission for post-surgical complications.[23] Nonetheless, additional studies will be needed to fully assess the association of perioperative IV iron repletion, averted ABT, and associated ABT complications.[28,32] Adverse events during hospitalization were recorded in three studies;[15,21,23] as either estimated blood loss,[21] number of blood transfusions,[23] or gastrointestinal AEs.[21,33] Post-operative mortality rates varied across the studies and was lowest in the study of benign uterine surgery and highest in the study of hip fractures.[16,20] Length of hospital stay and measures of QoL also varied across studies (Table 5). Advanced age, as well as multiple comorbidities may have played a role in the lack of significance between FCM and comparators in these studies.[7]

While all 10 studies were randomized controlled trials, clinical and methodological heterogeneity was evident across the studies (Table 1), along with variation in the design elements (eg, type of blinding, duration of follow-up, timing of preoperative and postoperative assessments, comparator used, dosage, treatment schedule), All of these factors are important considerations when evaluating the utility of the exposure for the reported primary and secondary outcomes. A meta-analysis was not deemed feasible for this SLR, given the heterogeneity across the studies. Nonetheless, our findings are consistent with two prior quantitative syntheses of IV iron repletion in surgical patients which reported the efficacy of FCM associated with postoperative hemoglobin in elective surgeries.[34,36] Additionally, for this SLR we conducted a thorough systematic qualitative assessment, which permitted a quality of evidence GRADE assessment (Table 6) for each of the primary and secondary outcomes. Although the overall quality of evidence scores were very low, future SLRs with GRADE assessments and consensus guideline considerations will likely benefit from assessment of iron repletion for elective surgeries partitioned as preoperative versus postoperative interventions. Together, the totality of data from these studies support the beneficial effect of FCM for hematological parameters in elective surgery, while continuing to inform on the role of iron repletion in perioperative patient management.[31]

Table 6 Quality of Evidence (GRADE) Assessment

Outcome	Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication Bias	Other Considerations	Quality of Evidence
Absolute or relative change in hemoglobin concentration								
Rationale	10/10	8/10 RCTs inadequately blinded outcome assessments; 8/10 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results.	Positive effect demonstrated in 10/10 RCTs. Both clinical and methodologic heterogeneity are high. Therefore, some inconsistency exists.	Secondary endpoint in 6/10 studies (primary in 4/10) and therefore may not be powered appropriately. The intervention was given preop. in 7/10 studies, postop. in 3/10, and intraop. in 1/10. Difficult to make direct comparisons because of difference in comparators and populations.	1 study terminated early; no CIs reported for 6/10 studies; wide CI/IQR for 5/10 studies. Insufficient sample sizes (<400 total) in 8/10 studies.	Undetected	There are potentially many subgroups in this analysis (eg, type of surgery, severity of condition, age) that may have affected findings that cannot be considered because of small sample sizes.	We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect. There generally appears to be a consistent effect with low risk of AEs. Most articles report positive outlook on use in future similar patient populations. Patients appear neutral to positive.
Absolute or relative change in iron stores (serum ferritin)								
Rationale	8/10 (Not Lee or Richards)	7/8 RCTs inadequately blinded outcome assessments; 7/8 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results.	Positive effect demonstrated in 8/8 RCTs. Both clinical and methodologic heterogeneity are high. Direction appears consistent but variation appears large and therefore inconsistent.	Secondary endpoint in 6/8 studies (primary in 1 study) and therefore not sufficiently powered. Difficult to make direct comparisons because of differences in comparators and populations.	1 study terminated early; no CI reported for 4/8; wide CI/IQR range for 4/4 studies. Insufficient sample sizes (<400 total) in 8/8 studies.	Undetected	Selective reporting in 2/8 studies (TSAT/ferritin endpoint not prespecified).	We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect

(*Continued*)

Table 6 (Continued).

Outcome	Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication Bias	Other Considerations	Quality of Evidence
Absolute or relative change in iron stores (TSAT)								
Rationale	6/10 (Not Lee, Bernabeau-Wittel, Padmanabhan, or Richards)	5/6 RCTs inadequately blinded outcome assessments; 6/6 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results	Positive effect demonstrated in 6/6 RCTs. Both clinical and methodologic heterogeneity are high. Direction appears consistent but variation appears large and therefore inconsistent.	Secondary endpoint in 5/6 studies (primary in 1 study) and therefore not sufficiently powered. Difficult to make direct comparisons because of difference in comparators and populations	1 study terminated early; no confidence interval reported for 3/6; wide CI/IQR for 3/3 studies. Insufficient sample sizes in each study (<400 total).	Undetected	Selective reporting in 2/6 studies (TSAT/ferritin endpoint not prespecified).	We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect.
Proportion of participants who received a perioperative ABT								
Rationale	10/10	5/10 RCTs inadequately blinded outcome assessments; 7/10 RCTs inadequately blinded participants; 6/10 inadequately blinded personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results.	Positive effect reported in 2/10 studies (no difference in 8/10 studies). Consistency in findings may be an artifact of the disease severity/age/complexity of the surgery. Large variations in degree to which outcome is affected.	Primary endpoint in 4/10; percentage of blood transfusions secondary endpoint in 3/10; number of transfused blood units primary endpoint (1/10); reported as an absolute number in 8/10. Difference in populations and intervention schedules may have influenced outcome.	1 study terminated early; no CI reported for 6/10; wide CI/IQR for 3/6. Uncertain if there was a large enough sample size despite a large enough number of events to calculate a precise effect estimate. Several studies had only 1 participant with the outcome of interest.	Undetected	N/A	We have very very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect. The studies in this review looked at populations who had a large range of bleed/transfusion risk perioperatively. Need to assess ABT risk and adequately power the studies to detect sufficient number of events. Event may be too rare in some cases.

Proportion of participants who experienced adverse events

| Rationale | 6/10 (Not Bernabeu-Wittel, Froessler, Khalafallah, Keeler;) | 5/6 RCTs inadequately blinded outcome assessments; 5/6 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results. | No SAE (4/6); no significant difference in safety reported for 4/6 studies. | Prespecified secondary endpoint (3/6). Safety was not assessed similarly within or between. Studies also had different comparators. | 1 study terminated early. Not clear how safety was assessed. | Undetected | N/A | We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect. Lack of evidence, high inconsistency in reporting, high indirectness, and high imprecision. |

Postoperative mortality

| Rationale | Directly reported in 6/10 studies; indirectly in 4/10 as apparently no deaths occurred during follow-up. | 8/10 RCTs inadequately blinded outcome assessments; 8/10 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results. | Results were not consistent; may be an artifact of severity of disease in population under study. | Not a surrogate outcome; prespecified secondary endpoint (2/6); primary endpoint composite (1/6) all-cause (2/6); 30-day (1/6). Death is dichotomous; however, the assumption in 4/10 studies is that no one had died. | Insufficient number of events to make a conclusion. Not a primary outcome in 9/10 studies, so likely underpowered to observe the true effect. | Undetected | N/A | We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect. |

(Continued)

Table 6 (Continued).

Outcome	Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication Bias	Other Considerations	Quality of Evidence
Length of hospital stay								
Rationale	8/10 (Not Lee or Parks)	4/10 RCTs inadequately blinded outcome assessments; 5/8 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results.	Both clinical and methodologic heterogeneity are high. Heterogeneity among healthcare systems may also have influenced this outcome. Inconsistent results.	Not a surrogate outcome; prespecified secondary endpoint in 4/10 studies.	Wide CIs present in most studies.	Undetected	N/A	We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect.
Any validated measure of quality of life								
Rationale	7/10 (Not Padmanabhan, Keeler, or Parks)	6/7 RCTs inadequately blinded outcome assessments; 5/7 RCTs inadequately blinded participants and personnel. The proportion of information from studies at high risk of bias is sufficient to affect the interpretation of results.	Both clinical and methodologic heterogeneity are high. Results reported inconsistently owing to different instruments. No difference of effect is consistent.	Surrogate marker; outcome not assessed similarly within and among studies, yielding very indirect comparisons.	Wide CIs present in most studies. Extreme imprecision present. Concentrated on certain subscores in some studies.	Undetected.	Selective reporting was present for this outcome in a few studies.	We have very little confidence in the effect estimate; the true effect is likely to be substantially different from the estimate of the effect.

Abbreviations: AE, adverse event; CI, confidence interval; IQR, interquartile range; RCT, randomized clinical trial; SAE, serious adverse event; TSAT, transferrin saturation.

Limitations

Several methodologic limitations in this systematic review are noteworthy. Physicians were not blinded in 5 studies (4 open-label[7,18,20,21] and 1 patient-blinded[19]). In 3 other studies, the physician was blinded to treatment allocation, but the comparators used in those studies (eg, oral iron[15] or usual care consisting of no treatment, oral iron recommendation, and transfusion[17,22]) might allow the physician to distinguish which patients were assigned to receive IV FCM vs the comparator in others. Overall, the risks of performance and biases were high or uncertain, highlighting the need for more effective blinding of patients and study personnel involved in the administration of study drug or outcome assessments. Blinding in some studies may not be feasible, despite the impact of blinding on the measurement of study outcomes. For example, a difference in route of administration between treatment arms, ethical considerations (possibility of exposing study patients to risks when blinding with placebo), or logistical hurdles (eg, limited economic support or authorization to use or access appropriate placebos and masking procedures) may be challenging in independent investigator-initiated studies.[7,35]

Other limitations of study heterogeneity may limit generalizability of the findings resulting from differing thresholds of Hb level, inclusion criteria, baseline clinical characteristics (eg, Hb, serum iron, serum ferritin, TSAT, ASA classification), IV FCM dosing and timing of administration, Hb thresholds for intervention (IV iron and transfusion). Heterogeneity or absence of reporting of secondary outcomes such as duration of follow-up, differences in criteria for hospital discharge (which would affect length of stay and may explain differences observed between the studies), and QOL instrument used (SF-36,[7,16] EQ-5D and Barthel questionnaire,[15] and EORTC QLQ-C30 and QLQ-STO22[19]) did not allow for a more in-depth review. Bias assessments in this review were qualitative, and meta-analysis was not feasible.

Conclusion

The preoperative correction of anemia and other hematological parameters with IV FCM appears to be a viable intervention to mitigate known risks associated with post-surgical anemia and consequent events such as the need for transfusion.[36] IPostoperative patients with IDA, in whom oral iron is contra-indicated who are not able to tolerate oral iron or achieve a satisfactory response to oral

iron may also benefit from FCM administration.[7,28] The benefits of IV iron suggested by this SLR support the recommendations of guidelines that incorporate the use of IV iron in patients undergoing elective surgery.

Abbreviations

ABT, allogeneic blood transfusion; AE, adverse event; ASA, American Society of Anesthesiologists; ENT, ear, nose, and throat; EORTC-QLQ-C30, the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30; EPO, erythropoietin; EQ-5D, EuroQol 5-dimension quality of life scale; FCM, ferric carboxymaltose; GRADE, Grading of Recommendations Assessment, Development, and Evaluation; Hb, hemoglobin; IDA, iron-deficiency anemia; IV, intravenous; NHMRC, National Health and Medical Research Council; NICE, National Institute for Health and Care Excellence; NS, not significant; PBM, perioperative blood management; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; QLQ-ST022, Quality of Life Questionnaire-Gastric Cancer Module; QOL, quality of life; RBC, red blood cell; SC, subcutaneous; SD, standard deviation; SLR, systematic literature review; TSAT, transferrin saturation.

Author Contributions

JJJ, LMM, NB, MS: Main contribution to the study concepts and study design as well as supervision and revision of the manuscript.

JJJ, MS, LMM: Main contribution to the data acquisition, analysis, and interpretation.

JJJ and LMM: Main contribution to manuscript preparation.

NB: Main contribution to statistical support.

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

References

1. Spahn DR. Anemia and patient blood management in hip and knee surgery: a systematic review of the literature. *Anesthesiology.* 2010;113(2):482–495. doi:10.1097/ALN.0b013e3181e08e97

2. Beattie WS, Karkouti K, Wijeysundera DN, Tait G. Risk associated with preoperative anemia in noncardiac surgery: a single-center cohort study. *Anesthesiology.* 2009;110(3):574–581. doi:10.1097/ALN.0b013e31819878d3

3. Foss NB, Kristensen MT, Kehlet H. Anaemia impedes functional mobility after hip fracture surgery. *Age Ageing.* 2008;37 (2):173–178. doi:10.1093/ageing/afm161

4. Napolitano LM. Perioperative anemia. *Surg Clin North Am.* 2005;85 (6):1215–1227. doi:10.1016/j.suc.2005.10.012

5. Avgerinos DV, Llaguna OH, Seigerman M, Lefkowitz AJ, Leitman IM. Incidence and risk factors for the development of anemia following gastric bypass surgery. *World J Gastroenterol.* 2010;16(15):1867–1870. doi:10.3748/wjg.v16.i15.1867

6. Theusinger OM, Leyvraz PF, Schanz U, Seifert B, Spahn DR. Treatment of iron deficiency anemia in orthopedic surgery with intravenous iron: efficacy and limits: a prospective study. *Anesthesiology.* 2007;107(6):923–927. doi:10.1097/01. anes.0000291441.10704.82

7. Khalafallah AA, Yan C, Al-Badri R, et al. Intravenous ferric carboxymaltose versus standard care in the management of postoperative anaemia: a prospective, open-label, randomised controlled trial. *Lancet Haematol.* 2016;3(9):e415–425. doi:10.1016/S2352-3026(16)30078-3

8. Munoz M, Gomez-Ramirez S, Campos A, Ruiz J, Liumbruno GM. Pre-operative anaemia: prevalence, consequences and approaches to management. *Blood Transfus.* 2015;13(3):370–379. doi:10.2450/2015.0014-15

9. Hofmann A, Ozawa S, Farrugia A, Farmer SL, Shander A. Economic considerations on transfusion medicine and patient blood management. *Best Pract Res Clin Anaesthesiol.* 2013;27(1):59–68. doi:10.1016/j.bpa.2013.02.001

10. Camaschella C. Iron-deficiency anemia. *N Engl J Med.* 2015;372 (19):1832–1843. doi:10.1056/NEJMra1401038

11. Shander A, Knight K, Thurer R, Adamson J, Spence R. Prevalence and outcomes of anemia in surgery: a systematic review of the literature. *Am J Med.* 2004;116(Suppl 7A):58s–69s. doi:10.1016/j.amjmed.2003.12.013

12. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* 2009;6(7):e1000097. doi:10.1371/journal.pmed.1000097

13. Higgins JP, Altman DG, Gotzsche PC, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ.* 2011;343:d5928. doi:10.1136/bmj.d5928

14. Balshem H, Helfand M, Schunemann HJ, et al. GRADE guidelines: 3. Rating the quality of evidence. *J Clin Epidemiol.* 2011;64 (4):401–406. doi:10.1016/j.jclinepi.2010.07.015

15. Bisbe E, Molto L, Arroyo R, Muniesa JM, Tejero M. Randomized trial comparing ferric carboxymaltose vs oral ferrous glycine sulphate for postoperative anaemia after total knee arthroplasty. *Br J Anaesth.* 2014;113(3):402–409. doi:10.1093/bja/aeu092

16. Bernabeu-Wittel M, Romero M, Ollero-Baturone M, et al. Ferric carboxymaltose with or without erythropoietin in anemic patients with hip fracture: a randomized clinical trial. *Transfusion.* 2016;56 (9):2199–2211. doi:10.1111/trf.13624

17. Froessler B, Palm P, Weber I, Hodyl NA, Singh R, Murphy EM. The important role for intravenous iron in perioperative patient blood management in major abdominal surgery: a randomized controlled trial. *Ann Surg.* 2016;264(1):41–46. doi:10.1097/SLA.0000000000001646

18. Keeler BD, Simpson JA, Ng O, Padmanabhan H, Brookes MJ, Acheson AG. Randomized clinical trial of preoperative oral versus intravenous iron in anaemic patients with colorectal cancer. *Br J Surg.* 2017;104(3):214–221. doi:10.1002/bjs.10328

19. Kim YW, Bae JM, Park YK, et al. Effect of intravenous ferric carboxymaltose on hemoglobin response among patients with acute isovolemic anemia following gastrectomy: the FAIRY randomized clinical trial. *JAMA.* 2017;317(20):2097–2104. doi:10.1001/jama.2017.5703

20. Lee S, Ryu KJ, Lee ES, Lee KH, Lee JJ, Kim T. Comparative efficacy and safety of intravenous ferric carboxymaltose and iron sucrose for the treatment of preoperative anemia in patients with menorrhagia: an open-label, multicenter, randomized study. *J Obstet Gynecol Res.* 2019;45(4):858–864. doi:10.1111/jog.13893

21. Padmanabhan H, Siau K, Nevill AM, et al. Intravenous iron does not effectively correct preoperative anaemia in cardiac surgery: a pilot randomized controlled trial. *Interact Cardiovasc Thorac Surg.* 2019;28(3):447–454. doi:10.1093/icvts/ivy226

22. Park HS, Kim TY, Kim HJ, Ro YJ, Jang HY, Koh WU. The effect of intraoperative ferric carboxymaltose in joint arthroplasty patients: a randomized trial. *J Clin Med.* 2019;8(10):1674. doi:10.3390/jcm8101674

23. Richards T, Baikady RR, Clevenger B, et al. Preoperative intravenous iron to treat anaemia before major abdominal surgery (PREVENTT): a randomised, double-blind, controlled trial [published online ahead of print, 2020 Sep 4]. *Lancet.* 2020;396(10259):1353–1361. doi:10.1016/S0140-6736(20)31539-7

24. American Society of Anaesthesiologists House of Delegates/Executive Committee. ASA physical status classification system. 2014. Available from: https://www.asahq.org/standards-and-guidelines/asa-physical-status-classification-system. Accessed August 12, 2019.

25. Bernabeu-Wittel M, Aparicio R, Romero M, et al. Ferric carboxymaltose with or without erythropoietin for the prevention of red-cell transfusions in the perioperative period of osteoporotic hip fractures: a randomized contolled trial. The PAHFRAC-01 project. *BMC Musculoskelet Disord.* 2012;13:27. doi:10.1186/1471-2474-13-27

26. Clevenger B, Richards T. Pre-operative anaemia. *Anaesthesia.* 2015;70(Suppl 1):20–28. doi:10.1111/anae.12918

27. Auerbach M. Intravenous iron in the perioperative setting [letter]. *Am J Hematol.* 2014;89(9):933. doi:10.1002/ajh.23793

28. Munoz M, Auerbach M. Postoperative intravenous iron: a simple strategy to improve outcomes. *Lancet Haematol.* 2016;3(9):e401–402. doi:10.1016/S2352-3026(16)30083-7

29. National Blood Authority. *Patient Blood Management Guidelines: Module 2 Perioperative.* Canberra, Australia: National Blood Authority; 2012.

30. Padhi S, Kemmis-Betty S, Rajesh S, Hill J, Murphy MF. Blood transfusion: summary of NICE guidance. *BMJ.* 2015;351:h5832. doi:10.1136/bmj.h5832

31. Thakrar SV, Clevenger B, Mallett S. Patient blood management and perioperative anaemia. *BJA Educ.* 2017;17(1):28–34. doi:10.1093/bjaed/mkw061

32. Maxwell MJ, Wilson MJA. Complications of blood transfusion. *Continuing Educ Anaesth Crit Care Pain.* 2006;6(6):225–229. doi:10.1093/bjaceaccp/mkl053

33. Tolkien Z, Stecher L, Mander AP, Pereira DI, Powell JJ. Ferrous sulfate supplementation causes significant gastrointestinal side-effects in adults: a systematic review and meta-analysis. *PLoS One.* 2015;10(2):e0117383. doi:10.1371/journal.pone.0117383

34. Koo CH, Shin HJ, Cho H, Ryu JH. The effect of perioperative intravenous iron on hemoglobin in surgical patients: a meta-analysis. *J Surg Res.* 2020;246:42–51. doi:10.1016/j.jss.2019.08.023

35. Laso-Morales MJ, Vives R, Vallejo-Tarrat A, Calo N, Valle-Beltran A, Pontes C. Single dose of intravenous ferric carboxymaltose infusion versus multiple fractionated doses of intravenous iron sucrose in the treatment of postoperative anaemia in colorectal cancer patients: study protocol for a randomised controlled trial. *Trials.* 2019;20(1):23. doi:10.1186/s13063-018-3125-2

36. Muñoz M, Gomez-Ramirez S, Cuenca J, et al. Very-short-term perioperative intravenous iron administration and postoperative outcome in major orthopedic surgery: a pooled analysis of observational data from 2547 patients. *Transfusion.* 2014;54(2):289–299. doi:10.1111/trf.12195

37. Page MJ, Moher D, Bossuyt PM, et al. PRISMA 2020 explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. *BMJ.* 2021;372:n160. doi:10.1136/bmj.n71

Relationship Between Estradiol and Hemostasis Determined Through Thromboelastography Profile in Controlled Ovarian Stimulation Cycles

Wiryawan Permadi, Mulyanusa Amarullah Ritonga, Hartanto Bayuaji, Niswan Helja, Corina Delarosa Khoirunnisa & Tono Djuwantono

ORIGINAL RESEARCH

Relationship Between Estradiol and Hemostasis Determined Through Thromboelastography Profile in Controlled Ovarian Stimulation Cycles

Wiryawan Permadi
Mulyanusa Amarullah Ritonga [ID]
Hartanto Bayuaji [ID]
Niswan Helja [ID]
Corina Delarosa Khoirunnisa
Tono Djuwantono

Department of Obstetrics & Gynecology, Dr. Hasan Sadikin Hospital, Faculty of Medicine, Padjadjaran University, Bandung, Indonesia

Purpose: We aimed to analyze the relationship between estradiol level and thromboelastography profile.

Patients and Methods: This prospective study with comparative analysis was performed on nineteen samples undergoing an IVF procedure in two fertility clinics in Bandung city. Blood samples taken on the second and twelfth days of the IVF cycle.

Results: There were significant differences in the value of estradiol, fibrin formation time (α), and coagulation index (CI) ($p<0.05$). Correlation tests showed no relationship and no significant correlation between an increased level of estradiol and coagulation index (CI) (r_s =0.054; p=0.827) and between an increased level of estradiol and fibrinolysis time (LY30) (r_s =−0.151; p=0.536). A moderate significant relationship was observed between age and coagulation index (CI) (r_s=−0.430; p=0.033) and between age and maximum amplitude (MA) (r_s=−0.494; p=0.032).

Conclusion: Supraphysiological estradiol levels in controlled ovarian stimulation cycles affect the coagulation index and change mainly the fibrin formation time.

Keywords: estradiol, hemostasis, thromboelastography, controlled ovarian cycles

Introduction

Recent technological advances and cultural conditions have started to cause fertility issues. These issues are increasing in number and have become a persistent problem. To date, the median prevalence of infertility is 9% worldwide, distributed evenly in both developing and developed countries. Statistics have shown recently that there are at least 72.4 million infertile couples and approximately 40.5 million were seeking health care solutions for infertility problems. Infertility's current definition refers to a condition where couples of reproductive age have not achieved conception after one year of marriage and have regular sexual intercourse without contraception.

Data on Indonesia's overall number of infertility cases are limited, as there has not been much literature covering this particular subject. The 2010 Basic Health Research Report stated that Indonesia's infertility cases range from around 5.5 to 5.9%. Out of 67 million couples during their productive age in Indonesia, 10–15% or 8 million couples experienced infertility or had fertility issues that made it difficult for them to have children. According to 2013 Indonesia Statistic data, the prevalence rate of infertility in the country is increasing every year. In 2013, the prevalence rate was 15–25% among all couples.[1,2]

Correspondence: Mulyanusa Amarullah Ritonga
Email mulyanusa@unpad.ac.id

The increase in infertility is currently associated with the postponement of marriage, an increasing number of male-factor infertility, increasing prevalence of obesity, and sexual infectious diseases.[3] Current advances in medical technology, providing a solution for infertility in assisted reproductive technology (ART),[4,5] include controlled ovarian stimulation (COS), followed by intrauterine insemination (IUI), or in vitro fertilization (IVF).[5,6]

Assisted reproductive technology is principally divided into three main stages, namely: 1) controlled ovarian stimulation; 2) trigger ovulation; and 3) fertilization. At the controlled ovarian stimulation stage, many follicles containing oocytes are recruited, and estrogen is increased supraphysiologically. This process was associated with ovarian hyperstimulation syndrome (OHSS).[3,7] Recent studies showed that the rapidly increasing plasma levels of estradiol due to the IVF cycle might lead to a procoagubility state in several hemostasis parameters.[8-10] These supraphysiological conditions can increase the risk of arterial and venous thrombosis which, if it persists, is associated with venous thromboembolism (VTE), OHSS, implantation failure, or repeated abortion.[11,12]

Hemostasis was measured by several tests such as clotting time, bleeding time, prothrombin time (PT), and activated partial prothrombin time (aPTT). However, the current test is static and takes a long time; and this may cause a delay in the administration of the next phase of treatment.[13] Another weakness of these tests is the fact that they have to be repeated to get a precise result. Although they provide information on platelet counts, they do not accurately describe the platelets' functional aspects.

Thromboelastography (TEG) is a hemostasis examination technique known since 1948 to assess the global physiology of hemostasis by examining the components of complete blood elasticity. Initially, TEG was used for hemostasis assessment in cases of liver transplants, heart surgery, and obstetric procedures, especially for assessing the volume of blood loss and potential for thrombosis. TEG examination can also be used to determine the type of blood components needed for transfusion in bleeding. The test assesses the fibrin formation time, the strength of clots, and coagulation indices, and the results are obtained in the form of an overview chart. In interpreting TEG examination, experts review whether the patient has hypercoagulability and whether this was associated with fibrinogen or platelet factors.[14,15]

A study by Harnett et al in 2002[11] and by Westerlund in 2012[12] found that on the IVF cycles, there was a tendency toward hypercoagulability on TEG examination, mainly on the decreased time of clot formation. This was an interesting finding in terms of TEG use in ART, since due to its real-time principle, it can detect more quickly and precisely. In this study, we analyzed the relationship and influence of thromboelastography profile and estradiol on IVF cycles.

Materials and Methods

This was a comparative prospective analytic study, observing the changes in coagulation index on the second and twelfth days of short-protocol IVF due to increased levels of estradiol.

This study was conducted on women aged 20–45 years undergoing a short-cycle IVF protocol in the Aster Infertility Clinic of Hasan Sadikin General Hospital Bandung and in Bandung Fertility Centre of Limijati Hospital Bandung between October and December 2014. The inclusion criteria were women aged 20–45 years, who do not smoke, were healthy, able to do daily activities independently, can communicate well and have no prior history of cardiovascular disease, not taking medications that may affect coagulation balance such as vitamin C or vitamin K, salicylic acid, tranexamic acid or mefenamic acid in the last four weeks, and willing to participate in this study after receiving an explanation and signing an informed consent. Subjects may resign or withdraw from the study at any time for any reason. Subjects who stopped undergoing the IVF protocol in the middle of the cycle or subjects with incomplete data were excluded from the study.

After obtaining approval from Hasan Sadikin General Hospital's ethics committee, the study team recruited nineteen people as subjects who will undergo the short-cycle IVF protocol. Recombinant FSH (Gonal F®, Merck-Serono Laboratories) was used for stimulation protocol, and Cetrorelix (Cetrotide® Merck-Serono Laboratories) was administered on the seventh day of stimulation. Around 4–5 mL of blood samples were taken on the second day of menstruation and put into Vacutainer® with citric acid (Becton Dickinson, Franklin Lakes, NJ, USA) to examine the levels of estradiol. For thromboelastography examination, 1 mL of sample was transferred into a 1% selit vial, and 2.34 mL of samples were then inserted into a TEG® analyzer cup (model 5000; Haemoscope Corp., Niles, IL, USA). In another cup, we

inserted 5 μL ReoPro® (Eli lilly, Indianapolis, IN, USA), a platelet glycoprotein IIB/IIIa receptor antagonist. Both cups were then warmed at 37 °C and analyzed.

This study used a 5% significance rate (α = 1.65) and 90% power of test (Z = 1.28). We obtained a sample of nineteen subjects. The variables tested in this study were the levels of estradiol on the second day, and the dependent variables were the level of estradiol on the twelfth day, and the assessment of the TEG result in the form of the R value (time needed for the formation of fibrin), the K value (time needed to maintain the strength of the clot), the α angle (the speed of fibrin formation), MA value (maximum amplitude, a direct function of fibrin bond dynamic component and platelets), coagulation index (CI, a mathematical formula describing hemostasis coagulation), and LY 30 value (the rates of fibrinolysis after MA is reached). Confounding variables include age, basal FSH levels, recombinant FSH dose used, and antral follicle count (AFC).

Before performing statistical tests, numeric data were tested for normality using the Shapiro–Wilk test. Wilcoxon tests were performed to determine differences in estradiol levels and hemostasis between patients. Spearman tests were used to determine the relationship between risk factors and the thromboelastography profile. Results were confirmed statistically significant if the *p*-value was <0.05.

Results

The baseline characteristics of our nineteen samples can be seen in Table 1, and the complete data description can be seen in Table 2. The age variable shows that the mean age in these data is 33.6 years old, while the median is 33 years old with a range from 28 to 42 years old. The FSH dose variable obtained the mean dose as 202.6 IU, median 225 IU, ranging from 100 to 300 IU. The basal FSH variable mean level is 8.5 IU with median 7.5 IU. The range level is 4.5−16.2 IU, and the antral follicle count variable obtained mean number is 8.2, the median is 8 with range 3–11.

Data obtained from the first and second measurements were calculated for their mean, median, standard deviation, and range. The results are presented in Table 3. Comparison between variables measured in two measurements in Table 3 shows the estradiol variable with a mean value on the second day of IVF cycle is 39.70 pg/mL and the twelfth day of IVF cycle is 2415.99 pg/mL, the median value on the second day of IVF cycle is 25 pg/mL and the

Table 1 Characteristic Data

Variable	n (%)
Age (years)	
Mean (SD)	33.6 (5.2)
Median	33
Range	26–42
FSH doses (IU)	
Mean (SD)	202.6 (64)
Median	225
Range	75–300
Basal FSH (IU)	
Mean (SD)	8.5 (2.9)
Median	8.3
Range	4.5–16.2
Antral follicle count	
Mean (SD)	8.2 (3)
Median	8
Range	3–13

twelfth day of IVF cycle is 2239 pg/mL, and range of the second day of IVF cycle is 5–231 pg/mL and the twelfth day of IVF cycle is 120–6290 pg/mL. The *P*-value in this variable is 0.001, which is statistically significant.

The R_{value} variable obtained the mean value on the second day of IVF cycle is 8.59 min and on the twelfth day of IVF cycle is 7.34 min, with the median value on the second day of IVF cycle is 7.9 min and on the twelfth day of IVF cycle is 7.5 min, and the range of minutes on the second day of IVF cycle is 4.2–19.4 min and the twelfth day of IVF cycle is 4–9.5 min. The *P*-value in this variable is 0.446 means no correlation statistically.

The K_{value} variable obtained the mean value on the second day of IVF cycle is 2.6 min and on the twelfth day of IVF cycle is 2.13 min, with the median value on the second day of IVF cycle is 2.2 min and on the twelfth day of IVF cycle is 2.1 min, and the range of the value on the second day of IVF cycle is 1.4–5.7 min and on the twelfth day of IVF cycle is 1.6–3.3 min. The *P*-value in this variable is 0.107 and not significant statistically.

The angle (α) variable obtained the mean value on the second day of IVF cycle is 56.07 degrees and on the twelfth day of IVF cycle is 60.64 degrees, with the median value on the second day of the IVF cycle is 58 degrees and on the twelfth day of IVF cycle is 60.6 degrees, and the angle range on the second day of IVF cycle is 38.5–70

Table 2 Original Data Results

No.	Age (Years)	FSH Dose (IU)	Basal FSH	AFC	2nd Day of IVF Cycle							
					Estradiol	R (min)	K (min)	Angle (deg)	MA (mm)	G (d/sc)	EPL (%)	A (mm)
I	39	300	16.19	4	5	7,7	1,8	64,2	70	11,7	0,1	68,9
2	35	225	12.5	5	6,09	9,5	3,3	48,5	53	5,6	1,9	47,2
3	31	150	8.3	11	5	10,5	2,8	53,3	61,4	7,9	0,5	59,5
4	28	100	4.5	10	20,6	14,8	5,1	36,7	49,9	5	1,6	46,2
5	29	150	9.15	8	61,3	4,6	1,8	65,9	66,7	10	1,6	61,9
6	41	225	4.7	6	33,94	4,9	1,4	70	70,4	11,9	0,1	66,5
7	42	300	10.6	3	48,74	9,3	2,3	58	65	9,3	0,4	57,5
8	29	225	7.07	8	13,15	7,9	1,8	63,6	65,3	9,4	0,3	59,8
9	30	150	6.31	10	95,85	6,8	2,1	61,3	65	9,3	0	66
10	41	300	3.61	9	26,23	7,7	1,8	63,6	64,7	9,2	0	65,7
11	39	225	7.29	13	15,88	7,9	1,9	62,4	64,5	9,1	0,1	51,3
12	32	225	8.2	12	231	6,9	2,2	59,2	61,5	8	0,1	60,5
13	33	225	7.5	6	25,14	7,4	2,1	51,2	65,8	9,5	0	62,1
14	38	225	7.03	5	34,35	8,3	2,5	55,3	60,6	7,7	0,5	60,9
15	34	225	9.1	6	5,6	8,7	2,9	52,5	58,9	7,2	0,5	52,8
16	26	150	6.5	10	20,6	10,5	3,4	44,3	59	7,2	0	59,6
17	26	75	6.01	13	28,63	6,3	2	62,7	63,8	8,8	0	54,7
18	36	225	10.5	7	15	19,4	5,7	35,8	57,5	6,8	0,1	51,9
19	30	150	9.5	10	25	4,2	2,5	56,8	59,5	7,4	1,3	59,1

degrees and on the twelfth day of IVF cycle is 51–70 degrees. The *P*-value in this variable is 0.027, which is statistically significant.

The MA variable obtained the mean value on the second day of IVF cycle is 62.23 mm and on the twelfth day of IVF cycle is 64.16 mm, with the median value on the second day of IVF cycle is 63.8 mm and the twelfth day of IVF cycle is 63.4 mm. The range on the second day of IVF cycle is 49.4–70.4 mm and on the twelfth day of the IVF cycle is 61.3–69.5 mm. The *P*-value in this variable is 0.136 showing no statistically significant correlation.

The G variable obtained the mean value on the second day of IVF cycle is 8.47 d/sc and on the twelfth day of IVF cycle is 8.68 d/sc, with the median value on the second day of IVF cycle is 8.8 d/sc and on the twelfth day of IVF cycle is 8.4 d/sc. The range from the second day of IVF cycle is 5–11.9 d/sc and the twelfth day of IVF cycle is 6–11.4 d/sc. The *P*-value in this variable is 0.589 showing no statistical correlation result.

The EPL variable obtained the mean value on the second day of IVF cycle is 0.48% and on the twelfth day of IVF cycle is 0.33%, with the median value on the second day of IVF cycle is 0.1% and twelfth day of IVF cycle is 0.1%. The range on the second day of the IVF cycle is 0–1.9% and the

twelfth day of the IVF cycle is 0–1.4%. The *P*-value in this variable is 0.379 resulting in no statistical correlation.

The A variable obtained the mean value on the second day of IVF cycle is 58.53 mm and on the twelfth day of IVF cycle is 61.39 mm, with the median value on the second day of IVF cycle is 59.6 mm and the twelfth day of IVF cycle is 61.7 mm. The range on the second day of IVF cycle is 46.2–68.9 mm and on the twelfth day of IVF cycle is 50.5–68.3 mm. The *P*-value in this variable is 0.038 showing a significant correlation statistically.

The Cl variable obtained the mean value on the second day of IVF cycle is 1.85 mm and on the twelfth day of IVF cycle is 2.35 mm, with the median value on the second day of IVF cycle is 2.2 mm and on the twelfth day of IVF cycle is 2.4 mm. The CI range on the second day of IVF cycle is −1.2 to 3.8 mm and on the twelfth day of IVF cycle is 1.2–3.5 mm. The *P*-value in this variable is 0.039 showing a significant correlation statistically.

The LY30 variable obtained the mean value on the second day of IVF cycle is 0.39% and on the twelfth day of IVF cycle is 0.31%, with the median value on the second day of IVF cycle is 0.1% and on the twelfth day of IVF cycle is 0.1%, and the range of percentage from the second day of IVF cycle is 0–1.9% and the twelfth day of IVF cycle is 0–1.8%. The *P*-value in this variable is 0.454 showing no statistical correlation.

		12th Day of IVF Cycle									
CI	LY30 (%)	Estradiol	R (min)	K (min)	Angle (deg)	MA (mm)	G (d/sc)	EPL (%	A (mm)	CI	LY30 (%)
3,2	0,1	182,6	9,1	1,6	67	68,4	10,8	0,2	67,3	2,5	0,2
0,3	1,9	120	9,5	1,8	70	65	8,2	0,1	62	1,2	0,1
1,3	0,5	1668	4	1,7	66,7	62,7	8,4	0,1	58,1	2,8	0,1
−1,2	1,6	3534	7,2	2,2	59,5	64,2	9	1	61	2,4	1
3,3	1,6	2242	5,8	1,8	65,1	69,5	11,4	0,1	68,3	3,5	0,1
3,8	0,1	1893	6,2	2,2	60,1	65,6	9,5	0,1	64,3	2,9	0,1
2,1	0,4	325,3	8,2	2,2	59,5	63,4	8,7	0,4	55,5	2,1	0,4
2,3	0,3	2239	6	1,6	67,6	68,2	10,7	0,1	64,5	3,2	0,1
2,6	0	3119	7,5	2	62,4	62,3	8,3	0	62	2	0
2,3	0	6290	8,6	2,5	56,2	61,7	6	0	62,9	1,8	0
2,2	0,1	3781	7,5	2,3	58,5	64,5	9,1	0	65,4	2,5	0
2,1	0,1	5650	7,4	3	51	61,3	7,9	0	60,7	2,1	0
2,5	0	3404	7,6	2,1	61,4	65,8	9,5	0,5	64,1	2,5	0,5
1,7	0	1363	8,5	2,3	54,1	62,3	7,2	0,4	61,5	1,5	0
1,4	0,5	1281	6,2	2,1	60,6	62	8,2	1,4	54,7	2,3	1,4
1,2	0	2560	8,1	2,7	55	65,1	9,3	0	61,7	2,5	0
2,5	0	2052	7,1	2,1	61,1	62,8	8,4	0	60,5	2,2	0
−1	0,1	2400	8,5	2,3	54,1	62,3	7,2	0,4	61,5	1,5	0
2,5	0,2	1800	6,5	2	62,3	62	7,2	1,4	50,5	3,2	1,8

Changes in the level of estradiol, fibrin formation time (α), fibrin formation time (A), and coagulation index (CI) were found to be statistically significant ($p<0.05$), as seen in Table 4. Correlation between baseline data and the difference among variables measured in Table 4 shows that the correlation value is efficient at the variable age, shows the coagulation index (CI) is −0.430 with a p-value of 0.033, the maximum amplitude (MA) is −0.494 with a p-value of 0.032, the time required for clot formation (R) is 0.152 with a p-value of 0.534, and the fibrin formation time (A) is −0.341 with a p-value of 0.153.

The correlation coefficient value on the estradiol variable shows the coagulation index (CI) is 0.054 with a p-value of 0.827 and fibrinolysis time (LY30) is −0.151 with a p-value of 0.536. The correlation coefficient value on basal FSH, namely fibrin formation time (A), is 0.351 with zp-value of 0.141, and clot stabilization time (K) is −0.352 with a p-value of 0.139.

The correlation coefficient value on the FSH-r dose variable, namely the coagulation index (CI) is −0.352 with a p-value of 0.140. The correlation coefficient value on the AFC variable, namely the coagulation index (CI) is 0.212 with a p-value of 0.383, and the time required for clot (R) is −0.057 with a p-value of 0.817.

After the correlation test, we performed another statistical analysis using Guilford Criteria to determine the relationship between the two groups and baseline data. We found no relationship and no significant correlation between the changes in the level of estradiol and coagulation index (CI) (r_s=0.054; p=0.827) as well as between changes in the level of estradiol and fibrinolysis time (LY30) (r_s=−0.151; p=0.536). Medium, statistically significant correlation was found between age and coagulation index (CI) (r_s =−0.430; p=0.033) and between age and maximum amplitude (MA) (r_s=−0.494; p=0.032).

Discussion

It is common knowledge that estrogen has an impact on coagulation and fibrinolysis, but knowledge and studies on the exact nature of this relationship are limited. Current information on this varies for both intrinsic or extrinsic estrogen.[16,17] Many studies linked the use of hormone replacement therapy or contraceptives to coagulation, but not many investigate the effects of estrogen alone on the physiological changes in the reproductive cycle.[16]

The mechanism with which estrogen affects hematological variables can be studied in the cycle of in vitro fertilization, especially in the controlled ovarian stimulation stage. Although assisted reproductive technique indications through the application of in vitro fertilization vary widely, samples were generally nearly homogeneous in terms of demographics. Unlike hormone replacement

Table 3 Comparison Between Variables Measured in Two Measurements

Variables	Measurement		P-value
	Second Day of IVF Cycle	**Twelfth Day of IVF Cycle**	
Estradiol (pg/mL) Mean Median Range	 39.70 25 (5–231)	 2415.99 2239 (120–6290)	 0.001*
R_{value} (min) Mean Median Range	 8.59 7.9 (4.2–19.4)	 7.34 7.5 (4–9.5)	 0.446
K_{value} (min) Mean Median Range	 2.6 2.2 (1.4–5.7)	 2.13 2.1 (1.6–3.3)	 0.107
α (angle) Mean Median Range	 56.07 58 (38.5–70)	 60.64 60.6 (51–70)	 0.027
MA (mm) Mean Median Range	 62.23 63.8 (49.4–70.4)	 64.16 2.1 (61.3–69.5)	 0.136
G (d/sc) Mean Median Range	 8.47 8.8 (5–11.9)	 8.68 8.4 (6–11.4)	 0.5829
EPL (%) Mean Median Range	 0.48 0.1 (0–1.9)	 0.33 0.1 (0–1.4)	 0.379
A (mm) Mean Median Range	 58.53 59.6 (46.2–68.9)	 61.39 61.7 (50.5–68.3)	 0.038
CI (mm) Mean Median Range	 1.85 2.2 (−1.2 to 3.8)	 2.35 2.4 (1.2–3.5)	 0.039
LY30 (%) Mean Median Range	 0.39 0.1 (0–1.9)	 0.31 0.1 (0–1.8)	 0.454

Note: *Paired *T*-test.

Table 4 Correlation Between Baseline Data and the Difference Among Variables Measured

Correlation		Correlation Coefficient (r_s)	p-value
Age	Coagulation index (CI) Maximum amplitude (MA) Time required for clot formation (R) Fibrin formation time (A)	−0.430 −0.494 0.152 −0.341	0.033* 0.032 0.534 0.153
Estradiol	Coagulation index (CI) Fibrinolysis time (LY30)	0.054 −0.151	0.827 0.536
Basal FSH	Fibrin formation time (A) Clot stabilization time (K)	0.351 −0.352	0.141 0.139
FSH-r doses	Coagulation index (CI)	−0.352	0.140
AFC	Coagulation index (CI) Time required for clot (R)	0.212 −0.057	0.383 0.817

Note: *r_s: Spearman rank correlation coefficient.

therapy and contraceptives, this technique had the advantage of measuring the impacts of changes in the concentration of estrogen and its effect on coagulation and fibrinolysis in the short term.[18]

The current study found a significant relationship between elevated levels of estradiol and thromboelastography profiles, especially in the fact that the speed of fibrin formation was faster on the second measurement compared to that on the first measurement (second day of stimulation). The hemostasis components cause a significant increase in the coagulation index.

These results were consistent with studies on thromboelastography in the IVF cycle conducted by Harnett et al in 2002[11] and Westerlund et al in 2013.[12] In contrast to the study by Harnett et al[11] who found that significant changes occurred in the reduction of clot formation time, we found no significant changes in the fibrin formation time. This might be related to the characteristics of our subjects. Our subjects were younger compared to the subjects in previous studies.[11] Age is the most important prognostic factor associated with folliculogenesis, successful pregnancy, and other reproductive functions.[19,20] We found in this study that age was inversely related to the levels of

intrinsic estradiol and proportionally related to the levels of FSH.

De Graaf follicles (antral follicles) are characterized by the formation of pockets of fluid near the oocyte referred to as the antrum. Granulose cells and theca cells continue to do mitosis along with the increase in antral volume. The growth of antral follicle size is influenced by FSH levels in the blood and is dependent on FSH. Theca cells will express luteinizing hormone (LH) receptors and will start producing androgens, predominantly androstenedione, and these will be aromatized by the granulose cells to produce estradiol. As a consequence of this, the estradiol levels will increase.[6,21]

This theory is still in line with what was found in this study, where we found a moderate and statistically significant relationship between elevated levels of estradiol and the antral follicle count (AFC). On the other hand, there was a fairly moderate correlation between age and the changes in coagulation index and between age and maximum amplitude (MA). Both age-related findings are statistically significant. This finding may lead to new allegations that age may also have a major impact on measurable changes in coagulation as measured through thromboelastography.

This is in contrast to Harnett et al's conclusion, which stated that the decrease in clotting time led to the quick formation of clots, while fibrinolysis is not affected.[11] Harnett et al's findings led to the recommendation to use thromboelastography as a modality to detect ovarian hyperstimulation syndrome as indicated by hypercoagulability. This suggested that further study is needed to explore the correlation between thromboelastography measurement and successful pregnancies and implantation rate of the in vitro fertilization cycle.

No previous study mentioned any such hypofibrinolysis as found in this study, although in general, changes in coagulation index were consistently found. Hypofibrinolysis will cause a disruption of the clot dissolution process.[22,23] The formation of antibodies against plasminogen activators such as tissue plasminogen activator (tPA) or components such as annexin A2 receptor fibrinolysis will result in major thrombosis, which may have an even greater impact today than ovarian hyperstimulation syndrome. Hypofibrinolysis is often found in antiphospholipid syndrome and is known to be associated with repeated abortion.[10,24] Hypofibrinolysis is also usually found in patients with hypothyroidism and alcoholic liver disease.

These findings gave new insights about the physiology of reproductive hemostasis. Limited time to conduct research has been the main problem in this study, and this study was also conducted in a small number of subjects. Similar to previous studies, the difficulty in controlling other factors that potentially interfered withthe assisted reproductive technology also presented as a challenge. These factors include the given regimen, the long duration of pituitary suppression using GnRH analogues, and the individual response as mentioned by Nelson.[25]

Considering that our findings on the thromboelastography component or hemostasis profiles differ from previous studies, future research may be necessary for successful pregnancies and thromboelastography profile associated with an ideal dose of FSH-r for the success of an assisted reproductive technology program. More extensive research about the thromboelastography component or hemostasis profiles with a large number of subjects is needed.

Conclusion

In conclusion, this study shows significant differences in the change of the estradiol level, the rate of fibrinogen formation, and the coagulation index in controlled ovarian stimulation cycles.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

References

1. Dasar RK. Badan Penelitian Dan Pengembangan Kesehatan Kementrian Kesehatan Republik Indonesia [Health Research and Development Council, Indonesian Ministry of Health]. National Basic Health Research; 2010. Indonesian.
2. Dasar RK. Badan Penelitian Dan Pengembangan Kesehatan Kementrian Kesehatan Republik Indonesia [Health Research and Development Council, Indonesian Ministry of Health]. National Basic Health Research; 2013. Indonesian.
3. Vloeberghs V, Peeraer K, Pexsters A, D'Hooghe T. Ovarian hyperstimulation syndrome and complications of ART. *Best Pract Res Clin Obstet Gynaecol*. 2009;23(5):691–709. doi:10.1016/j.bpobgyn.2009.02.006
4. For Women's, National Collaborating Centre, and U. K. Children's Health. *Fertility: Assessment and Treatment for People with Fertility Problems*. 2 ed. London: National Collaborating Centre for Women's and Children's Health Commissioned by the National Institute for Clinical Excellence; RCOG Press; 2013.

5. Anwar INC. Seleksi pasien menuju fertilisasi in vitro [Patient selection through In Vitro Fertilization]. In: Darmasetiawan MS, Anwar INC, Djuwantono T, Adenin I, Jamaan T, editors. *Fertilisasi in vitro Dalam Praktek Klinik* [In Vitro Fertilization in Clinical Practice]. 1 ed. Jakarta: Puspa Swara; 2006:2–37. Indonesian.

6. Balen AH. *Infertility in Practice*. 3 ed. London: Informa Healthcare, Ltd; 2008.

7. Balen A. Ovarian hyperstimulation syndrome. *Hum Fertil (Camb)*. 2013;16(3):143. doi:10.3109/14647273.2013.826044

8. Chan WS. The 'ART' of thrombosis: a review of arterial and venous thrombosis in assisted reproductive technology. *Curr Opin Obstet Gynecol*. 2009;21(3):207–218. doi:10.1097/GCO.0b013e328329c2b8

9. Huang JY, Rosenwaks Z. In vitro fertilisation treatment and factors affecting success. *Best Pract Res Clin Obstet Gynaecol*. 2012;26 (6):777–788. doi:10.1016/j.bpobgyn.2012.08.017

10. Bauersachs RM, Manolopoulos K, Hoppe I, Arin MJ, Schleussner E. More on: the 'ART' behind the clot: solving the mystery. *J Thromb Haemost*. 2007;5(2):438–439. doi:10.1111/j.1538-7836.2007.02339.x

11. Harnett MJ, Bhavani-Shankar K, Datta S, Tsen LC. In vitro fertilization-induced alterations in coagulation and fibrinolysis as measured by thromboelastography. *Anesth Analg*. 2002;95 (4):1063–1066. table of contents. doi:10.1097/00000539-200210000-00050

12. Westerlund E, Henriksson P, Wallen H, Hovatta O, Wallberg KR, Antovic A. Detection of a procoagulable state during controlled ovarian hyperstimulation for in vitro fertilization with global assays of haemostasis. *Thromb Res*. 2012;130(4):649–653. doi:10.1016/j. thromres.2011.11.024

13. Thakur M, Ahmed AB, Gaur A. A review of thromboelastography. *Int J Periop Ultrasound Appl Technol*. 2012;1(1):25–29. doi:10.5005/ jp-journals-10027-1006

14. Bolliger D, Seeberger MD, Tanaka KA. Principles and practice of thromboelastography in clinical coagulation management and transfusion practice. *Transfus Med Rev*. 2012;26(1):1–13. doi:10.1016/j. tmrv.2011.07.005

15. Chen A, Teruya J. Global hemostasis testing thromboelastography: old technology, new applications. *Clin Lab Med*. 2009;29 (2):391–407. doi:10.1016/j.cll.2009.04.003

16. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during aging: from periphery to brain. *Trends Mol Med*. 2013;19 (3):197–209. doi:10.1016/j.molmed.2012.12.007

17. Sahin M. Estrogen effects on platelets. In: Bartos JR, editor. *Estrogen: Production, Function and Applications*. 1 ed. New York: Nova Biomedical Books; 2009:215–230.

18. Brummel-Ziedins KE, Gissel M, Francis C, Queenan J, Mann KG. The effect of high circulating estradiol levels on thrombin generation during in vitro fertilization. *Thromb Res*. 2009;124(4):505–507. doi:10.1016/j.thromres.2009.02.006

19. Arnal JF, Gourdy P, Lenfant F. In vivo dissection of the estrogen receptor alpha: uncoupling of its physiological effects and medical perspectives. *Ann Endocrinol (Paris)*. 2013;74(2):82–89. doi:10.1016/j.ando.2013.03.001

20. Barrett-Connor E, Laughlin GA. Endogenous and exogenous estrogen, cognitive function, and dementia in postmenopausal women: evidence from epidemiologic studies and clinical trials. *Semin Reprod Med*. 2009;27(3):275–282. doi:10.1055/s-0029-1216280

21. Fritz M, Speroff L. *Clinical Gynecologic Endocrinology and Infertility*. 8 ed. Philadelphia: Lippincott Williams and Wilkins; 2011.

22. Chapin JC, Hajjar KA. Fibrinolysis and the control of blood coagulation. *Blood Rev*. 2014;29(1):17–24. doi:10.1016/j. blre.2014.09.003

23. Wright JG, Cooper P, Astedt B, et al. Fibrinolysis during normal human pregnancy: complex inter-relationships between plasma levels of tissue plasminogen activator and inhibitors and the euglobulin clot lysis time. *Br J Haematol*. 1988;69(2):253–258. doi:10.1111/j.1365-2141.1988.tb07630.x

24. Gardiner C, Hills J, Machin SJ, Cohen H. Diagnosis of antiphospholipid syndrome in routine clinical practice. *Lupus*. 2013;22 (1):18–25. doi:10.1177/0961203312460722

25. Nelson SM. Prophylaxis of VTE in women - during assisted reproductive techniques. *Thromb Res*. 2009;123(Suppl 3):S8–S15. doi:10.1016/S0049-3848(09)70127-6

Trends of Ordering Hypercoagulability Work-Up at an Academic Medical Center

Marjan Alidoost, Gabriella A Conte, Varsha Gupta, Swapnil Patel, Ishan Patel, Mohammed Shariff, Shreya Gor, Michael J Levitt, Arif Asif & Mohammad A Hossain

Published online: 28 May 2021.

Submit your article to this journal

View related articles

View Crossmark data

ORIGINAL RESEARCH

Trends of Ordering Hypercoagulability Work-Up at an Academic Medical Center

Marjan Alidoost
Gabriella A Conte
Varsha Gupta
Swapnil Patel
Ishan Patel
Mohammed Shariff
Shreya Gor
Michael J Levitt
Arif Asif
Mohammad A Hossain [ORCID]

Department of Medicine, Jersey Shore University Medical Center, Hackensack Meridian Health, Neptune, New Jersey, 07753, USA

Background: Venous thromboembolism is a significant clinical event, with an annual incidence of 1–2 per 1000 population. Risk factors include recent surgery, prolonged immobility, oral contraceptive use, and active cancer. Inherited risks include protein C and S deficiencies, antithrombin deficiency, factor V Leiden mutation and prothrombin. These factors can be tested to guide therapy, but current evidence suggests that testing for inherited thrombophilia is not recommended in most inpatient settings. In the era of high value care, hypercoagulable testing for VTE creates a financial burden for the hospital and patients. We performed a retrospective chart review of hypercoagulable orders on VTE patients at our institution.

Methods: Institutional Review Board approval was obtained. A total of 287 adult patients admitted over a 3-month period with the diagnosis of VTE were included. Patients were identified via ICD-10 codes and data were collected from electronic medical records. Patient characteristics, provoked versus unprovoked VTE, and relative contraindications for hypercoagulability work-up were analyzed. Our primary outcome was to assess the appropriateness of thrombophilia testing in VTE patients based on screening guidelines. Our secondary outcome was to analyze the cost burden of ordering these tests.

Results: A total of 287 patients were included in our data analysis. Patient risk factors for VTE were malignancy, previous DVT, immobilization, surgery 3 months prior, and central line placement. Fifty-seven of 287 patients had at least one hypercoagulable test ordered during hospitalization which did not adhere to guidelines. Misuse of testing occurred during active thrombosis, active anticoagulation, presence of risk factors, first episode of VTE, and malignancy. The cost of ordering these 5 thrombophilia tests totaled over $40,000.

Conclusion: In our study, numerous patients were tested without compliance to standard recommendations, which created financial and value-based burdens on our health care system. Increased awareness among clinicians is thus warranted to ensure high value care.

Keywords: venous thromboembolism, VTE, hypercoagulability, thrombophilia

Introduction

Venous thromboembolism (VTE) is an increasingly significant clinical event, with an annual incidence rate of approximately 1–2 per 1000 population.[1] Along with the choice of treatment for VTE, clinicians must also plan the duration of treatment by identifying whether the incident was provoked or unprovoked by known risk factors for VTE, such as recent surgery, prolonged immobility, oral contraceptive use, and active cancer.[2,3] Inherited risk factors include protein C deficiency, protein S deficiency, antithrombin deficiency, factor V Leiden (FVL) mutation, and prothrombin gene mutation G20210.[4] Testing for inherited risk factors can be used for guidance on therapy; however, improper timing of these tests can affect

Correspondence: Gabriella A Conte
Chief Resident, Internal Medicine Residency Program, Department of Medicine, Jersey Shore University Medical Center, Hackensack-Meridian Health, Neptune, NJ, 07753, USA
Tel +1 201-446-4611
Email gabriella.
conte@hackensackmeridian.org

thrombophilia results, leading to inappropriate anticoagulation therapy. The evidence currently available suggests that testing for inherited thrombophilia is not recommended in most clinical settings. In the era of high value care, it places a financial burden on both the hospital and its patients.[4] Given this, we performed a retrospective review of hypercoagulable testing that was ordered on patients admitted for VTE at our institution, Jersey Shore University Medical Center (JSUMC).

Methods

After receiving Institutional Review Board (IRB) approval, a retrospective chart review was conducted for 287 adult patients (aged 18 years and older) admitted to JSUMC between May 2018 and August 2018. All clinical and laboratory data were compiled from the EMR. Our inclusion criteria included any adult patient presenting to the emergency department with a diagnosis of VTE. Patients were identified using ICD-10 codes for VTE.

Baseline clinical characteristics were gathered for each patient (Table 1), including gender, BMI, medical history, and family history of various thrombophilias. Furthermore, information was collected on the presence or absence of VTE risk factors, site of VTE, and thrombophilia test results. The patterns for ordering thrombophilia testing were reviewed for the following inherited conditions:

1. APC resistance/factor V Leiden mutation
2. Prothrombin gene mutation
3. Protein S deficiency
4. Protein C deficiency
5. Antithrombin deficiency

In the absence of definitive guidelines for thrombophilia testing, we reviewed the literature and, in conjunction with the JSUMC hematology-oncology department, created our own guidelines for this study.[5–9] From this, we formulated contraindications for work-up (Table 2). Our guidelines for relative indications for hypercoagulability work-up are listed in Table 3. Our primary outcome was to assess the appropriateness of thrombophilia testing in patients with VTE in the inpatient setting based on published thrombophilia screening guidelines. Our secondary outcome was to analyze the cost burden of ordering these tests. In order to properly assess the financial impact of eliminating unwarranted thrombophilia testing, current market prices for thrombophilia tests were obtained from the inpatient laboratory department of the hospital. Simple

statistical analysis was performed for this study. We considered ordering the work-up untimely if it was ordered for a patient with a provoked VTE, if ordered while the patient was on anticoagulation medication, or if ordered during an acute VTE, as detailed in Figure 1.

Results

Included in this review were 287 patients with a diagnosis of VTE. The following risk factors for VTE were analyzed in the selected patients: malignancy (20.6%), previous DVT (19.9%), immobilization (19.2%), surgery in the past 3 months (10.8%), and central line placement (1%) (Table 1).

For 57 of the 287 patients, one or more type of hypercoagulable testing was performed (19.86%). All 57 patients for whom the thrombophilia work-up was performed were found to have been unnecessarily tested as per our compiled guidelines (Table 2). Reasons for non-adherence were found to be: active thrombosis (57/57), active anticoagulation (47/57), presence of risk factors/provoked VTE (22/57), first episode of VTE (17/57), and malignancy (2/57) (Figure 2). We also evaluated the number of relative indications in these tested patients (Figure 3). These findings indirectly suggest the reasons for non-adherence to guidelines by the ordering physicians. Upon analyzing the financial burden related to improper testing, we calculated the total cost of ordering these five tests based on current market prices (Table 4). Based on current market prices, we found that the overall cost of ordering these five common tests – APC resistance/factor V Leiden mutation, prothrombin gene mutation, protein S deficiency, protein C deficiency, and antithrombin deficiency – totaled more than $40,000.

Discussion

Thrombophilia is a condition that predisposes an individual to experience clot formation in the circulatory system. The etiology of thrombosis may be multifactorial, and the presence of a nidus causing thrombophilia is only one of many elements that may determine the risk of recurrence. Thrombophilia can be acquired or inherited. Inherited thrombophilia refers to conditions in which a genetic mutation affects the amount or function of a protein in the coagulation system. These factors include deficiencies of natural anticoagulants, such as protein S, protein C, antithrombin, and the two-point mutations – factor V Leiden and the prothrombin gene.[9] Activated protein C resistance (APCR) due to FVL mutation is the most common hereditary thrombophilia among the Caucasian

Table 1 Patient Demographics

Total Patients		287
Age		63.7 years
Gender		
	Male	136 (47.4%)
	Female	151 (52.6%)
Race		
	White	234 (81.5%)
	Black	80 (27.9%)
	Asian	9 (3.1%)
	Other	40 (14.0%)
BMI		29.71
Medical History		
	Immobilization	55
	Hospitalization in last 3 months	80
	Malignancy	59
	Previous DVT	57
	Prolonged travel	26
	Chronic kidney disease	27
	Atrial fibrillation	34
	Inflammatory bowel disease	3
	Liver disease	1
	Heparin induced thrombocytopenia	3
	Antiphospholipid syndrome	1
	Cerebrovascular accident	14
	Surgery in past 3 months	31
	History of central line placement	6
	Present pregnancy	1
	Nephrotic syndrome	1
	Congestive heart failure	19
	PCOS	0
	PNH	0
	Warfarin induced skin necrosis	0
	History of recurrent abortions	0
	Substance abuse	14
	Diabetes mellitus	40
	Hypertension	136
	Coronary artery disease	38
	Chronic obstructive pulmonary disease	21
	Active malignancy	30
Family History		
	Factor V mutation	4
	Prothrombin gene mutation	1
	Protein S deficiency	0
	Protein C deficiency	0
	Antithrombin III deficiency	0

Table 2 Criteria for "No Adherence" to Guidelines for Inherited Thrombophilia

Provoked VTE (Presence of Major Transient Risk Factor Like Surgery, Trauma or Prolonged Immobility)
1st episode of VTE, regardless of provoked v/s unprovoked
Active malignancy
Acute/active VTE
Active VTE treatment/Anticoagulation

Table 3 Relative Indications for Hypercoagulability Work-Up

Recurrent VTE/Prior History of VTE
Family history of hypercoagulable state
VTE at unusual sites
VTE at age <45 years

population.[10] Acquired thrombophilia includes antiphospholipid syndrome (APS), among other autoimmune disorders such as Behcet's disease.[11] The primary instance in which testing for thrombophilia leads to a change in treatment is the presence of APS, for which warfarin is recommended rather than a direct oral anticoagulant (DOAC).[12] The presence of APS is furthermore distinguished from the significant determination of provoked versus unprovoked VTEs.

Provoked VTEs have a much lower recurrence rate, estimated to be less than 1% annually, than unprovoked VTEs, which have a recurrence rate ranging from 5–27%.[13] Sometimes, clinicians will obtain thrombophilia work-ups by citing their possible use as a risk stratification model for determining duration of anticoagulation. However, the literature suggests that other models may be more beneficial in this situation. For instance, in a multicenter prospective cohort study, Rodger et al analyzed patients with unprovoked VTE and their recurrence rates. The study found that men have a higher recurrence rate than women, estimated to be 13.7% annually. Yet, for women, the recurrence rate varied depending on the following characteristics: hyperpigmentation, edema/redness of the lower extremities,
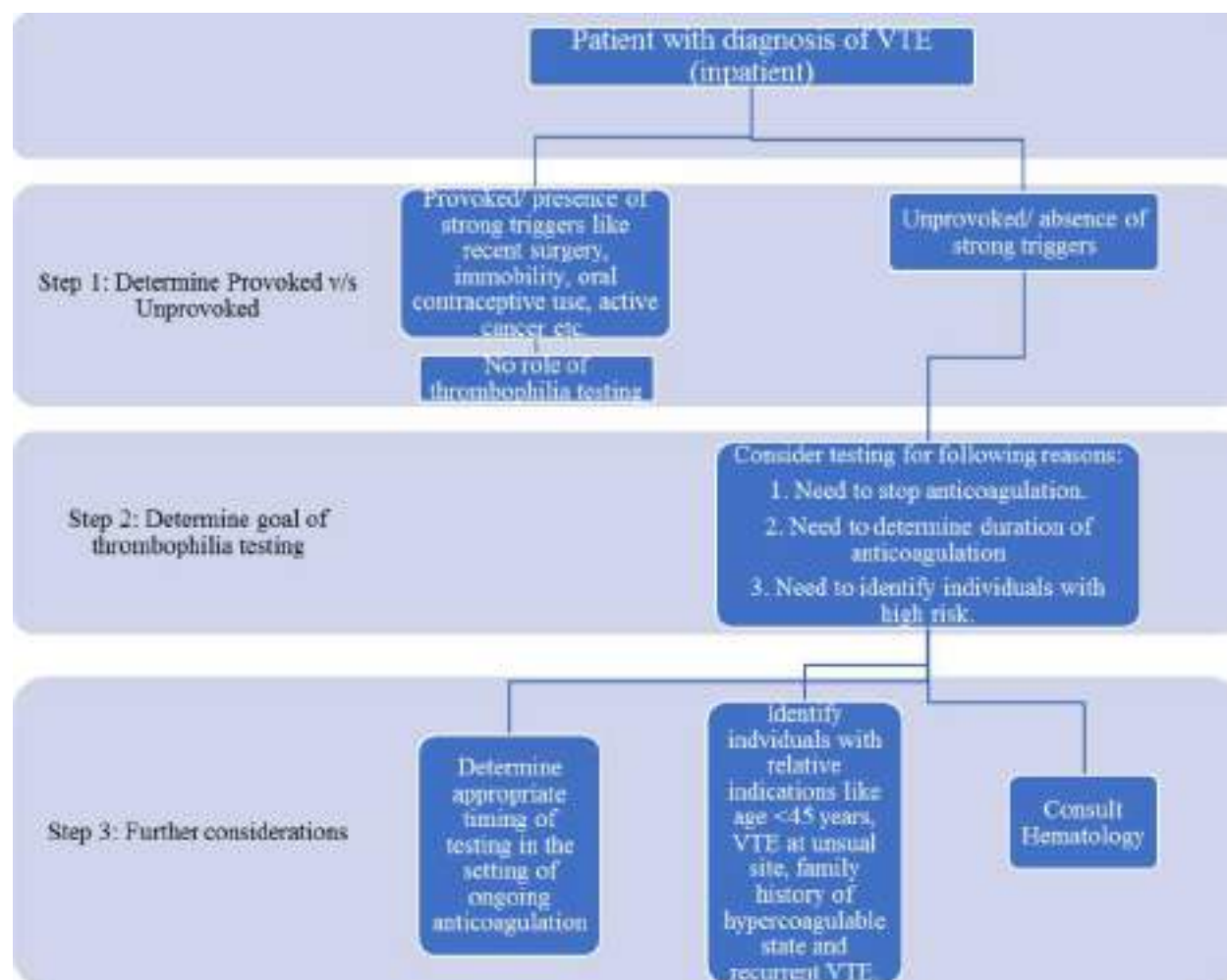
Figure 1 Suggested algorithm for determining the need for a hypercoagulability work-up.
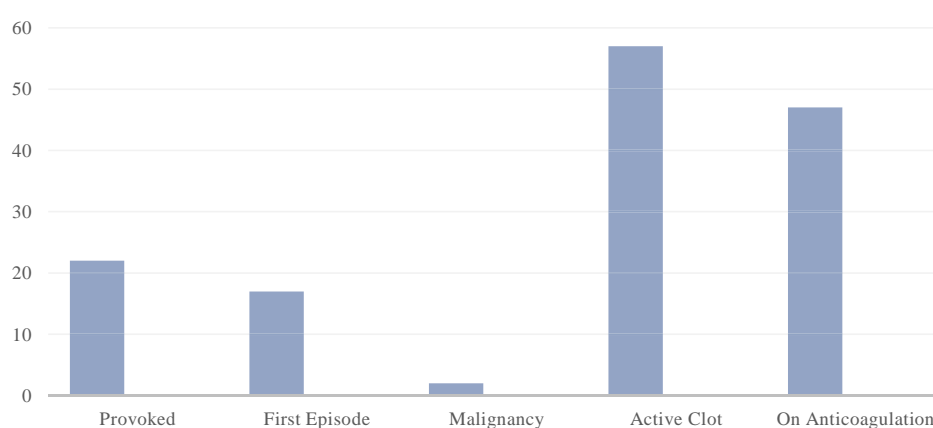
Figure 2 Analysis of why the hypercoagulability work-up was ordered unnecessarily.
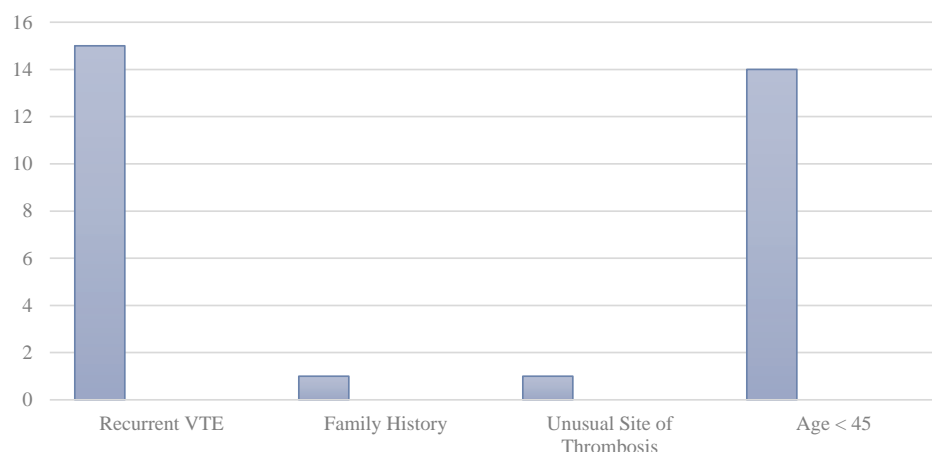
Figure 3 Analysis of relative indications present in patients who received thrombophilia work-up.

D-dimer >250, BMI >30, and age >65 years. Women with 0–1 of these characteristics had a 1.6% recurrence rate, whereas women with 2 or more traits had a recurrence rate of 14.1%, like men.[13] The authors concluded that women with unprovoked VTE and 0–1 risk characteristics as listed above could discontinue anticoagulation after treatment of their acute VTE.

There are differing opinions regarding anticoagulation and thrombophilia, since the literature is unclear if they confer a higher risk of recurrence.[11] Some, such as Stevens et al, recommended not performing thrombophilia testing following an episode of provoked VTE, as a positive test is not enough to determine the duration of anticoagulation and may subject patients to otherwise avoidable bleeding risks.[8] They also reported that thrombophilia testing should not be performed in patients after a single episode of unprovoked VTE because a negative result is not enough to stop anticoagulation in a patient with low bleeding risk and willingness to continue therapy. Females with a personal history of unprovoked VTE or estrogen/

pregnancy-related VTE also carry an indication for prophylaxis and are unlikely to benefit from testing.

Others, such as Connors et al, recommended thrombophilia testing at the completion of anticoagulation therapy for provoked VTE.[9] Conversely, for unprovoked VTE, they recommended testing after the event if cessation of therapy is being considered, and if the test would change management strategy. Current medications and the presence of an active VTE should be accounted for. Several reports in the literature recommend waiting 6 weeks post-acute VTE as this entity may decrease protein C, protein S, and factor VIII, thus interfering with test results.[14] Regarding timing of the testing, Connors et al recommend waiting until 2 weeks after vitamin K antagonists (VKA) are stopped, 2 days after a DOAC is stopped, and 1 day after unfractionated heparin or low-molecular-weight heparin is stopped.[9,10] Heparin products such as UFH and LMWH interfere with antithrombin levels. Pruthi et al noted that a DOAC may result in a false positive APCR. VKAs decrease protein S and protein C levels and

Table 4 Cost Analysis

Type of Test	No. of Times Ordered	Current Price of Test	Total Cost
APC resistance/Factor V Leiden mutation	43	$241	$10,363
Prothrombin gene mutation	13	$237	$3081
Protein S deficiency	37	$180	$6660
Protein C deficiency	36	$231	$8316
Antithrombin deficiency	36	$226	$8136
			$36,556

thus may also yield false positive or indeterminate assay results.[15] Various studies in the past have found that diagnosing a heritable thrombophilia does not typically predict recurrence and that a significant risk of bleeding does not justify extending the duration of anticoagulation.[16–18]

Official guidelines, such as those of the "Choosing Wisely" campaign of the American Society of Hematology (ASH), recommend that patients diagnosed with VTE do not undergo thrombophilia testing in the context of major transient VTE risk factors such as surgery, trauma, or prolonged immobility.[5] The rationale for this recommendation is that testing will not influence the duration or intensity of treatment. ASH thus recommends guidance from an expert in the field regarding testing for thrombophilia.

In 2010, the British Society for Haematology and the British Committee for Standards in Haematology published recommendations for testing heritable thrombophilia.[6] They recommended testing of selected patients, such as those presenting with VTE at an early age (under 40 years of age) or those with a significant family history of thrombosis, defined as more than two family members. Other groups they reported as possibly benefiting from testing include children with purpura fulminans and pregnant women at risk for VTE. Relevant grade I recommendations from the study include the following:[6]

1. Initiation and intensity of anticoagulation therapy following a diagnosis of acute VTE should be the same in patients with or without heritable thrombophilia
2. Indiscriminate testing for heritable thrombophilia in unselected patients presenting with a first episode of VTE is not indicated
3. Decisions regarding the duration of anticoagulation, ie short-term versus lifelong, should be decided based on whether the VTE was provoked, along with other risk factors including a known history of heritable thrombophilia
4. Case findings of asymptomatic relatives with high risk thrombophilia deficiencies, such as protein C, protein S, and antithrombin deficiencies, should be considered in selected patients with a significant family history of thrombosis
5. If a first-degree relative with VTE has not been tested, then suggest that female patients consider

an alternative means of contraception to oral contraceptive pills
6. Testing for heritable thrombophilia is not indicated with arterial thrombosis

As per the National Institute for Health and Clinical Excellence (NICE) guidelines, thrombophilia testing may be considered for individuals when considering discontinuation of anticoagulation following an unprovoked VTE.[7] For unprovoked events, the decision whether to continue or discontinue anticoagulation should be made 3 months post-VTE episode. Some literature does suggest that hereditary thrombophilias have a higher risk of recurrence, estimated to be elevated by a factor ranging from 4 to 30,[19] however, the need for a hypercoagulability work-up should ultimately be determined by a hematologist-oncologist.

The available evidence suggests that testing for inherited thrombophilia is not recommended in most clinical settings. The testing of hypercoagulability requires a planned and thoughtful approach and may require consulting an expert in the field of hematology-oncology. Clinicians should avoid ordering thrombophilia testing in hospitalized patients for the following reasons: (1) many tests are inaccurate in the setting of acute VTE and ongoing anticoagulation; (2) results will not influence management; (3) it is a cost burden to both patients and hospitals; and (4) a positive test result may lead to patient anxiety and an improperly prolonged course of anticoagulation. Based on the results of our study and literature review, we created an algorithm by which physicians can more effectively decide when to perform thrombophilia testing (Figure 1). We aim to conduct a 5-year follow-up retrospective chart review at our institution after educating our clinicians on how to use the devised thrombophilia algorithm. We plan to analyze a similar cohort of patients using similar ICD-10 codes and thereafter analyze adherence to testing guidelines based on our institutional algorithm along with its financial impact. This additional retrospective study will assess improvement in ordering practices at our institution for the betterment of high value patient care.

This study was limited by its nature as a retrospective, single center study with a relatively small sample size. The information was obtained via chart review of patients identified with ICD-10 codes, but it is possible there may have been patients with VTE who were not identified and therefore not included. It is also possible that patients who did not follow-up at our center were later diagnosed with

malignancy and thus the provoked VTE rate may have been higher than reported in our study. Additionally, no hospital-based protocol was followed.

Conclusion

Our study demonstrates that there is a low degree of adherence to currently available thrombophilia testing recommendations in our institutional inpatient setting, which is an ineffective use of resources and can cause an unnecessary financial burden. An increased awareness among clinicians is thus warranted when ordering hypercoagulable work-up to adopt high value care and cost-effective testing. Additionally, there are other proposed algorithms for determining length of anticoagulation, which warrant clinicians' attention. We furthermore propose an algorithm by which we aim to guide and assess the appropriateness of thrombophilia testing at our institution.

Statement of Ethics

Ethical approval to conduct this study was obtained from the Institutional Review Board (IRB). This study was approved and issued under the Federal Wide Assurance with the Office for Human Research Protections. Patient data acquired was anonymized.

Consent for Publication

An informed consent waiver was approved for this study by the IRB.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed on the journal to which the article will be submitted; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

This project was not supported by any grants or funding agencies.

Disclosure

Dr Michael J Levitt is a member of the Speakers' Bureau for Amgen, Takeda, and GlaxoSmithKline, outside the submitted work. The authors declare that there is no other conflict of interest regarding the publication of this paper.

References

1. Naess IA, Christiansen SC, Romundstad P, et al. Incidence and mortality of venous thrombosis: a population-based study. *J Thromb Haemost*. 2007;5(4):692–699. doi:10.1111/j.1538-7836.2007.02450.x PMID: 17367492.
2. Kearon C, Akl EA, Ornelas J, et al. Antithrombotic therapy for VTE disease: CHEST guideline and expert panel Report [published correction appears in Chest. 2016 Oct;150(4):988]. *Chest*. 2016;149(2):315–352. doi:10.1016/j.chest.2015.11.026
3. Pollak AW, McBane RD. Succinct review of the new VTE prevention and management guidelines. *Mayo Clin Proc*. 2014;89(3):394–408. doi:10.1016/j.mayocp.2013.11.015
4. Middeldorp S. Inherited thrombophilia: a double-edged sword. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):1–9. doi:10.1182/asheducation-2016.1.1
5. Hicks LK, Bering H, Carson KR, et al. The ASH Choosing Wisely® campaign: five hematologic tests and treatments to question. *Hematology Am Soc Hematol Educ Program*. 2013;2013(1):9–14. doi:10.1182/asheducation-2013.1.9
6. Baglin T, Gray E, Greaves M, et al. Clinical guidelines for testing for heritable thrombophilia. *Br J Haematol*. 2010;149(2):209–220. doi:10.1111/j.1365-2141.2009.08022.x
7. Howard LS, Hughes RJ. NICE guideline: management of venous thromboembolic diseases and role of thrombophilia testing. *Thorax*. 2013;68(4):391–393. doi:10.1136/thoraxjnl-2012-202376
8. Stevens SM, Woller SC, Bauer KA, et al. Guidance for the evaluation and treatment of hereditary and acquired thrombophilia. *J Thromb Thrombolysis*. 2016;41(1):154–164. doi:10.1007/s11239-015-1316-1
9. Connors JM, Longo DL. Thrombophilia testing and venous thrombosis. *N Engl J Med*. 2017;377(12):1177–1187. doi:10.1056/NEJMra1700365
10. Singh D, Natarajan A, Nand S, Mai HP. Genetics of hypercoagulable and hypocoagulable states. *Neurosurg Clin N Am*. 2018;29(4):493–501. doi:10.1016/j.nec.2018.06.002
11. Rybstein MD, DeSancho MT. Hypercoagulable states and thrombophilias: risks relating to recurrent venous thromboembolism. *Semin Intervent Radiol*. 2018;35(2):99–104. doi:10.1055/s-0038-1642037
12. Garcia D, Erkan D. Diagnosis and management of the antiphospholipid syndrome. *N Engl J Med*. 2018;378(21):2010–2021. doi:10.1056/NEJMra1705454
13. Rodger MA, Kahn SR, Wells PS, et al. Identifying unprovoked thromboembolism patients at low risk for recurrence who can discontinue anticoagulant therapy. *CMAJ*. 2008;179(5):417–426. doi:10.1503/cmaj.080493
14. Heit JA. Thrombophilia: common questions on laboratory assessment and management. *Hematology Am Soc Hematol Educ Program*. 2007;2007(1):127–135. doi:10.1182/asheducation-2007.1.127
15. Pruthi RK. Optimal utilization of thrombophilia testing. *Int J Lab Hematol*. 2017;39(Suppl 1):104–110. doi:10.1111/ijlh.12672
16. Baglin T, Luddington R, Brown K, Baglin C. Incidence of recurrent venous thromboembolism in relation to clinical and thrombophilic risk factors: prospective cohort study. *Lancet*. 2003;362(9383):523–526. doi:10.1016/S0140-6736(03)14111-6
17. Christiansen SC, Cannegieter SC, Koster T, Vandenbroucke JP, Rosendaal FR. Thrombophilia, clinical factors, and recurrent venous thrombotic events. *JAMA*. 2005;293(19):2352–2361. doi:10.1001/jama.293.19.2352
18. Ho WK, Hankey GJ, Quinlan DJ, Eikelboom JW. Risk of recurrent venous thromboembolism in patients with common thrombophilia: a systematic review. *Arch Intern Med*. 2006;166(7):729–736. doi:10.1001/archinte.166.7.729
19. Campello E, Spiezia L, Adamo A, Simioni P. Thrombophilia, risk factors and prevention. *Expert Rev Hematol*. 2019;12(3):147–158. doi:10.1080/17474086.2019.1583555

Anaemia Among Children Who Attended the Children's Teaching Hospital in Karbala, Iraq

Khansaa Albaroodi

Published online: 28 May 2021.

Submit your article to this journal

Article views: 269

View related articles

View Crossmark data

Citing articles: 2 View citing articles

ORIGINAL RESEARCH

Anaemia Among Children Who Attended the Children's Teaching Hospital in Karbala, Iraq

Khansaa Albaroodi [ID]

Pharmacy Department, Al Zahrawi
University College, Karbala, Iraq

Introduction: The World Health Organization (WHO) reported a moderate incidence of anaemia among pregnant and nonpregnant women and among children younger than 59 months in 2011.

Purpose: The aim of this study was to investigate anaemia among children younger than 14 years submitted to haematological exams at the Children's Teaching Hospital in Karbala, Iraq.

Patients and Methods: This was a cross-sectional study carried out in the Children's Teaching Hospital Laboratory Department in Karbala, Iraq, from 1 July 2019 until 1 September 2019.

Results: The prevalence of anaemia among children aged 0–14 years in Karbala was 9.9%. There was no significant relationship between the type of anaemia diagnosed and age or sex. However, there was a significantly positive relationship between the type of anaemia diagnosed and each ferritin level, mean corpuscular volume, and mean corpuscular haemoglobin ($p < 0.0001$). The study participant skull diameter and length in relation to sex were compared to the WHO reference values for child growth standards, and the study values were less than the normal range for children below 5 years of age.

Conclusion: A high prevalence of anaemia among children was reported with its apparent consequence on their health. This study highlights the prevalence of anaemia among children up to 14 years of age in Karbala, and future research is encouraged.

Keywords: anaemia, children with anaemia, prevalence, thalassemia

Introduction

Anaemia is a widespread health problem that affects children in both developing and well-developed countries and has major consequences for human health in addition to social and economic development; anaemia can occur at any age.[1] Anaemia is a health condition in which there is an inadequate number of red blood cells or their oxygen-carrying capacity to meet the body's physiological needs. Iron deficiency is the main reason for anaemia; however, there are other causes of anaemia, such as folate, vitamin B12 and vitamin A deficiencies, chronic inflammation, parasitic infections, and inherited disorders, which can all cause anaemia.[1,2] Haemoglobin or haematocrit levels are the most common tools used to assess the severity of iron deficiency in any population. The health consequences of anaemia can lead to many undesired outcomes, such as poor pregnancy outcomes, impaired physical and cognitive development, increased risk of morbidity in children and reduced work productivity in adults.[1–3]

The WHO determined that approximately two billion people are anaemic, which is defined as haemoglobin (Hb) concentrations that are below recommended thresholds.[2]

Correspondence: Khansaa Albaroodi
Pharmacy Department, Al Zahrawi
University College, Karbala Al-Najaf
Highway, Karbala, 56001, Iraq
Tel +9647727009050
Email khansaa.albaroodi@hotmail.com

Iron deficiency-induced anaemia is an important public health problem in the Eastern Mediterranean region. In the "Assessment of the Food and Nutrition Situation in Iraq" of May-June 2000, approximately 800,000 children under 5 were chronically malnourished. The report also indicated a high prevalence of anaemia in school children; additionally, the numbers of infants with low birth weight and women with severe anaemia have increased.[4] It has been estimated that more than one-third of the population in the region is anaemic. Pregnant women and young children are the most at risk: approximately 50% of pregnant women and 63% of children under 5 have iron deficiency-induced anaemia. Recent data on anaemia rates in preschool children, pregnant women and women of childbearing age show no improvement in the overall situation.[2] The WHO considers anaemia a severe public health problem in Iraq (55.9% of the preschool population in Iraq had Hb <110 g/L);[1] however, these results had no clinical evidence to rely on because of a lack of research and data in this area during that time.[1] In 2011, the WHO reported that Iraq had a moderate incidence of anaemia among pregnant and nonpregnant women and children younger than 59 months.[5] Many studies have investigated the incidence of anaemia among children worldwide.[6–9]

By reviewing studies that focus on anaemia in Iraq, we found a study that investigated the classification of anemia in Iraq.[10] Another study by Taj-Eldin investigated thalassemia in Iraq.[11] A trial to estimate the prevalence of sickle cell disease was conducted among primary school children.[12] More recently, studies were carried out in Iraq to estimate the prevalence of anaemia;[13–16] thalassemia;[17] anaemia and hookworm;[18] anaemia and pregnancy;[19–21] anemia among rheumatoid arthritis patients;[22] and Helicobacter pylori and iron deficiency anaemia.[23] It is obvious that these studies are limited and inadequate to support any updates in the documentation regarding anaemia in Iraq. Therefore, this research was carried out to emphasize the prevalence of anaemia in one of Iraq governorates, Karbala. This study aimed to investigate anaemia among children younger than 14 years submitted to haematological exams at the Children's Teaching Hospital in Karbala, Iraq.

Patients and Methods

This was a cross-sectional study carried out in the Children's Teaching Hospital Laboratory Department in Karbala, Iraq, from 1 July 2019 until 1 September 2019. During July 2019, 1179 outpatients attended the hospital, which was close to the number of outpatients who attended the hospital during August 2019 (1170 outpatients). Among

those patients, we collected data on 234 anaemic patients, and those patients agreed to participate in the study by signing a consent form. As the Children's Teaching Hospital is the only hospital for children in Karbala, we can calculate the prevalence of anaemia among children aged 0–14 years in Karbala, which was 9.9%.

Researchers collected patient information during their visit to the laboratory department by abstracting the patients' haematology results from their records (haemoglobin, haematocrit (HCT), Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and red blood cell (RBC) were measured by using Sysmex; ferritin was measured by using Cobas e 411; and thalassemia was measured by using Bio Rad D10). Researchers measured the patient skull diameter (by using a measuring tape wrapped around the widest possible circumference) and length (also by using a measuring tape) to compare the growth of these children with the standard average. Data were analysed by using the SPSS (version 20) software package (SPSS Inc., Chicago, IL) and are presented as the frequency in tables and figures as appropriate. The Shapiro–Wilk test for normality was applied to the continuous data (skull diameter and length), and these data were normally distributed ($p>0.05$). Pearson's chi-square test and one-way ANOVA were used to find the association and differences, respectively, between variables. The skull diameter and length in relation to the child's sex were compared to the WHO reference value child growth standards.[24]

A p value <0.05 was considered significant.

Results

After collecting information from the participant's records, data were analysed, revealing that the participant mean age was 4.1 ± 3.4 years, and the mean skull diameter and length were 48.6 ± 4.6 cm and 98.6 ± 24.5 cm, respectively. Table 1 summarizes the study participant demographic data; more than two-thirds of the participants were younger than 5 years. Approximately, two-thirds of the study participants were recently diagnosed with anaemia, and more than half of them had mild iron deficiency-induced anaemia, which makes it reasonable that most of them were not using any medications to treat anaemia.

Patients with thalassemia had a separate centre in the hospital, and by reviewing centre records, we found that the proportion of these patients increased over time (Figure 1).

As shown in Table 2, laboratory values were categorized according to the Children's Teaching Hospital Laboratory Department in Karbala, Iraq. The vast majority of the study

Table I Study Participant Demographic Data

	Frequency (%)
Sex	
Male	132 (56.4)
Female	102 (43.6)
Total	234 (100)
Age	
Less than 5 years	166 (70.9)
5–11 years	54 (23.1)
12–14 years	14 (6)
Total	234 (100)
Time of diagnosis	
Recently diagnosed	152 (65)
Less than 6 months	42 (17.9)
More than 6 months	40 (17.1)
Type of anaemia diagnosed	
Mild IDA*	138 (59)
Moderate IDA*	73 (31.2)
Severe IDA*	9 (3.8)
Thalassemia	5 (2.1)
Normochromic normocytic anaemia	9 (3.8)
Total	234 (100)
Using medications for anaemia	
Yes	72 (30.8)
No	162 (69.2)
Total	234 (100)

Abbreviation: *IDA, iron deficiency anaemia.

participants had low levels of ferritin, haemoglobin, MCV, MCH and HCT, which is logical as far as they are anaemic.

Pearson's chi-square test was used to examine whether there was a relationship between the type of anaemia diagnosed and each of the following: age, sex, ferritin, red blood cells (RBCs), MCV, MCH, and HCT. The results showed that there was no significant relationship between the type of anaemia diagnosed and age or sex. On the other hand, the type of anaemia diagnosed had a significantly ($P<0.0001$) positive relationship with ferritin level (chi-square value=234, df=8, R=0.35), MCH (chi-square value=107.5, df=8, R=0.435), and MCV (chi-square value=46.1, df=4, R=0.26). However, the relationship with RBC count was significantly negative (chi-square value=55.3, df=8, R= - 0.29) (Table 3).

Table 4 displays the mean ± SD of the study participant skull diameter and length in relation to sex and compared to WHO standards. It was obvious that the mean participant skull diameter and length were smaller than the normal range for children below 5 years of age.

Discussion

This study revealed that the most common type of anaemia in Karbala was iron deficiency-induced anaemia, and patients did not use any medications to treat it. Microcytic and hypochromic anaemia can be caused by IDA and thalassemia to the same extent;[25,26] some patients had both conditions and received treatment for only one of them, as the other was not diagnosed.[27] Differentiation between thalassemia and IDA can be carried out effectively with serum ferritin, serum iron and HbA_2 level estimation; however, recent data suggest that the red cell distribution width (RDWI) is more advantageous, as all discriminating factors, including RBC count, MCV and RDW, are incorporated.[28] Another study used the mean corpuscular haemoglobin concentration and red blood cell
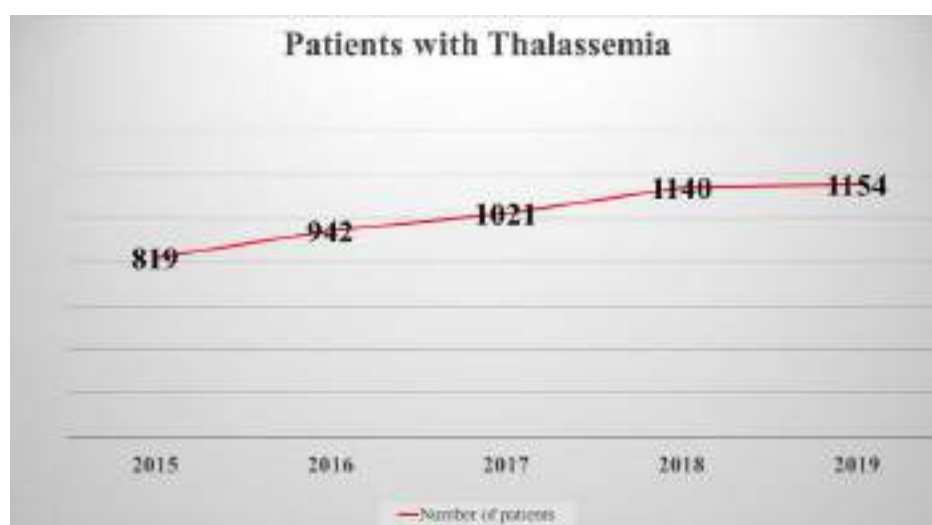
Figure I Patients with thalassemia.

Table 2 Study Participant Haematology Laboratory Values

	Frequency (%)
Ferritin level	
Low <30 (µg/L)	229 (97.9)
Normal (30–400) (µg/L)	4 (1.7)
High >400 (µg/L)	1 (0.4)
Total	234 (100)
RBC level	
Low <4.06 (10^6/uL)	26 (11.1)
Normal (4.06–5.30) (10^6/uL)	206 (88)
High >5.30 (10^6/uL)	2 (0.9)
Total	234 (100)
Haemoglobin	
Low <12 (g/dl)	234 (100)
Normal (12–16) (g/dl)	0
MCV level	
Low <76 fL	207 (88.5)
Normal (76–96) fL	27 (11.5)
Total	234 (100)
MCH level	
Low <26 pg	218 (93.2)
Normal (26–32) pg	15 (6.4)
High >32 pg	1 (0.4)
Total	234 (100)
HCT level	
Low <38%	233 (99.6)
Normal (38–52)%	1 (0.4)
Total	234 (100)

Abbreviations: RBCs, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; HCT, haematocrit.

count to differentiate iron deficiency-induced anaemia from thalassemia.[29] However, a review article showed that despite the excellent performances of the Green and King index (IGK), the Ehsani index, and RBC count, none of them presented sufficient sensitivity and specificity to establish a diagnosis between thalassemia and IDA, and these tests require more time-consuming and costly methods.[30] As mentioned earlier, there is a limitation in studies that investigate the prevalence of anaemia in Iraq; to the best of our knowledge, this is the first study to investigate this aspect among children in Karbala, Iraq. Our findings showed that the prevalence of anaemia among children aged 0–14 years in Karbala was 9.9%, which is considered high. A study by Faraj, Lami et al showed similar findings to ours; a high prevalence of anaemia among children in Wasit, Iraq, indicates a major nutritional and health problem.[15] Another study carried out in Baghdad in 2003 found that anaemia among

adolescents was a health problem of moderate severity.[13] These results show that anaemia in Iraq may be related to malnutrition and poverty. A cross-sectional study was conducted to determine the overall and age- and sex-specific prevalence of anaemia in the city of Mashhad, Iran. The findings show that anaemia is a considerable public health problem in Mashhad, Iran, especially among preschool children, adult women and the elderly.[8]

Our study findings revealed that there was no relation between the type of anaemia and age or sex. In agreement with these results, a cross-sectional study was carried out to determine the prevalence of anaemia among preschool children in a rural village in the Northern State of Sudan and showed that the prevalence of anaemia was not significantly associated with any of the studied demographic and socio-economic factors (sex, economic status of the family, mother's literacy or family size) or the health of the child.[6] Additionally, another study in Istanbul showed no significant relationship between the prevalence of anaemia and student age or sex.[31] On the other hand, a study was carried out to assess the health and nutrition of Syrian refugees affected by the conflict; the results showed that global acute malnutrition is relatively low in the assessed Syrian refugee populations. However, these study results indicate a serious public health problem among women and children, especially in the Zaatari camp.[7] Another study investigated the prevalence of ID and IDA among Syrian children, the effectiveness of oral iron supplements in the management of ID, and the diagnostic effectiveness of conventional iron markers. The results revealed a high prevalence of anaemia, ID, and IDA among a group of apparently healthy Syrian children.[9] However, this aspect can be considered a limitation in our study, as we did not investigate nutritional levels among the participants.

Our study findings showed that there were more male participants than females in the study sample, indicating that anaemia is more common among males than females; agreeing with our findings, Gür, Yıldız et al reported a markedly higher risk for anaemia and Fe deficiency in men, indicating higher Fe requirements in boys than in girls,[31] and another study carried out in Wasit showed similar findings.[15] In addition, many studies have investigated anaemia and pregnancy; a study in Baquba found that anaemia during pregnancy is a major health problem, and iron deficiency anaemia was common.[19] On the other hand, another study among emigrated Yazidis people in the Khanki camp in Duhok, a city in Iraqi Kurdistan, found that iron deficiency-induced anaemia had a high prevalence among residents of this camp, especially among pregnant women. In addition, there was a strong

Table 3 Relationship Between the Type of Anaemia Diagnosed and Haematology Parameters

		Type of Anaemia Diagnosed					Pearson's Chi-Square Value	df	P value	Pearson's R Value
		Mild IDA	Moderate IDA	Severe IDA	Thalassemia	Normochromic Normocytic Anaemia				
Sex	Female	56	34	6	2	4	2.75	4	0.66	−0.055
	Male	82	39	3	3	5				
Age	<5 years	95	51	9	5	6	14.6	8	0.066	−0.006
	5–11 years	38	15	0	0	1				
	12–14 years	5	7	0	0	2				
Ferritin	Low	138	73	9	0	9	234	8	<0.0001	0.35
	Normal	0	0	0	4	0				
	High	0	0	0	1	0				
RBC	Low	6	10	4	2	4	55.3	8	<0.0001	−0.29
	Normal	132	62	5	2	5				
	High	0	1	0	1	0				
MCV	Low	120	73	9	4	1	64.1	4	<0.0001	0.26
	Normal	18	0	0	1	8				
MCH	Low	131	72	9	5	1	107.5	8	<0.0001	0.435
	Normal	7	1	0	0	7				
	High	0	0	0	0	1				
HCT	Low	137	73	9	5	9	0.69	4	0.951	−0.042
	Normal	1	0	0	0	0				

Abbreviations: IDA, iron deficiency anaemia; RBCs, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; HCT, haematocrit.

significant relationship between iron deficiency-induced anaemia and low serum zinc.[20] Furthermore, a cross-sectional study was carried out in Al-Diwaniyah, Iraq, and showed that maternal anaemia affects the anthropometric measurements of newly delivered full-term neonates.[21] Our study showed an increase in the incidence of thalassemia over time, a warning sign to work towards minimizing its risks. Conversely, another study investigated the prevalence of thalassemia minor among college students in Kurdistan (7.7%).[17] In Basrah, a study investigated haemoglobinopathies and glucose-6-phosphate dehydrogenase (G6PD) deficiency and found that there was an association between G6PD deficiency and haemoglobinopathies and thalassemia.[14] Many studies were carried out in Iraq to find any relationship between anaemia and other diseases.[16,18,22,23] However, poor quality of life in Iraq can be the main reason for this high prevalence, as indicated by a recent study that found an association between selected water and sanitation indicators and anaemia.[32] Our study findings show that the mean participant skull diameter and length were under the normal range for children below 5 years of age, which indicates that poor nutrition and anaemia affected child growth in this sample.

Conclusion

In conclusion, participants with IDA took over the essential element among other types of anaemia existing in Karbala Iraq, and their need to be treated is one of the priorities. This study highlights the prevalence of anaemia among children up

Table 4 Mean Participant Skull Diameter and Length in Relation to Age and Sex

Sex	Age Group	N	Skull Length		Skull Diameter	
			Study Participants Mean ±SD (cm)	Reference Length (cm) Less Than 5 Years	Study Participants Mean ±SD (cm)	Reference Skull diameter (cm) Less Than 5 Years
Female	Less than 5 years	63	85.1±15.2	118.4	46.3±4.6	52.6
	5–11 years	32	126.9±12.1		52.6±2.2	
	12–14 years	7	145±9.7		55.6±4.3	
	Total	102	102.3±26.4		48.9±5.2	
Male	Less than 5 years	100	85.8±15.1	118.7	47±3.8	53.5
	5–11 years	28	124.7±9.9		52±1.6	
	12–14 years	4	140.5±8.8		52.5±2.6	
	Total	132	95.7±22.6		48.3±1	

to 14 years of age in Karbala, and future studies should be encouraged to link anaemia with other environmental and nutritional factors that play an important role in its incidence.

Acknowledgments

The author would like to thank Rusul Abdulkareem and Rusul Mohammad for their outstanding efforts as well as Dr. Mohammad Al Mousawy for his support and assistance with his valuable and profound comments.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of Al-Zahrawi University College (ZUC Approval at 01-09-2019). All parents or legal guardians of the children provided informed consent before participating in the study.

Disclosure

The author has no conflicts of interest to declare.

References

1. World Health Organization. Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia; 2008. Available from: https://apps.who.int/iris/bitstream/handle/10665/43894/9789241596657_eng.pdf?sequence=1. Accessed December 01, 2019.
2. World Health Organization, Centers for Disease Control and Prevention. *Assessing the Iron Status of Populations: Including Literature Reviews*. Geneva, Switzerland: World Health Organization, Centers for Disease Control and Prevention; 2004.
3. Hoffbrand AV, Moss PAH. *Hoffbrand's Essential Haematology.* 7th ed. Wiley-Blackwell; 2016.
4. Kreisel W. *Health Situation in Iraq*. Brussels: World Health Organization; 2001.
5. World Health Organization. The global prevalence of anaemia in 2011; 2015; Available from: https://apps.who.int/iris/bitstream/handle/10665/177094/9789241564960_eng.pdf. Accessed December 18, 2019.
6. Hussein MD, Mohamed S. Prevalence of anaemia in preschool children in Karma Albalad area, Northern State, Sudan. *East Mediterr Health J*. 2014;20(1):33–38. doi:10.26719/2014.20.1.33
7. Hossain SMM, Leidman E, Kingori J, Al Harun A, Bilukha OO. Nutritional situation among Syrian refugees hosted in Iraq, Jordan, and Lebanon: cross sectional surveys. *Confl Health*. 2016;10(1):26. doi:10.1186/s13031-016-0093-6
8. Iman EM, Reza HMM, Farhad F, Reza BH, Khosro SS. Anemia as a public health issue in Mashhad, Iran: evidence from the first population-based study. *Acta Med Iran*. 2015;53(3):186–190.
9. Albaroudi IN, Khodder M, Al Saadi T, Turk T, Youssef LA. Prevalence, diagnosis, and management of iron deficiency and iron deficiency anemia among Syrian children in a major outpatient center in Damascus, Syria. *Avicenna J Med*. 2018;8(3):92–103. doi:10.4103/ajm.AJM_169_17
10. Jalili MA, Demarchi M. Classification of anemia in Iraq. *J Fac Med Baghdad*. 1952;16(3/4):71–100.
11. Taj-Eldin S, Al-Rabii H, Jawad J, Fakhri O. Thalassaemia in Iraq. *Ann Trop Med Parasitol*. 1968;62(2):147–153. doi:10.1080/00034983.1968.11686542
12. Alkasab FM, Al-Alusi FA, Adnani MS, Alkafajei AM, Al-Shakerchi NH, Noori SF. The prevalence of sickle cell disease in Abu-AL-Khasib district of southern Iraq. *J Trop Med Hyg*. 1981;84(2):77–80.
13. Al-Sharbatti SS, Al-Ward NJ, Al-Timimi DJ. Anemia among adolescents. *Saudi Med J*. 2003;24(2):189–194.
14. Hassan MK, Taha JY, Al-Naama LM, Widad NM, Jasim SN. Frequency of ß-thalassaemia, haemoglobin S and glucose-6- phosphate dehydrogenase deficiency in Basra governorate, Iraq. *East Mediterr Health J*. 2003;9.
15. Faraj SA, Lami JKA, Jabar HNA, Hassan UMAA. Epidemiological character of anemia among Wasit Province children, Iraq. *Adv Med Dent Res*. 2016;1(1):11–14.

16. Aboud SF, Al-Tu'ma FJ, Alkarem LA. Correlation of vitamin D deficiency and hyperparathyroidism with anemic in Female Iraqi. *Iraqi Natl J Chem*. 2017;17(1):34–43.

17. Alnakshabandi AA, Muhammad HA. Prevalence of B-thalassemia carriers among a cohort of university students in Hawler Province of Iraqi Kurdistan. *Iraqi J Pharm Sci*. 2009;18(2).

18. Kamil ZH, Rashid KH, Al-Awad AS. The prevalence of anemia and hookworm ancylostoma duodenale infection in Mishamish Village, Al-Hashimya District, Babylon Province, Iraq. *Med J Babylon*. 2014;11(4).

19. Salman ST, Hussein AA. Determine morphological types of anemia in anemic pregnant women in albatool teaching hospital for maternity and children in Baqubah City, Iraq. *Inte J Adv Res*. 2016;4 (5):934–939. doi:10.21474/IJAR01/448

20. Ahmad HM. Anemia and iron deficiency anemia prevalence with serum zinc assessment among emigrated yazidis people in khanki camp in Duhok city, Kurdistan Region, Iraq.. *J Dent Med Sci*. 2016;15(10):105–110.

21. Al-Hajjiah NN, Almkhadree MA. The effect of maternal anemia on the anthropometric measurements in fullterm neonates. *Asian J Pharm Clin Res*. 2018;11(4):422–424. doi:10.22159/ajpcr.2018. v11i4.25579

22. Getta HA, Khoshnaw N, Alwan AF, Mirza RR. Types of anaemia and its correlation with disease activity in patients with rheumatoid arthritis among kurdish population of Iraq. *Iraqi J Hematol*. 2016;5 (1).

23. Rashid NG, Ahmed FF, Abdulla BK, Yassin AK, Al Ani MH. The relation between helicobacter pylori seropositivity and iron deficiency anaemia in Iraq-Kurdistan-Sulaimani City. *J Sulaimani Coll Med*. 2018;8(1):17–21. doi:10.17656/jsmc.10146

24. World Health Organization. *WHOChild Growth Standards Growth Velocity Based on Weight, Length and Head Circumference. Methods and Development*. Geneva: World Health Organization; 2009:262.

25. Lin CK, Chen LP, Chang HL, Sung YC. Underestimation of the coexistence of iron deficiencies and thalassemia minors: a single institution experience in Taiwan. *Kaohsiung J Med Sci*. 2014;30 (8):409–414. doi:10.1016/j.kjms.2014.03.010

26. Akers AS, Howard D. Distinguishing iron deficiency anaemia from thalassemia trait in clinical obstetric practice. *J Pregnancy Reprod*. 2017.

27. Verma S, Gupta R, Kudesia M, Mathur A, Krishan G, Singh S. Coexisting iron deficiency anemia and beta thalassemia trait: effect of iron therapy on red cell parameters and hemoglobin subtypes. *ISRN Hematol*. 2014;2014:293216. doi:10.1155/2014/293216

28. Jameel T, Baig M, Ahmed I, Hussain MB, Alkhamaly MBD. Differentiation of beta thalassemia trait from iron deficiency anemia by hematological indices. *Pak J Med Sci*. 2017;33(3):665–669. doi:10.12669/pjms.333.12098

29. Matos JF, Dusse LMS, Borges KBG, Castro RLV, Coura-Vital W, Carvalho MG. A new index to discriminate between iron deficiency anemia and thalassemia trait. *Rev Bras Hematol Hemoter*. 2016;38:214–219. doi:10.1016/j.bjhh.2016.05.011

30. Reis FMP, Andrade RR, Rodrigues CF, Barbosa FT. Discriminant indexes to simplify the differential diagnosis between iron deficiency anemia and thalassemia minor in individuals with microcytic anemia. *Rev Assoc Med Bras*. 2020;66:1277–1282. doi:10.1590/1806-9282.66.9.1277

31. Gür E, Yıldız I, Celkan T, et al. Prevalence of anemia and the risk factors among school children in Istanbul. *J Trop Pediatr*. 2005;51 (6):346–350. doi:10.1093/tropej/fmi032

32. Kothari MT, Coile A, Huestis A, Pullum T, Garrett D, Engmann C. Exploring associations between water, sanitation, and anemia through 47 nationally representative demographic and health surveys. *Ann N Y Acad Sci*. 2019;1450(1):249–267. doi:10.1111/nyas.14109

Chlamydia pneumoniae-Induced IFN-Gamma Responses in Peripheral Blood Mononuclear Cells Increase Numbers of CD4+ but Not CD8+ T Effector Memory Cells

Tamar A Smith-Norowitz, Sarah Shidid, Yitzchok M Norowitz & Stephan Kohlhoff

Open Access Full Text Article

ORIGINAL RESEARCH

Chlamydia pneumoniae-Induced IFN-Gamma Responses in Peripheral Blood Mononuclear Cells Increase Numbers of CD4+ but Not CD8+ T Effector Memory Cells

Tamar A Smith-Norowitz [iD]
Sarah Shidid
Yitzchok M Norowitz
Stephan Kohlhoff

Department of Pediatrics, Division of Infectious Diseases, State University of New York Downstate Medical Center, Brooklyn, NY, 11203, USA

Background: *Chlamydia pneumoniae* causes respiratory infection in adults and children. Previous studies in our laboratory identified significantly higher in vitro T lymphocyte responses to *C. pneumoniae* in children with asthma compared to healthy controls which may indicate the presence of T effector memory (TEM) lymphocytes.

Aim: In the present study, healthy subjects were screened for the presence of TEM cells and their cytokines. CCR7 negative effector TEMs may indicate persistent infection with *C. pneumoniae*.

Methods: Peripheral blood mononuclear cells (PBMC) (1×10^6/mL) from adult non-asthmatic subjects were infected for 1h ± *C. pneumoniae* TW-183 at a multiplicity of infection (MOI) = 0.1 and cultured (48 hrs). Distributions of lymphocytes (CD4+, CD8+) and TEM cells (CD4+CCR7+CD45RA+CD154+, CD8+CCR7+CD45RA+CD154+) were determined. Levels of intracellular interleukin (IL)-2, IL-4, and interferon (IFN)-gamma were measured (flow microfluorimetry); IFN-gamma was measured in supernatants (ELISA).

Results: *C. pneumoniae* infection led to a decrease in numbers of CD8+ TEM and CD8+CD154+ cells; CD4+TEM and CD4+CD154+ cells did not change. Numbers of TEM cells (CD4+IL-2+, CD8+ IL-2+) also decreased. However, number of TEM cells (CD4+IL4-+, CD8+ IL-4+) and (CD4+ IFN-gamma+, CD8+IFN-gamma+) did not change. When stratified according to IFN-gamma+ status, numbers of CD4+ IL-2+ and CD4+IL-4+ TEMs increased; CD8+IL-2+ and CD8+ IL-4+ TEMs decreased.

Conclusion: *C. pneumoniae*-induced PBMC IFN-gamma+ responses increased numbers of CD4+ IL-2+ and CD4+IL-4+ TEM cells, while CD8+IL-2+ and CD8+IL-4+ TEMs decreased. Production of IFN-gamma by *C. pneumoniae* infected PBMC should be further studied as a biomarker of persistent infection in humans.

Keywords: *C. pneumoniae*, T effector memory lymphocytes, interleukin-2

Correspondence: Tamar A Smith-Norowitz
SUNY Downstate Medical Center, Department of Pediatrics, Box 49, 450 Clarkson Ave., Brooklyn, NY, 11203, USA
Tel +1 718 270-1295
Fax +1 718 270-3289
Email tamar.smith-norowitz@downstate.edu

Introduction

Chlamydia pneumoniae (*C. pneumoniae*) is an intracellular bacterium that infects humans; it causes respiratory infections[1,2] in asthmatic and non-asthmatic subjects.[2–5] *C. pneumoniae* activation of immune cells (eg, monocytes/macrophages, epithelial cells) in vitro produce cytokines that may contribute to the pathology observed in asthma; protective immune responses against respiratory infection are also reduced.[2] However, in children with chronic respiratory disease

(CRD), *C. pneumoniae* infection triggers the production of pathogen-specific Immunoglobulin (Ig) E and contributes to inflammatory responses.[6] *C. pneumoniae* infection treatment includes tetracyclines or erythromycin; tetracyclines have been reported to have beneficial effects in patients with asthma.[2,7,8]

Previous studies in our laboratory demonstrated that *C. pneumoniae* was present in 11% of wheezing children.[3] Other studies in our laboratory found an association of specific IgE responses to *C. pneumoniae* in 86% of *C. pneumoniae* positive children with asthma compared with 9% of culture positive patients with pneumonia who did not have asthma.[9,10] Prior literature reported that *C. pneumoniae* could enter a persistent state in vitro spontaneously, after treatment with cytokines such as interferon (IFN)-gamma, antibiotics or nutrient restriction.[11–13] Following acute respiratory infection, long-term culture positivity in nasopharyngeal (NP) specimens can occur for up to 6 months,[3,14] and has been associated with continuing clinical symptoms of asthma.[3]

Diagnostic testing for *C. pneumoniae* using NP specimens may underestimate persistent infection; the presumed focus of infection in asthma is the lower airway. The chlamydial load may also be low. Other studies reported that after acute infection with *C. pneumoniae*, positive lymphocyte proliferation and secretion of IFN-gamma were detected in vitro.[15] In that study, lymphocyte responses to *C. pneumoniae* declined following the acute phase of infection.[15] Persistence of IFN-gamma responses was observed up to 16 weeks following acute infection in a subset of *C. pneumoniae* positive subjects.[15]

Earlier studies in our laboratory demonstrated important immunological differences between asthmatics and non-asthmatic subjects in regard to the host response to *C. pneumoniae*.[16] In peripheral blood mononuclear cells (PBMC) obtained from adult asthmatics, *C. pneumoniae* stimulation induced predominant production of T helper (Th) 2 responses and IgE.[16] These responses may indicate chronic inflammation, which can be related to persistent infection. In addition, we found that *C. pneumoniae*-induced IFN-γ production in vitro was more prevalent in pediatric patients with asthma compared with non-asthma; this is consistent with the presence of circulating T memory lymphocytes.[17] Since cell-mediated immunity (CMI) of the Th1 type is required for optimal control of chlamydial infections in vivo one might expect persistent infection in individuals with decreased Th1 and/or increased Th2 type responses.[18,19] However, the observation of a positive IFN-gamma PBMC response in the presence of *C. pneumoniae*-specific T effector memory cells (TEMs) would indicate persistent *C. pneumoniae* infection.

Prior literature has reported that circulating memory cells responsive to chlamydial antigens are found in humans;[19,20] they are specialized to handle infection arising within peripheral organs due to their cytotoxicity and ability to localize to tissues. TEMs may play a role in immunoprotection and immunopathology following recognition of *C. pneumoniae*-infected cells. Effector memory T lymphocytes have been demonstrated in other human latent infections (eg, tuberculosis).[21] Thus, the presence of TEMs may indicate either past or persistent infection depending on their characteristics.

The aim of the current study was to expand the existing knowledge of effector memory T lymphocytes through identification of *C. pneumoniae* specific CD4+ and CD8+ TEMs and their cytokines (IL-4, IL-2, IFN-gamma) in *C. pneumoniae*-stimulated PBMC in non-asthmatic subjects without respiratory illness. When stratified according to IFN-gamma+ status, numbers of CD4+ IL-2+ and CD4+IL-4+ TEMs increased while CD8+IL-2+ and CD8+ IL-4+ TEMs decreased.

Materials and Methods
Study Population

The study population consisted of patients (≥18 years old) who visited the outpatient clinic of the Department of Medicine at SUNY Downstate Medical Center (Brooklyn, NY) between June and August 2019. The inclusion criteria were: (1) non-asthmatic adult without clinically defined persistent asthma symptoms,[22] (2) low serum IgE levels (<100 IU/mL) and (3) no other chronic or severe respiratory disease (eg, tuberculosis, emphysema, bronchiolitis or diffuse lung disease). Exclusion criteria included subjects with asthma, severe respiratory disease or human immunodeficiency virus (HIV)-1. All subjects had an NP swab tested for *C. pneumoniae* (determined by PCR), and peripheral blood (10mL) was collected. All demographic and clinical data were obtained from electronic medical records (EMRs) and reviewed at the time of enrollment. The study was approved by the SUNY Downstate Medical Center Institutional Review Board (Brooklyn, NY) and human studies adhered to the World Medical Association Declaration of Helsinki. Written informed consent for participation and publication was obtained from all participants.

Immunoglobulin Determination: Total Serum IgE

Total serum IgE levels were determined in serum using the UniCap Total IgE fluoroenzyme immunoassay (Pharmacia and Upjohn Diagnostics, Freiburg, Germany) as previously described.[17,23] Tests were performed in the Clinical Diagnostic Laboratory at SUNY Downstate Medical Center (Brooklyn, NY).

Preparation of *C. pneumoniae*

C. pneumoniae TW-183 (ATCC; Manassas, VA) was propagated in HEp-2 cells as previously described.[24,25]

Cell Cultures

PBMC were separated from blood on a Ficoll-Paque (GE Healthcare, Sweden) gradient (density 1.077) and put into cell culture as previously described,[23] at 37°C in cRPMI medium in a humidified 5% CO_2 atmosphere for 2 days. Cell viability was determined at 0 and 48 hrs (>98% and 95%, respectively), in the absence of infection with *C. pneumoniae*.

In vitro Infection with *C. pneumoniae*

Following a 2 hr incubation to allow adherence, cell cultures were infected with *C. pneumoniae* (by adding purified EB for 1hr), or mock-infected (MI) for 48 hrs (IFN-gamma) at 37°C in cRPMI in a humidified 5% CO_2 atmosphere, as previously described.[23] The cytokine assay (IFN-gamma) was run using supernatants collected from above cultures. The multiplicity of infection (MOI; 0.1) and time points (48h p.i. for cytokines)[26] used for analysis were selected based on kinetic and dose response studies (using MOI of 0.01–10) for optimization of the assay. Two types of controls were used in infection experiments: identical volumes of heat-inactivated purified *C. pneumoniae*[26] and identical volumes of HEp-2 cell cultures not containing any bacteria processed the same way as the purified *C. pneumoniae*[25] based on dose-response experiments.

Cytokine (IFN-Gamma) Determination: ELISA

For the in vitro quantitative determination of human cytokine content in cell culture supernatants, a solid-phase sandwich ELISA assay was performed using the Human IFN-gamma ELISA kit (Abcam, Cambridge, MA), according to the manufacturer's recommended procedure. Cell culture supernatants were collected at 48 hr p.i. by centrifugation, and samples were stored at −80° until analysis. Sensitivity for cytokine assay was <15.0 pg/mL.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) of Bacteria in Swabs and Cultures

Extractions of bacterial DNA from NP swab specimens[27] and PBMC were performed using a QIAAmp DNA Mini-Kit (Qiagen Inc., Valencia, CA), according to manufacturer's recommendations, as previously described.[27,28] Specimens were tested for the presence and quantification of *C. pneumoniae* and *M. pneumoniae* DNA, using TAQMan technology-based qPCR (Light Cycler 2.0 platform; software version 4.0, Roche Diagnostics Corp, Indianapolis, IN), as previously described.[27,28] A specimen from either nostril that was positive for *C. pneumoniae* defined a positive result.

Determination of Cell Surface Markers

Single- and dual-color immunophenotyping of lymphocytes was performed at 48hr as described in our previous studies[16,29] with modifications for intracellular staining. Gating strategies included forward and side scatter, single parameter histograms, two-parameter density plots, and back-gating to confirm gating strategies. Antibodies (Abs) used included mouse anti-human monoclonal antibodies (mAbs) (of the IgG1 isotype): fluorescein isothiocyanate (FITC)-conjugated CD3, CD14, CD45RO, CD45RA, CD154, CCR7, IL-2, IL-4, IFN-gamma; phycoerythrin (PE)-conjugated CD8 and CD4-perCP; and the following Simultest (FITC/PE-conjugated) reagents: CD4/CD8, CD3/CD19. All mAbs were purchased from BD Biosciences (San Diego, CA) or R&D Systems (Minneapolis, MN), and titrated to obtain maximum staining efficiency according to manufacturer's recommendation. Flow cytometric analysis was performed on a Coulter Epics XL/MCL Flow Cytometer using System II software (Coulter, Miami, FL) and CytoComp (Coulter). The total numbers of lymphocytes were calculated from the white blood cell (WBC) count (total lymphocytes/mm^3 or percentage total lymphocytes).

Determination of *C. pneumoniae* Activated TEMs

TEMs are commonly found in the presence of persistent viral infections and can be characterized by expression of either CD4, CD8, CD45RO, CD45RA, CCR7, CD154, IFN-gamma, IL-2, IL-4. Based on recent experiments,[30]

we established a cut-off (50% increase in TEMs 48 hrs post-stimulation).

Statistical Analysis

Data are expressed as means ± SD unless otherwise indicated. All statistical analyses were performed using Windows v.12.0 software (SPSS Inc., Chicago, IL).

Results
Study Population

The population studied included blood and plasma samples from five non-asthmatic adult patients: 2 males (ages 24, 51) and 3 females (ages 51, 59, 65). Total serum IgE levels were low (<100 IU/mL) in all subjects. Subjects tested negative for *C. pneumoniae*.

Phenotypic Determination of *C. pneumoniae*-Activated TEMs .

TEMs are cells that are either CD4+ or CD8+ and are CCR7-, CD45 RO+ and CD154+. These T cells are activated by known antigen (ie, *C. pneumoniae*). We then characterized the cytokine profile of the *C. pneumoniae*-activated CD4+154+ and CD8+CD154+ T cells by analyzing distributions of IL-2+, IL-4+ and IFN-gamma+ cells.

Total CD4+ and CD8+ TEMs and CD154 + TEMs

C. pneumoniae infection (48 hr) led to a decrease in average numbers of CD8+ TEM and CD8+CD154+ cells (50%, 33%, respectively) (Figure 1A and B); numbers of CD4+TEMs and CD4+CD154+ cells did not change (at

1:10 or 1:100) (Figure 1A and B). However, numbers of CD4+CD154+ TEMs were 4-fold higher than numbers of CD8+CD154+ TEMs (Figure 1B).

CD4+ IL-2+, CD8+ IL-2+ TEMs

C. pneumoniae infection (48 hr) led to a decrease in numbers of CD4+IL-2+ and CD8+ IL-2+ TEMs (~27%, 50%, respectively) (Figure 2A)

CD4+IL-4+, CD8+IL-4+ TEMs and CD4 +IFN-Gamma+, CD8+IFN-Gamma+ TEMs

C. pneumoniae infection (48 hr) did not change numbers of CD4+IL-4+ or CD8+IL-4+ TEMs. Numbers of CD4+ IFN-gamma+ and CD8+IFN-gamma+ were low and did not change (at 1:10 or 1:100) (Figure 2B and C).

C. pneumoniae-Induced IFN Gamma Levels
IFN-Gamma Levels in Supernatants

C. pneumoniae induced IFN-gamma responses were measured on days 3, 5 and 7 post-infection. PBMC were infected using 2 different concentrations of *C. pneumoniae* (1:10, 1:100). At each time point, IFN-gamma levels were higher at the 1:10 compared with 1:100 concentration. IFN-gamma levels ranged from 204 pg/mL ± 85 to 541 pg/mL± 467, with levels peaking on day 5 post-infection (Figure 3). IFN-gamma levels (1:10) were >50% higher on day 5, than on day 3.
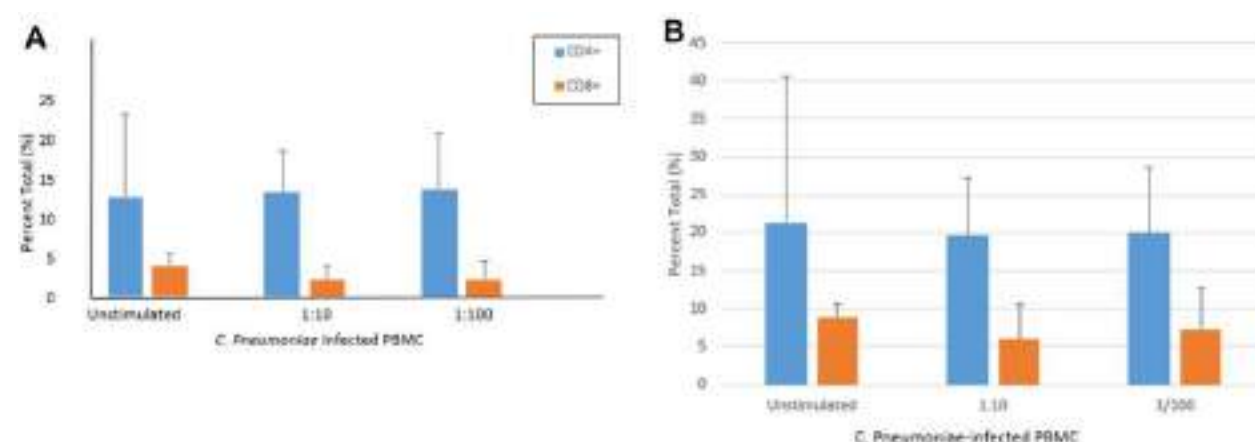
Figure 1 Phenotypic determination of *C. pneumoniae*-activated TEMs. (**A**) Total CD4+ and CD8+ TEMs. (**B**) CD4+CD154+ and CD8+CD4+ TEMs. See materials and methods. Blue box: CD4+ T cells. Red box: CD8+ T cells.

388

Journal of Blood Medicine 2021:12
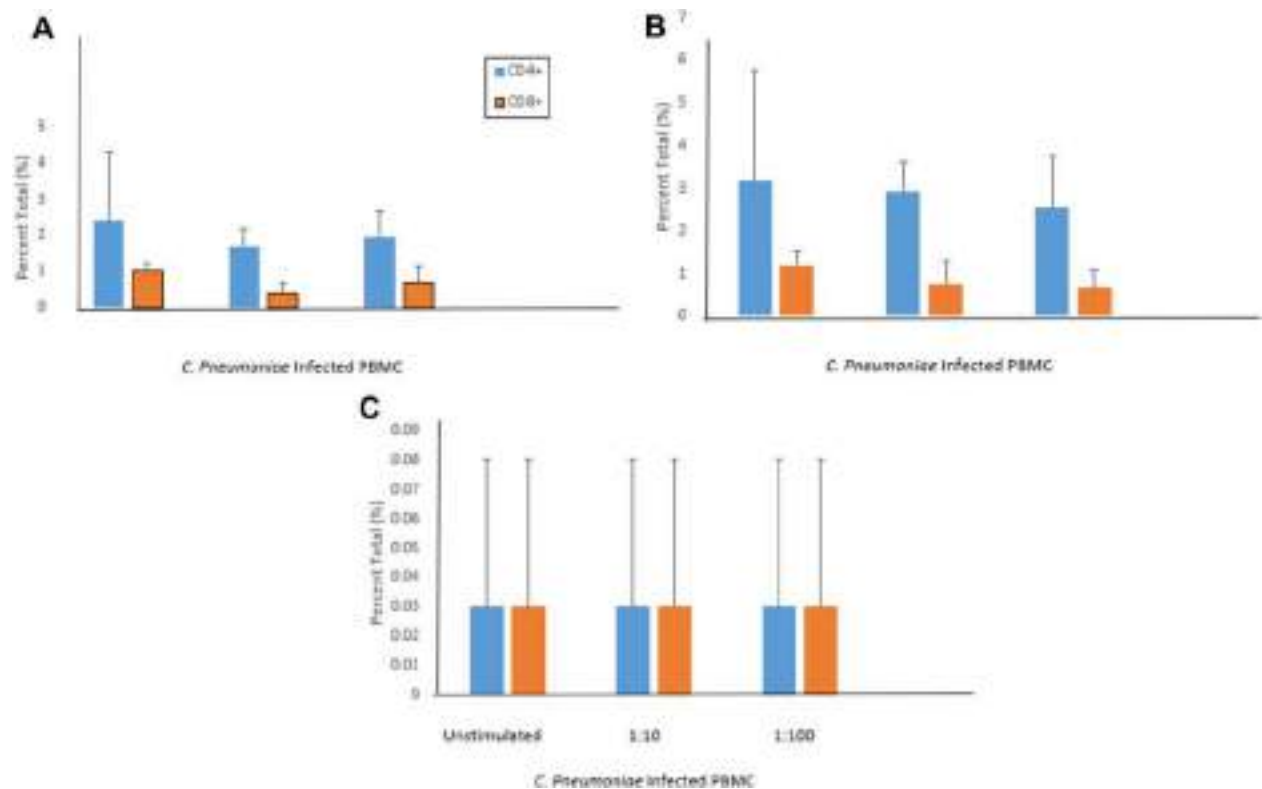
Figure 2 Phenotypic determination of *C. pneumoniae*-activated TEMs. (**A**) CD4+ IL-2+, CD8+ IL-2+ TEMs. (**B**) CD4+IL-4+, CD8+IL-4+ TEMs. (**C**) CD4+IFN-γ+, CD8+IFN-γ+ TEMs. See materials and methods. Blue box: CD4+ T cells. Red box: CD8+ T cells.
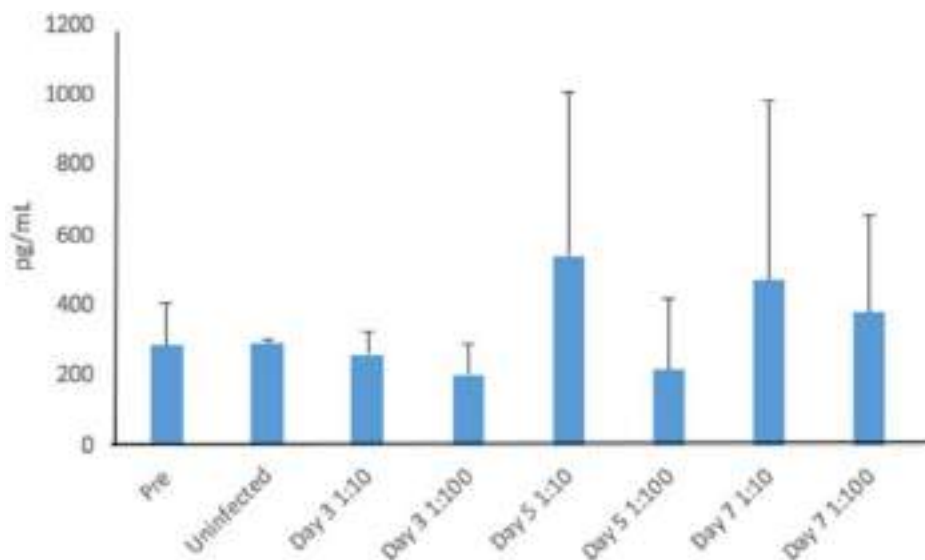
Figure 3 *C. pneumoniae*-induced IFN–γ levels in cell culture supernatants. *C. pneumoniae* induced IFN-γ responses were measured on days 3, 5 and 7 post-infection (ELISA) (N=4). See materials and methods. Data are reported as pg/mL.

IFN-Gamma Levels Stratified According to CD4+ and CD8+ TEMs

In each subject, IFN-gamma responses were then stratified according to CD4+CD154+ and CD8+CD154+ TEMs. Numbers of CD4+CD154+ and CD8+CD154+ TEMs were elevated in 25% of subjects (Figure 4A). In subject with high IFN-gamma response (represented in Figure 4A, subject 1), numbers of CD4+ CD154+ and CD8+CD154+ TEMs levels
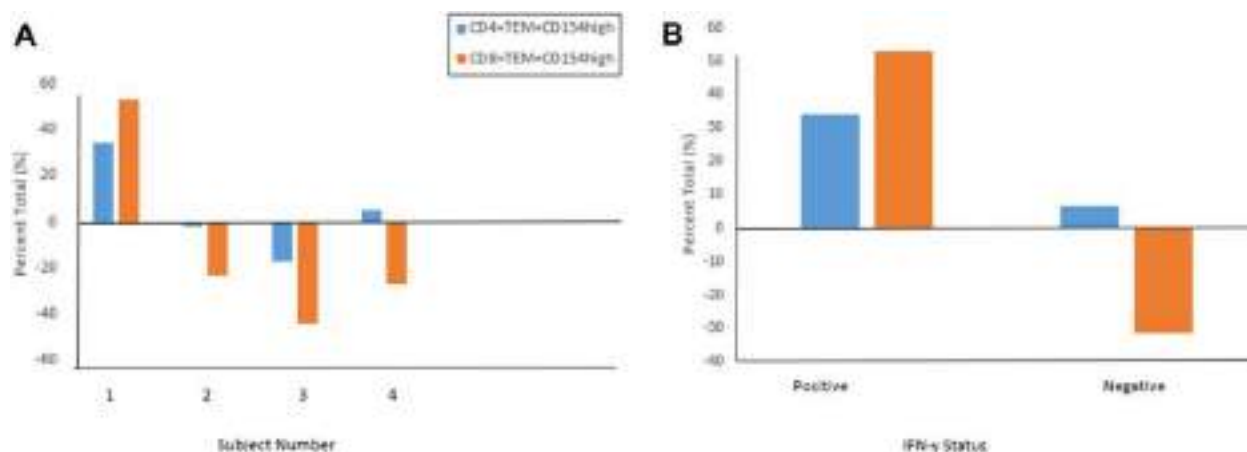
Figure 4 *C. pneumoniae*-induced IFN–γ levels stratified according to CD4+ and CD8+ TEMs. (**A**) Numbers of CD4+154+ and CD8+CD154+ TEMs (N=4), (**B**) numbers of CD4+154+ and CD8+CD154+ TEMs in representative IFN-γ positive and IFN-γ negative subject. See materials and methods. Blue box: CD4+ T cells. Red box: CD8+ T cells.

were higher than in subject with low IFN-gamma response (represented in Figure 4A, subject 4) (Figure 4B).

CD4+ and CD8+ TEMS Stratified According to IFN-Gamma Levels in Subjects
IFN-Gamma Positive
Subject who was IFN-gamma positive had an increase in CD4+IL-2+ and CD4+IL-4+ TEMs (Figure 5A), while numbers of CD8+IL-2+ and CD8+IL-4+ TEMs decreased from baseline (Figure 5B). Numbers of CD4+IFN-gamma+ and CD8+IFN-gamma+ TEMs were low and did not change.

IFN-Gamma Negative
Subjects who were IFN-gamma negative had a decrease in total number of TEMs and CD4$^+$IL-2+, CD4+IL-4+ TEMs, CD8+IL-2+ and CD8+IL4+ TEMs from baseline

(Figure 6A and B). Numbers of CD4+IFN-gamma+ and CD8+IFN-gamma+ TEMs were low and did not change.

Discussion

In this study *C. pneumoniae*-induced PBMC IFN-gamma + responses increased numbers of CD4+ IL-2+ and CD4 +IL-4+ TEMs, while numbers of CD8+IL-2+ and CD8 +IL-4+ TEMs decreased. The observation of a combined IFN-gamma and CD4+ T cell memory response in healthy individuals may be associated with specific responses against *C. pneumoniae* antigens associated with persistent infection. Little is known about the role of *C. pneumoniae*-specific TEMs in humans; additional data are needed to provide an understanding of the role of *C. pneumoniae*-specific TEMs and the cytokines
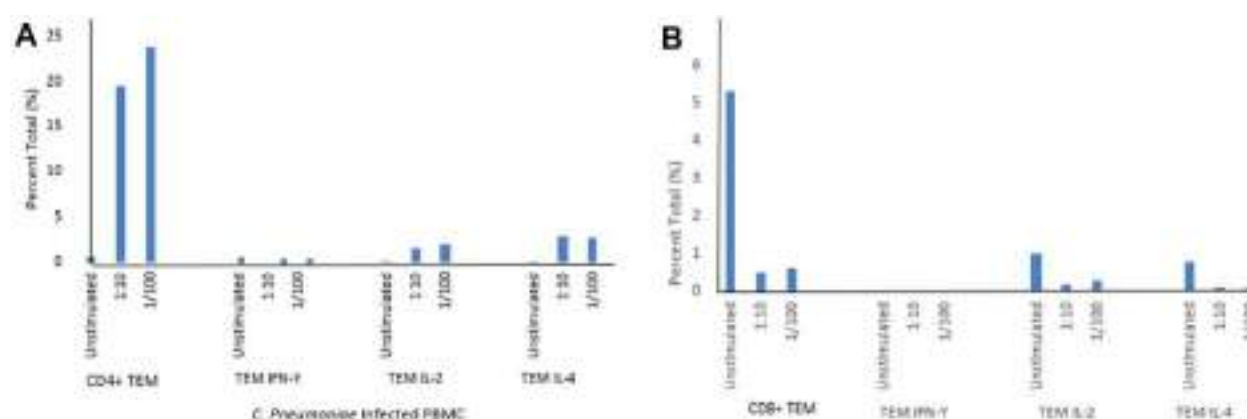
Figure 5 CD4+ and CD8+ TEMs stratified according to IFN-γ levels in IFN-γ positive subject. (**A**) CD4+, CD4+ IFN-γ+, CD4+IL-2+ and CD4+IL-4+ TEMs. (**B**) CD8+, CD8+ IFN-γ+, CD8+IL-2+ and CD8+ IL-4+ TEMs. See materials and methods.
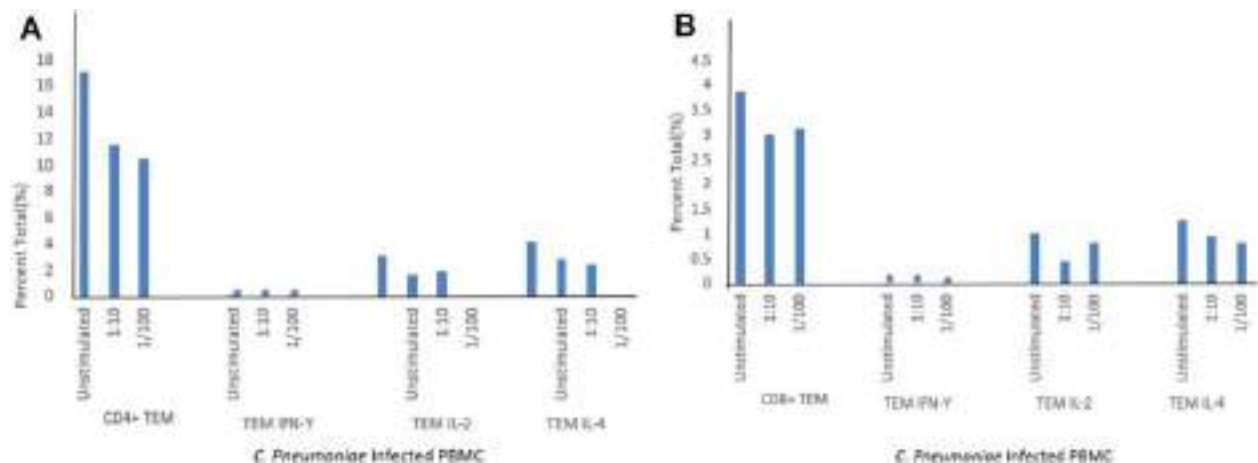
Figure 6 CD4+ and CD8+ TEMS stratified according to IFN-γ levels in IFN-γ negative subjects. (**A**) CD4+, CD4+ IFN-γ +, CD4+IL-2+ and CD4+IL-4+ TEMs. (**B**) CD8+, CD8+IFN-γ +, CD8+IL-2+ and CD8+IL-4+ TEMs. See materials and methods.

required for identification of past or persistent infection in mammalian cells.

Humoral and CMI contribute to protection against viral infections.[31] The anti-viral effect of CMI is through virus-specific CD4+ and CD8+ T cells by cytokine production and killing virus-infected cells.[32] Virus-specific CD4+ T-cell responses against chronic viruses are characterized by secretion of interferon-gamma (Th1 type response).[32] Virus-specific CD8+ T-cell responses produce many factors, including IFN-gamma, tumor necrosis factor (TNF)-α, and macrophage inflammatory protein-1β (MIP-1β).[32] During primary *C. pneumoniae* infection and reinfection, CD4+ and CD8+ T cells play a protective role in mouse models of infection.[33–35] This observation may suggest development of *C. pneumoniae*-specific memory T cells.[20] Virus-specific memory T cells have also been reported in other persistent infections (cytomegalovirus, varicella-zoster virus, Epstein-Barr virus and HIV).[32]

In the current study, *C. pneumoniae* infection led to a decrease in total numbers of CD8+TEM and CD8+CD154+ cells, but numbers of CD4+TEM and CD4+CD154+ cells did not change. In addition, numbers of TEM cells (CD4+IL-2+, CD8+ IL-2+) decreased. However, numbers of TEM cells (CD4+IL-4+, CD8+IL-4+) did not change in our stimulation system. This may indicate the low prevalence of *C. pneumoniae*-specific circulating memory cells in our subjects. Another possible explanation may be that *C. pneumoniae* inhibits *C. pneumoniae*-specific CD8+ memory T cells; these cells might be altered during infection and become functionally defective. However, IFN-gamma secretion has been reported as a necessary component for controlling *C. pneumoniae*

infection. Thus, overall, our data argue that CD4+TEM and CD4+ CD154+ T cells are necessary for sustaining T cell effector activity in activated Th1 cells. Our studies are in agreement with earlier studies of Bunk et al who reported that *Chlamydia pneumoniae*-induced memory CD4+ T cell responses involving the production of IFN-gamma and/or IL-2 were found in PBMC of healthy humans without acute respiratory infection.[20] When cells were restimulated with *C. pneumoniae*, the majority of cytokine-producing CD4+ T cells expressed CD154, a marker for CD4+ T cells activated with antigen.[20] Studies of others also demonstrated that CD154 is a specific marker of Ag-specific CD4+ T cells producing IFN-γ, IL-2, or TNF-α.[36,37]

IFN-gamma contributes to protective immunity against infectious diseases.[38] However, the role of IFN-gamma on the protective immunity to influencing TEM expression and activity in effector CD4+ or CD8+ T cells during *C. pneumoniae* infection is unclear. Our next observation was that when subjects were stratified according to IFN-gamma+ status, subjects who were IFN-gamma negative had a decrease in total number of TEMs and CD4+IL-2+, CD4+IL-4+ TEMs, CD8+IL-2+ and CD8+IL-4+ TEMs. Subjects who were IFN-gamma positive had an increase in CD4+ IL-2+ and CD4+IL-4+ TEMs, while numbers of CD8+IL-2+ and CD8+ IL-4+ TEMs decreased. These findings are suggestive of an important role for CD4+ and CD8+TEMs in IFN-gamma positive producing PBMC in response to *C. pneumoniae* infection. It could be speculated that these TEM cells and cytokines generate multifunctional inflammatory responses against *C. pneumoniae* infection and that specific TEMs have the potential to control cytokine-induced inflammatory responses in the presence of infection

or during recovery from infection. In asymptomatic persons, the presence of CD4+TEMs and IFN-gamma secretion by PBMC may indicate persistent infection because effector memory cells should disappear from peripheral blood once someone has recovered from acute infection.

In infectious diseases, toll like receptors (TLRs) induced by IFN-gamma help phagocytic cells recognize pathogen.[38] IFN-gamma contributes to CD4+ T cell differentiation to a Th1-type phenotype by activating signal transducer and activator of transcription (STAT) proteins that increase the T cell response to IL-2.[39] It is well established that control and clearance of bacterial and viral infections depend on antigen-specific IFN-gamma secreting T helper cells.[40,41]

There are limitations that should be mentioned including small sample size of the study population. Small sample size may contribute to decreased power and increased type II error; future larger studies may help verify the findings of this study. Other limitations include the presence of only one subject that was IFN-gamma positive. This is a preliminary pilot study; future studies will include more subjects with positive IFN-gamma responses to reach proper conclusions.

In this preliminary study, *C. pneumoniae*-induced PBMC increased CD4+ but not CD8+ TEM responses in asymptomatic subjects characterized by production of high levels of IFN-gamma. These results have important clinical implications for use of this IFN-gamma assay as a potential biomarker of persistent infection in humans.

Abbreviations

C. pneumoniae,*Chlamydia pneumoniae*; PBMC, peripheral blood mononuclear cells; TEM, T effector memory cell.

Data Sharing Statement

Upon request from authors.

Ethics Approval

This study was approved by the SUNY Downstate Medical Center Institutional Review Board (Brooklyn, NY) and human studies adhered to the World Medical Association Declaration of Helsinki. Written informed consent for participation and publication was obtained from all participants.

Consent to Participate

Written consent to participate was obtained.

Consent for Publication

Written consent for publication was obtained.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors report no conflicts of interest in this work.

References

1. Hammerschlag MR, Kohlhoff SA, Gaydos CA. Chlamydia pneumoniae. In: Mandell GL, Bennett JE, Dolin R, editors. *Principles and Practice of Infectious Diseases*. 8th ed. Philadelphia, PA: Elsevier, Inc.; 2014:2174–2182.

2. Johnston SL, Martin RJ. *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* a role in asthma pathogenesis? *Am J Respir Crit Care Med*. 2005;172:1078–1089. doi:10.1164/rccm.200412-1743PP

3. Emre U, Roblin PM, Gelling M, et al. The association of *Chlamydia pneumoniae* infection and reactive airway disease in children. *Arch Pediatr Adolesc Med*. 1994;148:727–731. doi:10.1001/archpedi.1994.02170070065013

4. Hammerschlag MR, Chirgwin K, Roblin PM, et al. Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. *Clin Infect Dis*. 1992;14:178–182. doi:10.1093/clinids/14.1.178

5. Martin RJ, Kraft M, Chu HW, et al. A link between chronic asthma and chronic infection. *J Allergy Clin Immunol*. 2001;107:595–601. doi:10.1067/mai.2001.113563

6. Ikezawa S. Prevalence of *Chlamydia pneumoniae* in acute respiratory tract infection and detection of anti-*Chlamydia pneumoniae*-specific IgE in Japanese children with reactive airway disease. *Kurume Med J*. 2001;48(2):165–170. doi:10.2739/kurumemedj.48.165

7. Kohlhoff SA, Hammerschlag MR. Treatment of chlamydial infections: 2014 update. *Expert Opin Pharmacother*. 2015;16:205–212. doi:10.1517/14656566.2015.999041

8. Khan AA, Slifer TR, Araujo FG, et al. Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. *Int J Antimicrobial Agents*. 1999;11:121–132. doi:10.1016/S0924-8579(98)00091-0

9. Emre U, Sokolovskaya N, Roblin PM, et al. Detection of anti-*Chlamydia pneumoniae* IgE in children with reactive airway disease. *J Infect Dis*. 1995;172:265–267. doi:10.1093/infdis/172.1.265

10. Kohlhoff SA, Joks R, Kamath T, et al. *Chlamydophila pneumoniae* mediated IgE production by peripheral blood mononuclear cells of allergic asthmatics is suppressed by doxycycline. *J Allergy Clin Immunol*. 2007;119(2):p525. doi:10.1016/j.jaci.2006.12.633

11. Beatty WL, Morrison RP, Byrne GL. Persistent chlamydiae: from cell culture to a paradigm form chlamydial pathogenesis. *Microbiol Rev*. 1994;58:689–694. doi:10.1128/MR.58.4.686-699.1994

12. Kutlin A, Flegg C, Stenzel D, et al. Ultrastructural study of *Chlamydia pneumoniae* in a continuous infection model. *J Clin Microbiol*. 2001;39:3721–3723. doi:10.1128/JCM.39.10.3721-3723.2001

13. Kohlhoff SA, Kutlin A, Riska P, et al. In vitro models of acute and long-term continuous infection of human respiratory epithelial cells with *Chlamydophila pneumoniae* have opposing effects on host cell apoptosis. *Microb Pathogen*. 2008;44:34–42. doi:10.1016/j.micpath.2007.08.003

14. Chirgwin K, Roblin PM, Gelling M, et al. Infection with *Chlamydia pneumoniae* in Brooklyn. *J Infect Dis*. 1991;163:757–761. doi:10.1093/infdis/163.4.757

15. Halme S, Latvala J, Karttunen R, et al. Cell-mediated immune response during primary *Chlamydia pneumoniae* infection. *Infect Immun*. 2000;68:7156–7158. doi:10.1128/IAI.68.12.7156-7158.2000

16. Smith-Norowitz TA, Chotikanatis K, Erstein DP, et al. *Chlalmydia pneumoniae* enhances the Th2 profile of stimulated peripheral blood mononuclear cells from asthmatic patients. *Hum Immunol*. 2016;77:382–388. doi:10.1016/j.humimm.2016.02.010

17. Smith-Norowitz TA, Weaver D, Chorny V, et al. *Chlamydia pneumoniae* induces interferon gamma responses in peripheral blood mononuclear cells in children with allergic asthma. *Scan J Immunol*. 2017;86:59–64. doi:10.1111/sji.12561

18. Igietseme JU, Ramsey KH, Magee DM, et al. Resolution of murine chlamydial genital infection by the adoptive transfer of a biovar-specific, Th1 lymphocyte clone. *Reg Immunol*. 1993;5:317–324.

19. Holland MJ, Bailey RL, Conway DJ, et al. T-helper type 1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of *Chlamydia trachomatis* in subjects with severe trachomatous scarring. *Clin Exp Immunol*. 1996;105:429–435. doi:10.1046/j.1365-2249.1996.d01-792.x

20. Bunk S, Schaffert H, Schmid B, et al. *Chlamydia pneumoniae*-induced memory CD4+ T-cell activation in human peripheral blood correlates with distinct antibody response patterns. *Clin Vaccine Immunol*. 2010;17:705–712. doi:10.1128/CVI.00209-09

21. Tonaco MM, Moreira JD, Nunes FFC, et al. Evaluation of profile and functionality of memory T cells in pulmonary tuberculosis. *Immunol Lett*. 2017;192:52–60. doi:10.1016/j.imlet.2017.10.014

22. Cowen MK, Wakefield DB, Cloutier MM. Classifying asthma severity: objective versus subjective measures. *J Asthma*. 2007;44:711–715. doi:10.1080/02770900701595576

23. Smith-Norowitz TA, Weaver D, Norowitz YM, et al. Doxycycline suppresses *Chlamydia pneumoniae* induced interferon gamma responses in peripheral blood mononuclear cells in children with allergic asthma. *J Infect Chemother*. 2018;24:470–475. doi:10.1016/j.jiac.2018.02.004

24. Wang SP, Grayston JT. Microimmunofluorescence serological studies with the TWAR organism. In: Oriel D, Ridgeway G, editors. *Chlamydial Infections: Proceedings of the Sixth International Symposium on Human Chlamydial Infections*. Cambridge: Cambridge University Press; 1986:329–332.

25. Roblin PM, Dumornay W, Hammerschlag MR. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J Clin Microbiol*. 1992;30:1968–1971. doi:10.1128/JCM.30.8.1968-1971.1992

26. Dzhindzhikhashvili MS, Joks R, Smith-Norowitz TA, et al. Doxycycline suppresses *Chlamydia pneumoniae*-mediated increases in ongoing immunoglobulin E and interleukin-4 responses by peripheral blood mononuclear cells of patients with allergic asthma. *J Antimicrob Chemother*. 2013;68:2363–2368. doi:10.1093/jac/dkt179

27. Apfalter P, Barousch W, Nehr M, et al. Comparison of a new quantitative *omp*A-based real-time PCR TaqMan assay for detection of *Chlamydia pneumoniae* DNA in respiratory specimens with four conventional PCR assays. *J Clin Microbiol*. 2003;41:592–600. doi:10.1128/JCM.41.2.592-600.2003

28. Waring AL, Halse TA, Csiza CK, et al. Development of a genomics-based PCR assay for detection of *Mycoplasma pneumoniae* in a large outbreak in New York State. *J Clin Microbiol*. 2001;39:1385–1390. doi:10.1128/JCM.39.4.1385-1390.2001

29. Smith-Norowitz TA, Bluth MH, Drew H, et al. Effect of minocycline and doxycycline on IgE responses. *Ann Allergy Asthma Immunol*. 2002;89:172–179. doi:10.1016/S1081-1206(10)61934-5

30. Shidid S, Kohlhoff S, Norowitz Y, et al. Chlamydia pneumoniae decreases CD4+ and CD8+ T effector IL-2 responses in stimulated peripheral blood mononuclear cells in non-asthmatic subjects. *J Allergy Clin Immunol*. 2020;145(2):AB163. doi:10.1016/j.jaci.2019.12.430

31. Doherty PC, Christensen JP. Accessing complexity, the dynamics of virus-specific T cell responses. *Annu Rev Immunol*. 2000;18:561–592. doi:10.1146/annurev.immunol.18.1.561

32. Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasquier RA, Pantaleo G. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol Rev*. 2006;211:236–254. doi:10.1111/j.0105-2896.2006.00395.x

33. Rothfuchs AG, Kreuger MR, Wigzell H, et al. Macrophages, CD4 + or CD8 + cells are each sufficient for protection against *Chlamydia pneumoniae* infection through their ability to secrete IFN-γ. *J Immunol*. 2004;172:2407–2415. doi:10.4049/jimmunol.172.4.2407

34. Rottenberg ME, Gigliotti Rothfuchs AC, Gigliotti G, et al. Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae* as analyzed in genetically modified mice. *J Immunol*. 1999;162:2829–2836.

35. Pentilla JM, Anttila M, Puolakkainen M, et al. Local immune responses to *Chlamydia pneumoniae* in the lungs of BALB/mice during primary infection and reinfection. *Infect Immun*. 1998;66:5113–5118. doi:10.1128/IAI.66.11.5113-5118.1998

36. Frentsch M, Arbach O, Kirchhoff D, et al. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat Med*. 2005;11:1118–1124. doi:10.1038/nm1292

37. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. *Nat Med*. 2005;11:1113–1117. doi:10.1038/nm1293

38. Borges da Silva H, Fonseca R, Alvarez JM, et al. IFN- γ priming effects on the maintenance of effector memory CD4 + T cells and on phagocyte function: evidences from infectious diseases. *J Immunol Res*. 2015;2015:202816. doi:10.1155/2015/202816

39. Matikainen S, Sareneva T, Ronni T, et al. Interferon-α activates multiple STAT proteins and upregulates proliferation-associated IL-2Rα, cmyc, and pim-1 genes in human T cells. *Blood*. 1999;93:1980–1991. doi:10.1182/blood.V93.6.1980.406k20_1980_1991

40. Stubbe M, Vanderheyde N, Goldman M, et al. Antigen-specific central memory CD4 + T lymphocytes produce multiple cytokines and proliferate in vivo in humans. *J Immunol*. 2006;177:8185–8190. doi:10.4049/jimmunol.177.11.8185

41. Shtrichman R, Samuel CE. The role of gamma interferon in antimicrobial immunity. *Curr Opin Microbiol*. 2001;4:251–259. doi:10.1016/S1369-5274(00)00199-5

Dynamics of COVID-19 Lockdown on Blood Indices and Its Impact on Individuals' Immunological Health Status: A Cohort Study in Madinah, Saudi Arabia

Bandar A Suliman

Published online: 31 May 2021.

Submit your article to this journal 🗗

Article views: 108

View related articles 🗗

View Crossmark data 🗗

Citing articles: 2 View citing articles 🗗

ORIGINAL RESEARCH

Dynamics of COVID-19 Lockdown on Blood Indices and Its Impact on Individuals' Immunological Health Status: A Cohort Study in Madinah, Saudi Arabia

Bandar A Suliman [ID]

College of Applied Medical Sciences, Taibah University, Madinah, Saudi Arabia

Objective: The complete blood count (CBC) is an essential blood test that has been used for decades to assess individuals' overall health status. This study aimed to investigate the contributions of lockdown conditions to individuals' overall health status using blood indices as biological markers. During lockdown, people are limited to confined spaces, have access to limited nutritional supply options, experience increased stress, and are exposed to other environmental factors.

Methods: Our study's target population included all outpatients who were severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-negative and requested CBC assessments as part of their routine health checks. A total of 2414 CBC results were included, covering a period from February 2019 to December 2020. The average of different blood indices during the COVID-19 lockdown was compared to the 10-month period preceding the lockdown.

Results: The average counts of RBCs, hemoglobin, and hematocrit showed a significant increase during the lockdown period, which lasted from May 2020 to September 2020. Reductions were observed for the RBC distribution width, total white blood cell count, platelets, and platelet distribution width.

Conclusion: Our findings suggested that the overall health status of individuals improved during the lockdown period in the short term, but health status might be adversely affected under these conditions of a longer period. Both RDW and PDW could be used as indicators for the overall health status when assessed against other blood indices.

Keywords: COVID-19, CBC, lockdown, platelet distribution width, red cell distribution width

Introduction

The complete blood count (CBC) has been used for decades as an essential blood test to assess individuals' overall health status.[1] Specific blood indices, such as the total count of red blood cells (RBCs) which is important to understand the production capacity of the bone marrow, mean corpuscular volume (MCV) which reflects the cell's size, mean corpuscular hemoglobin (MCH), cell distribution width (RDW) which represent the variation in size between different RBCs, and hemoglobin concentration (HGB) which is used to monitor the blood's oxygenation capacity, have a significant diagnostic value in reflecting bone marrow's ability to produce hematopoietic-driven cells.[2,3] Other

Correspondence: Bandar A Suliman
College of Applied Medical Sciences, Taibah University, Madinah, Saudi Arabia
Tel +966-504772422
Email bsuliman@taibahu.edu.sa

indices related to white blood cells (WBC) and the differential count of each one of them had been used as markers for inflammatory reactions such as bacterial infections, viral infections, parasitic infections, or hematological malignancies.[4,5]

The immune system is composed of many intertwined cells and organs working in harmony to create the overall protection against pathogens. White blood cell count is very important indicator of how the immune system handles certain clinical and medical situations such as inflammatory reactions or infections by foreign organisms.[6–9]

SARS-CoV-2 is a single-strand RNA virus belonging to the Coronaviridae family of viruses. It is the primary cause of respiratory symptoms worldwide since the outbreak in Wuhan in January 2020.[10] Viral infections are represented in the blood work as a marked increase in total WBC count underlying a significant surge in lymphocytes, which are produced by the immune system as an antiviral response driven by interferons.[11–13]

The balance in different whole blood indices is essential to maintain the equilibrium of many important blood components such as albumin and plasma proteins. That is why a CBC is ordered and considered an essential blood test in the first line of the diagnostic process in many clinical complications.[14]

In this study, we aim to investigate the role of COVID-19 and the accompanying lockdown that was enforced between May 2020 and September 2020 on patients' blood indices in Medina region. We ask if these indices can be used to measure the overall health status of individuals and if a significant difference in blood indices is noticed that may be attributed directly to certain lifestyle changes associated with the lockdown.

Materials and Methods
Ethics Statement
This study was approved by the Internal Review Board of Taibah University in Madinah, Saudi Arabia (No. 2021/90/114/MLT). Written informed consent was obtained from each patient prior to participation in the study.

Study Population
Our study's target population were all out-patients who were SARS-CoV-2 negative and requested a complete blood count (CBC). Any patient showing respiratory symptoms or was less than 18 years were excluded from the study. A total of 2414 CBC results were obtained from

Table 1 Target Population Used for This Study (n = 2414)

Gender	Male	Female
Max Age	91	82
Min Age	18	18
Average Age	27	29
Count	772	1642

the laboratory information system (Table 1). These results were from 772 males (aged between 18 and 91 years) and 1642 females (aged between 18 and 82 years).

Complete Blood Count
Three milliliters of whole blood samples were collected in Lavender top tubes containing EDTA K3 (Advanced Medical Co., Riyadh) and then placed on a roller (12 RPM) until the analysis. CBC analysis was performed between 10 minutes and 3 hours after sample collection on the XP-300 Automated Hematology Analyzer (Sysmex Corporation, Hyogo).

Statistical Analysis
Blood indices were accumulated for each month to calculate the average, SD, and SEM and then blotted as graphical reorientations. Significance in the difference between monthly averages was calculated using the 2-sample t-test, which is commonly used to compare two independent parametric groups and tests the null hypothesis, assuming that both means are identical.[15] Additionally, the Violin plot was used to compare the averages of the entire time periods. The unpaired t-test, and the F-test were performed to calculate the p-value. Statistical analysis and graphical representation of data were performed using Prism 8.2 (GraphPad Software, Inc., San Diego).

Results
Iron-Related CBC Indices are Increased During the Lockdown Period
For the 10 months preceding the lockdown, the average RBC count was $4.32 \times 10^6/\mu L$ (Figure 1). This average showed a significant increase to $4.59 \times 10^6/\mu L$, an approximate 7% increase (Figure 2), for the lockdown period, which started in May 2019 and lasted for 5 months. This increase was determined to be significant using both the unpaired t-test and the F-test with a p-value of <0.0001 in both statistical tests. This observation was not limited to only RBCs. We showed that Hemoglobin and Hematocrit (Figure 1) showed
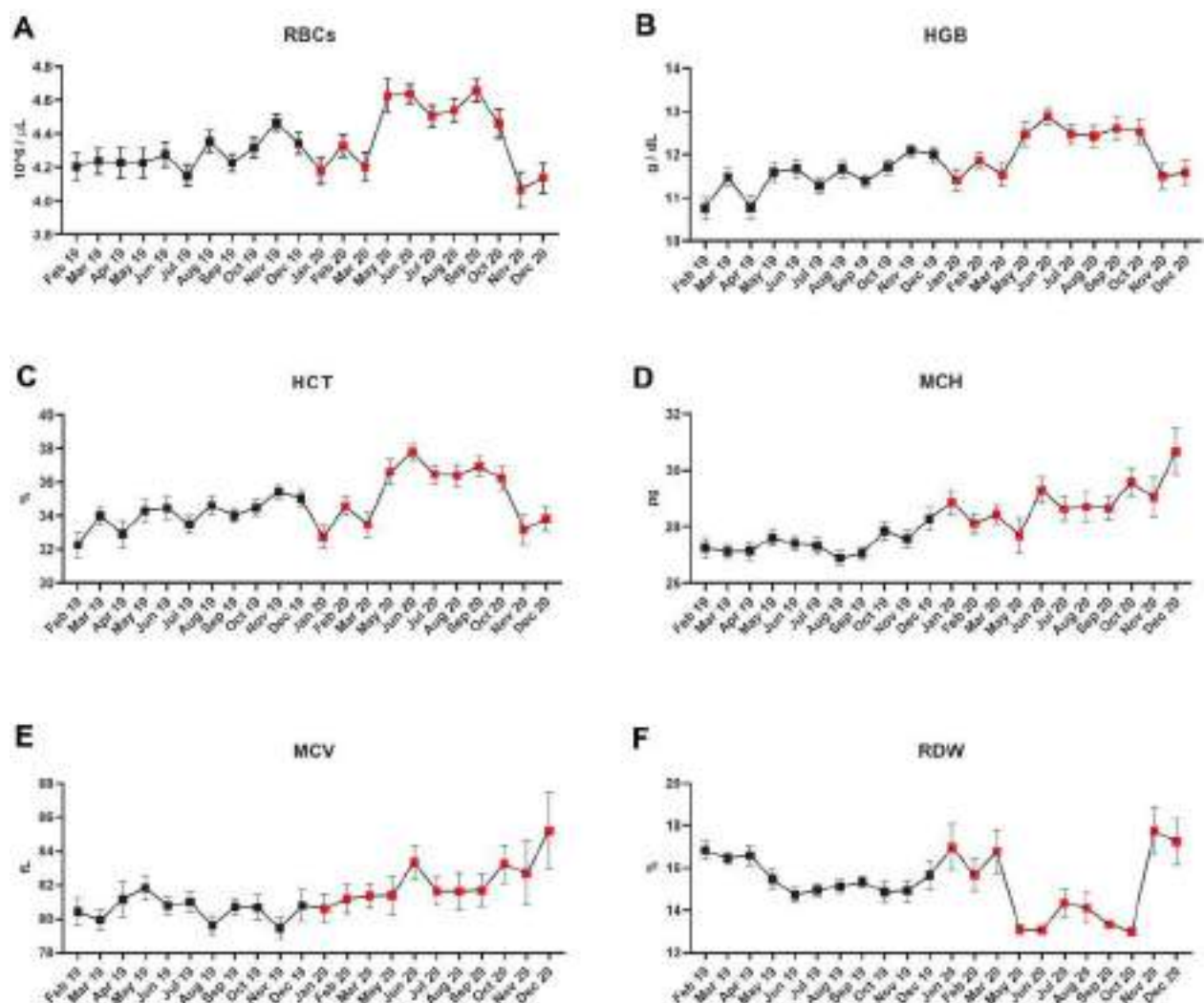
Figure 1 Accumulative representation of RBC-related blood indices for 2414 patients shown in months from February 2019 until December 2020. (**A**) Red blood cells (RBC) counts are shown as average for each month ±SEM in 10^6/μL; (**B**) Hemoglobin levels are shown as average for each month ±SEM in g/dL; (**C**) Hematocrit levels are shown as average for each month ±SEM in percentage; (**D**) Mean corpuscular hemoglobin (MCH) levels are shown as average for each month ±SEM in pg; (**E**) Mean corpuscular volume (MCV) levels are shown as average for each month ±SEM in fL; (**F**) RBC distribution width (RDW) levels are shown as average for each month ±SEM in percentage.

very similar results as RBCs (Table 2). Hemoglobin concentration increased significantly from 11.69 g/dL to 12.61 g/dL (t= 7.511; p-value= <0.0001), as well as Hematocrit which increased significantly from 34.33% to 36.91% (t= 7.876; p-value= <0.001; F= 1.223; p-value= <0.01). This clearly indicates that iron-related CBC indices are increased during the lockdown when compared to before that time period (Figure 2). RBC mean corpuscular volumes as well as corpuscular hemoglobin concentrations did not show any significant difference between the two time periods. The MCV average remained steady at 80.3 fL as well as the MCH average at 27.4 pg. The RBC distribution width (RDW-CV), on the other hand, showed a marked decrease from 15.27%

to 14.40% in the lockdown period (t= 3.988; p-value= <0.001; F= 1.662; p-value= <0.01).

White Blood Cell Counts are Decreased During the Lockdown Period

We then compared total WBC counts and the percentage of neutrophil and lymphocyte to the total counts (Figure 3). Total WBC was significantly lower during the lockdown period than in the previous 10 months (Table 3). The average WBC count before the lockdown was 8.423 × 10^3/μL which dropped to 7.156 × 10^3/μL during the lockdown. This decrease was determined to be statistically significant (t= 5.581; p-value= <0.001; F= 3.841; p-value= <0.001). However, lymphocyte
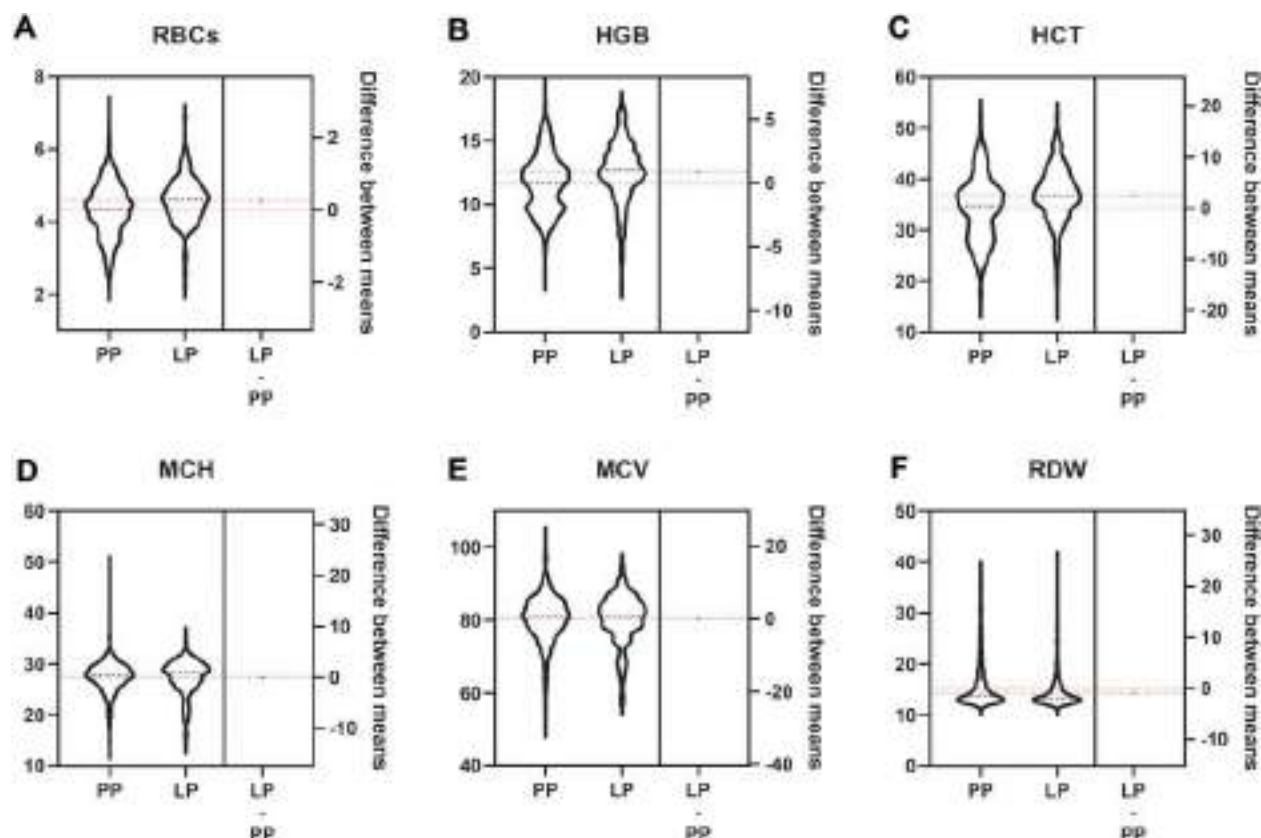
Figure 2 Difference between average means of blood indices. (**A**) RBCs: red blood cells. (**B**) HGB: hemoglobin. (**C**) HCT: hematocrit. (**D**) MCH: mean corpuscular hemoglobin. (**E**) MCV: mean corpuscular volume. (**F**) RDW: RBC distribution width.
Abbreviations: PP, period preceding the lockdown; LP, lockdown period.

and neutrophil counts remained on a relatively steady average (Table 3) throughout the year (Figure 4).

Platelet Counts are Decreased During the Lockdown Period

After that, we looked at Platelet counts, which showed a similar drop during the lockdown (Figure 5). Platelet counts showed an average of $361 \times 10^3/\mu L$ before the lockdown (Table 4). This average was significantly lowered to $318 \times 10^3/\mu L$ ($t= 4.659$; p-value= <0.001; $F= 2.732$; p-value= <0.001). More interestingly, the Platelet distribution width (PDW) was also lowered (Figure 6) from 13.18% to 12.58% ($t= 4.439$; p-value= <0.001; $F= 1.700$; p-value= <0.001).

Table 2 Statistical Analysis for Average Counts of RBC-Related Indices

Parameter	Mean PP	Mean LP	95% Confidence Interval	Unpaired t Test			F-Test		
				p-value	*t*	*df*	*p*-value	*F*	DFn
RBC	4.32	4.59	0.1936 to 0.3500	<0.001	6.817	1615	<0.001	1.359	1183
HGB	11.69	12.61	0.6781 to 1.157	<0.001	7.511	1715	**NS**	1.044	440
HCT	34.33	36.91	1.940 to 3.227	<0.001	7.876	1722	<0.01	1.223	1279
MCH	27.38	27.44	−0.2725 to 0.4048	**NS**	0.383	1704	**NS**	1.184	437
MCV	80.35	80.39	−0.6482 to 0.7249	**NS**	0.109	1709	**NS**	1.070	441
RDW	15.27	14.40	−1.301 to −0.4431	<0.001	3.988	1703	<0.001	1.662	1265

Notes: Comparison was performed between the "Lockdown Period: LP" which lasted for five months (from May 2020 until September 2020), and the "Preceding Period: PP" which lasted for 10 months (from July 2019 until April 2020). Mean: average value of all data in that particular time period.
Abbreviation: NS, not significant.
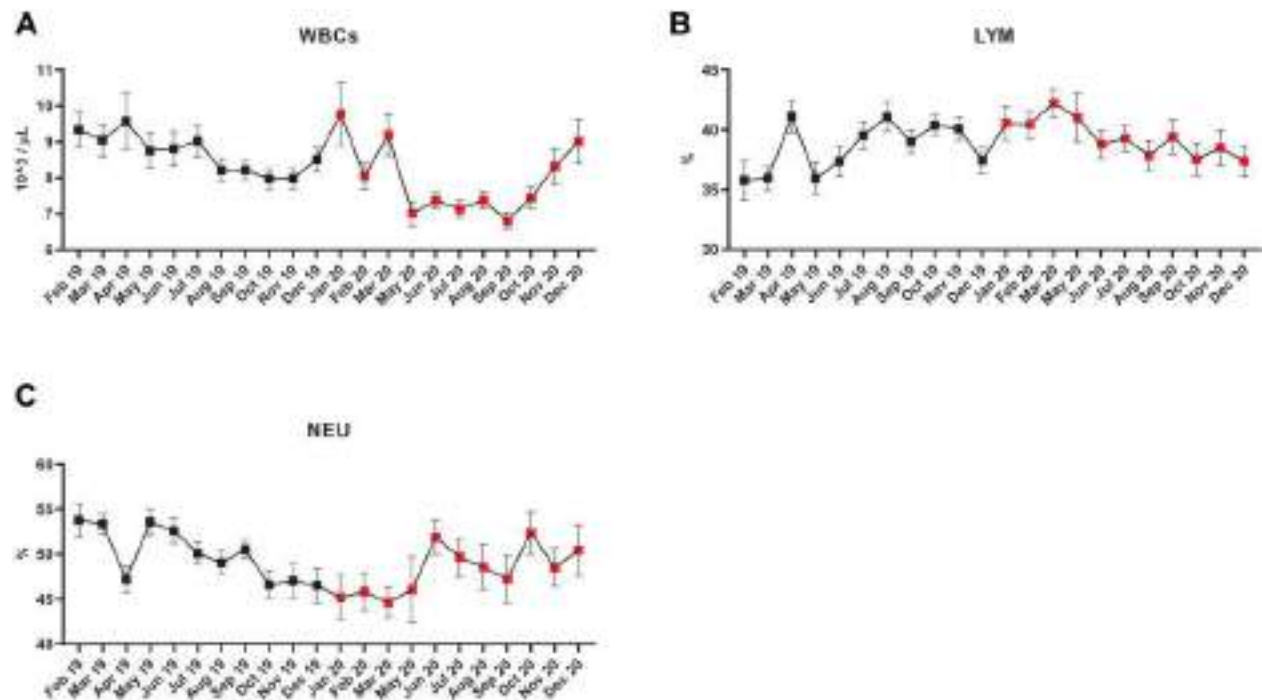
Figure 3 Accumulative representation of WBC-related blood indices for 2414 patients shown in months from February 2019 until December 2020. (**A**) Total white blood cells (WBC) counts are shown as average for each month ±SEM in $10^3/\mu L$; (**B**) Lymphocyte percentages are shown as average for each month ±SEM in percentage; (**C**) Neutrophil percentages levels are shown as average for each month ±SEM in percentage.

Discussion

Many factors are involved in regulating the cellular composition in our blood. These factors come from the environment we share, the food we eat, the atmosphere we breathe, and the pathogens transmitted between individuals in a particular community. The Corona pandemic showed us that many elements are still hidden from our understanding of our bodies' essential biological functions. One of the immune system's primary clinical facts is the noticeable increase of cellular and humoral factors upon acquiring an infection. SARS-CoV-2, such as any pathogen, should show similar effects on the parameters of our immune system. However, a question arises about the people who were not infected with the virus or those who have recovered (for more than 30 days or more).

Our study aimed to understand the overall individuals' immunological status during the lockdown period in Madinah, Saudi Arabia, which lasted for about 5 months, starting from April 2020 until September 2020. People in lockdown practice their daily routines in an entirely different extent than their regular routine which can be clearly observed from our results. Basic daily routines such as physical activity, smoking frequency, type and quantity of food, mental and physiological stress are just a few factors shaping how our body reacts to the external environment.

Table 3 Statistical Analysis for Average Counts of WBC-Related Indices

Parameter	Mean PP	Mean LP	95% Confidence Interval	Unpaired t Test			F Test		
				p-value	t	df	p-value	F	DFn
WBC	8.423	7.156	−1.712 to −0.8216	<0.001	5.581	1718	<0.001	3.841	1277
LYM	39.98	39.15	−2.217 to 0.5505	NS	1.181	1703	NS	1.110	1266
NEU	50.03	50.91	0.4100 to 3.350	NS	2.508	1702	NS	1.079	1265

Notes: Comparison was performed between the "Lockdown Period: LP", which lasted for 5 months (from May 2020 until September 2020), and the "Preceding Period: PP" which lasted for 10 months (from July 2019 until April 2020). Mean: average value of all data in that particular time period.
Abbreviation: NS, not significant.
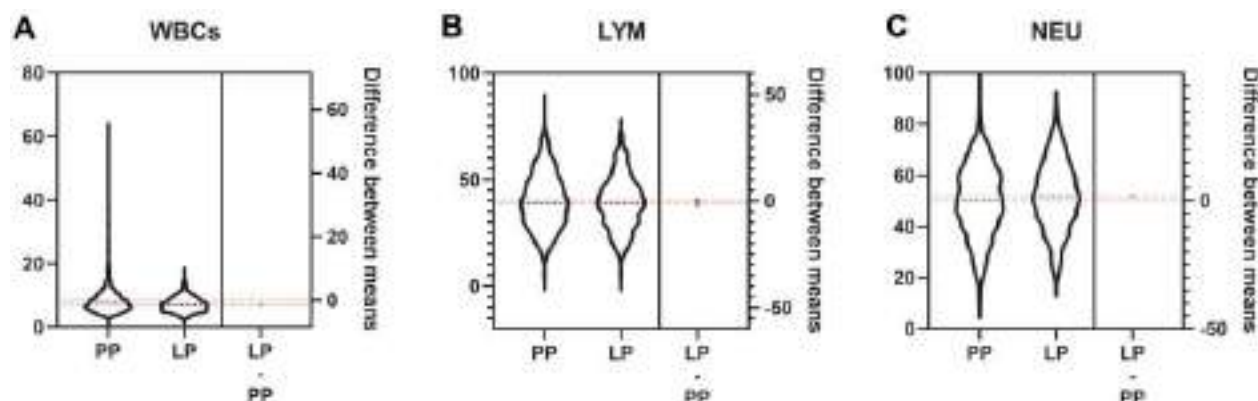
Figure 4 Difference between average means of blood indices. (**A**) WBCs: white blood cells. (**B**) LYM: lymphocytes. (**C**) NEU: neutrophils.
Abbreviations: PP, period preceding the lockdown; LP, lockdown period.
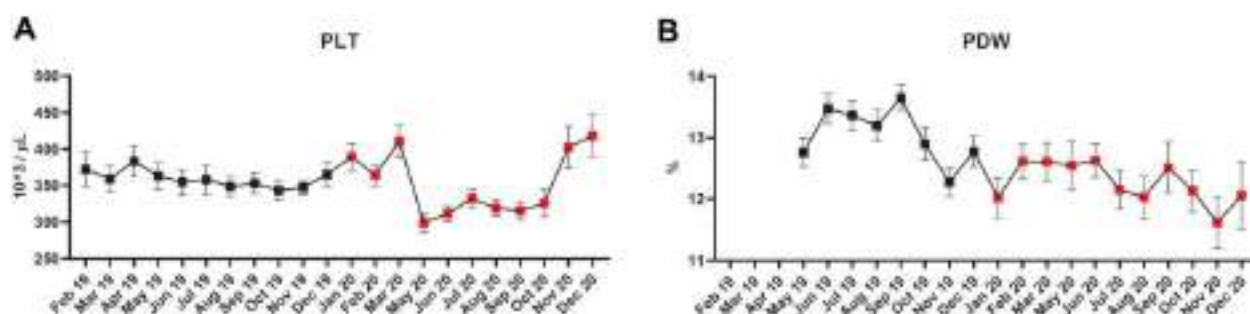
Figure 5 Accumulative representation of platelet-related blood indices for 2414 patients shown in months from February 2019 until December 2020. (**A**) Platelet counts are shown as average for each month ±SEM in $10^6/\mu L$; (**B**) Platelet distribution width (PDW) levels are shown as average for each month ±SEM in percentage.

Here we show that RBC counts along with hemoglobin and hematocrit levels showed a significant increase in our target population during the lockdown period. This increase can be attributed to many factors, most notably smoking. Tobacco smoking is directly linked to an increase in RBC counts and related indices. More importantly, many published studies showed that smoking routine increased considerably among people during the lockdown as a coping mechanism against physical and mental stress.[16] Interestingly, the moving average of both

MCV and MCH showed no significant deviation throughout the year. This was in agreement with previously published studies showing that smoking had no significant effect on those two hematological parameters.[17,18]

Moreover, RDW rates were lower during the lockdown period than before the lockdown. RDW represents the uniformity of RBC size, where lower percentages mean that all cells are almost the same size.[19] This uniformity in size may indicate that the overall oxygenation requirement levels of those individuals are remarkably similar. Also,

Table 4 Statistical Analysis for Average Counts of Platelet-Related Indices

Parameter	Mean PP	Mean LP	95% Confidence Interval	Unpaired t Test			F Test		
				p-value	t	df	p-value	F	DFn
PLT	361.7	318.2	−61.80 to −25.18	<0.001	4.659	1703	<0.001	2.732	1266
PDW	13.18	12.58	−0.8642 to −0.3345	<0.001	4.439	1587	<0.001	1.700	1167

Notes: Comparison was performed between the "Lockdown Period: LP", which lasted for 5 months (from May 2020 until September 2020), and the "Preceding Period: PP", which lasted for 10 months (from July 2019 until April 2020). Mean: average value of all data in that particular time period.
Abbreviation: NS, not significant.
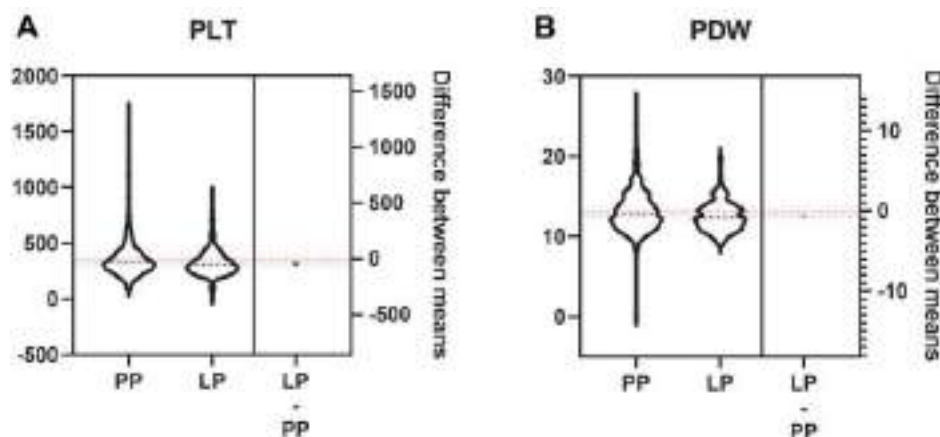
Figure 6 Difference between average means of blood indices. (**A**) PLT: platelets. (**B**) PDW: platelet distribution width.
Abbreviations: PP, period preceding the lockdown; LP, lockdown period.

many researchers have proposed the use of high RDW as a pathological marker in many disease models.[20–25] RDW is an actual representation of volumetric differences between many red blood cell populations including mature red cells, immature red cells and retics. The same technological concept applies to PDW in the context of platelets. Lower RDW might therefore indicate a healthy status, or in other terms, the absence of these pathological conditions. This finding also contradicts the notion of smoking as a detrimental factor of the high levels of RBC, HGB, and HCT.

Immunological status of individuals is very hard to assess. Many biological factors must be considered, such as a healthy diet, physical exercise, proper adherence to medical management, and other factors. The collection of blood specimens from different individuals before and during the lockdown would also generate some statistical bias that needs to be taken into consideration. Recent studies from Saudi Arabia showed that compliance with the medicinal regiment and following a healthy dietary routine were both diminished during the lockdown period.[26,27] Our results may shed some light on the impact of reduced physical activity accompanied with high mental stress on individuals' immune systems by using blood indices as a supporting factor.

Disclosure

The author reported no conflicts of interest for this work.

References

1. Young GP. CBC or not CBC? That is the question. *Ann Emerg Med*. 1986;15(3):367–371. doi:10.1016/S0196-0644(86)80587-X

2. Barnes P, McFadden S, Machin S, Simson E. The international consensus group for hematology review: suggested criteria for action following automated CBC and WBC differential analysis. *Lab Hematol*. 2005;11(2):83–90. doi:10.1532/LH96.05019

3. Sandhaus LM, Meyer P. How useful are CBC and reticulocyte reports to clinicians? *Am J Clin Pathol*. 2002;118(5):787–793. doi:10.1309/CQGG-HY0U-LRKL-GLMP

4. Al-Gwaiz LA, Babay HH. The diagnostic value of absolute neutrophil count, band count and morphologic changes of neutrophils in predicting bacterial infections. *Med Princ Pract*. 2007;16 (5):344–347. doi:10.1159/000104806

5. Todd JK. Childhood infections: diagnostic value of peripheral white blood cell and differential cell counts. *Am J Dis Child*. 1974;127 (6):810–816. doi:10.1001/archpedi.1974.02110250036005

6. Carel RS, Eviatar J. Factors affecting leukocyte count in healthy adults. *Prev Med*. 1985;14(5):607–619. doi:10.1016/0091-7435(85) 90081-7

7. Farhangi MA, Keshavarz S-A, Eshraghian M, Ostadrahimi A, Saboor-Yaraghi -A-A. White blood cell count in women: relation to inflammatory biomarkers, haematological profiles, visceral adiposity, and other cardiovascular risk factors. *J Health Popul Nutr*. 2013;31 (1):58. doi:10.3329/jhpn.v31i1.14749

8. Wang F, Hou M, Wu X, Bao L, Dong P. Impact of enteral nutrition on postoperative immune function and nutritional status. *Genet Mol Res*. 2015;14(2):6065–6072. doi:10.4238/2015.June.8.4

9. Nishitani N, Sakakibara H. Association of psychological stress response of fatigue with white blood cell count in male daytime workers. *Ind Health*. 2014;52(6):531–534. doi:10.2486/indhealth.2013-0045

10. Wu D, Wu T, Liu Q, Yang Z. The SARS-CoV-2 outbreak: what we know. *Int J Infect Dis*. 2020;94:44–48. doi:10.1016/j.ijid.2020.03.004

11. Peltola V, Mertsola J, Ruuskanen O. Comparison of total white blood cell count and serum C-reactive protein levels in confirmed bacterial and viral infections. *J Pediatr*. 2006;149(5):721–724. doi:10.1016/j.jpeds.2006.08.051

12. Korppi M, Kröger L, Laitinen M. White blood cell and differential counts in acute respiratory viral and bacterial infections in children. *Scand J Infect Dis*. 1993;25(4):435–440. doi:10.3109/00365549309008524

13. Vieira R, Diniz E, Vaz F. Clinical and laboratory study of newborns with lower respiratory tract infection due to respiratory viruses. *J Matern Fetal Neonatal Med*. 2003;13(5):341–350. doi:10.1080/jmf.13.5.341.350

14. Park SK. An interpretation on abnormal finding of CBC. *Korean J Med*. 2010;78(5):531–539.

15. Vetter TR, Mascha EJ. Unadjusted bivariate two-group comparisons: when simpler is better. *Anesth Analg*. 2018;126(1):338–342. doi:10.1213/ANE.0000000000002636

16. Yach D. Tobacco use patterns in five countries during the COVID-19 Lockdown. *Nicotine Tob Res*. 2020;22(9):1671–1672. doi:10.1093/ntr/ntaa097

17. Asif M, Karim S, Umar Z, et al. Effect of cigarette smoking based on hematological parameters: comparison between male smokers and non-smokers. *Turk J Biochem*. 2013;38(1).

18. Nadia M, Shamseldein H, Sara A. Effects of cigarette and Shisha smoking on hematological parameters: an analytic case-control study. *Int Multispecialty J Health*. 2015;10(1).

19. Evans TC, Jehle D. The red blood cell distribution width. *J Emerg Med*. 1991;9:71–74. doi:10.1016/0736-4679(91)90592-4

20. Wang C, Zhang H, Cao X, et al. Red cell distribution width (RDW): a prognostic indicator of severe COVID-19. *Ann Transl Med*. 2020;8(19).

21. Li N, Zhou H, Tang Q. Red blood cell distribution width: a novel predictive indicator for cardiovascular and cerebrovascular diseases. *Dis Markers*. 2017;2017:1–23. doi:10.1155/2017/7089493

22. Tham T, Bardash Y, Teegala S, Herman WS, Costantino PD. The red cell distribution width as a prognostic indicator in upper aerodigestive tract (UADT) cancer: a systematic review and meta-analysis. *Am J Otolaryngol*. 2018;39(4):453–458. doi:10.1016/j.amjoto.2018.04.013

23. Pedrazzani C, Tripepi M, Turri G, et al. Prognostic value of red cell distribution width (RDW) in colorectal cancer. Results from a single-center cohort on 591 patients. *Sci Rep*. 2020;10(1):1–9. doi:10.1038/s41598-020-57721-4

24. Emans ME, van der Putten K, van Rooijen KL, et al. Determinants of red cell distribution width (RDW) in cardiorenal patients: RDW is not related to erythropoietin resistance. *J Card Fail*. 2011;17(8):626–633. doi:10.1016/j.cardfail.2011.04.009

25. Vayá A, Alis R, Hernández J-L, et al. RDW in patients with systemic lupus erythematosus. Influence of anaemia and inflammatory markers. *Clin Hemorheol Microcirc*. 2013;54(3):333–339. doi:10.3233/CH-131738

26. Alshareef R, Al Zahrani A, Alzahrani A, Ghandoura L. Impact of the COVID-19 lockdown on diabetes patients in Jeddah, Saudi Arabia. *Diabetes Metab Syndr*. 2020;14(5):1583–1587. doi:10.1016/j.dsx.2020.07.051

27. Aljohani NE. The effect of the lockdown for the Coronavirus (COVID-19) pandemic on body weight changes and eating habits in Saudi Arabia. *J Saudi Soc Food Nutr*. 2020;13(1):103–113.

Use of Plerixafor for Stem Cell Mobilization in the Setting of Autologous and Allogeneic Stem Cell Transplantations: An Update

Yavuz M Bilgin

REVIEW

Use of Plerixafor for Stem Cell Mobilization in the Setting of Autologous and Allogeneic Stem Cell Transplantations: An Update

Yavuz M Bilgin ⓘ

Department of Internal Medicine/
Hematology, Admiraal de Ruijter
Hospital, Goes, the Netherlands

Abstract: Mobilization failure is an important issue in stem cell transplantations. Stem cells are yielded from the peripheral blood via apheresis. Granulocyte colony-stimulating factor (G-CSF) is the most commonly used mobilization agent among patients and donors. G-CSF is administered subcutaneously for multiple days. However, patients with mobilization failure cannot receive autologous stem cell transplantation and, therefore, cannot be treated adequately. The incidence rate of mobilization failure among patients is about 6–23%. Plerixafor is a molecule that inhibits the binding of chemokine receptor-4 with stromal-cell-derived factor-1, thereby resulting in the release of CD34+ cells in the peripheral blood. Currently, plerixafor is used in patients with mobilization failure with G-CSF and is administered subcutaneously. Several studies conducted on different clinical settings have shown that plerixafor is effective and well tolerated by patients. However, more studies should be conducted to explore the optimal approach for plerixafor in patients with mobilization failure. The incidence of mobilization failure among donors is lower. However, plerixafor is not approved among donors with mobilization failure. Moreover, several clinical studies in donors have shown a beneficial effect of plerixafor. In addition, the adverse events of plerixafor are mild and transient, which can overcome the adverse events due to G-CSF. This review assessed the current role and effects of plerixafor in stem cell mobilization for autologous and allogeneic stem cell transplantations.

Keywords: stem cell mobilization, apheresis, autologous stem cell transplantation, allogeneic stem cell transplantation

Introduction

Stem cell transplantation (SCT) is an established treatment for many hematologic malignancies. The proportion of patients requiring SCT is still increasing. In 2010 >30,000 patients received SCT in Europe. Meanwhile in 2019 >48,000 SCTs were performed; 59% were autologous and 41% allogeneic SCT. Autologous SCT was commonly performed for plasma cell disorders (55%) and lymphoma (36%) and the main indications for allogeneic SCT were acute leukemia (54%) followed by myelodysplastic syndrome (MDS) (12%).[1]

Hematopoietic stem cells are collected from the bone marrow or the peripheral blood. Hematologic recovery is faster and morbidity is lower if stem cells are collected from the peripheral blood. In addition, promoting patient's comfort the use of peripheral blood is preferred for a SCT. CD34, a surface marker, is expressed on progenitor stem cells. The number of CD34+ cells in the peripheral blood is used for monitoring

Correspondence: Yavuz M Bilgin
Department of Internal Medicine/
Hematology, Admiraal de Ruijter
Hospital, PO Box 15, Goes, 4460 AA, the
Netherlands
Tel +31-88-1250000
Email y.bilgin@adrz.nl

the collection time of the peripheral blood stem cells and is a reliable predictor for a successful stem cell mobilization. Peripheral CD34+ cell count is correlated with the number of collected CD34+ cells.[2,3] CD34+ cells can be yielded from peripheral blood after mobilization via apheresis. The most commonly used agent for stem cell mobilization is granulocyte colony-stimulating factor (G-CSF); it is injected subcutaneously for several days until sufficient CD34+ cells are measured in the peripheral blood.

Under normal conditions the number of CD34+ cells in the peripheral blood is negligible (only <0.05% of the total leukocyte count). After mobilization with G-CSF this increases up to 5–15 times and the CD34+ cells accounted for up to 6% of the total leukocyte count.[4] If the pre-apheresis CD34+ count is $<5\times10^9$/L, sufficient stem cell collection is not likely. If the CD34+ count is $>20\times10^9$/L the chance of collecting sufficient stem cells in one apheresis session is >90%.[5] A position statement by the European Group for Blood and Marrow Transplantation mentioned that a pre-apheresis CD34+ count $>20\times10^9$/L is sufficient to start stem cell collection.[6] Whereas in patients with a pre-apheresis CD34+ count between 10–20 $\times10^9$/L the collection of sufficient stem cells can be frequently achieved with >1 apheresis sessions.[6,7]

Stem cells can be mobilized either with (chemomobilization) or without chemotherapy. In mobilization without chemotherapy, G-CSF is administered for 4 days after

disease-specific chemotherapy and stem cells are collected by apheresis on day 5, if the CD34+ cells in the peripheral blood are $> 20\times10^9$/L (Figure 1A). In chemomobilization, G-CSF is administered after the mobilization-chemotherapy until there are sufficient CD34+ cells for a successful stem cell collection (Figure 1B). Therefore, with chemomobilization the timing of stem cell collection is unpredictable. In addition, there is a higher risk of bone marrow damage and toxicity resulting in more hospitalization. Nonetheless, chemomobilization results in a higher mobilization yield and has an anticancer effect.[8] In patients with non-Hodgkin's lymphoma (NHL) disease-specific chemotherapy followed by stem cell mobilization is the preferred strategy, which is effective and avoids additional chemomobilization in these heavily treated patients.[6] In patients with multiple myeloma (MM) chemomobilization with high-dose (≥3 g/m^2) cyclophosphamide is the most commonly used strategy. However, a recent study reported that chemomobilization with low-dose cyclophosphamide (2 g/m^2) is a safe mobilization regimen with stem cell collection rates comparable to that of high-dose cyclophosphamide.[9] Further, healthy donors are mobilized with G-CSF 1–2 daily subcutaneous injections for 4–5 days (Figure 1C).

A collection of CD34+ cell count of $\geq2\times10^6$/kg is considered as sufficient for a SCT. Nevertheless, transplantations with a CD34+ cell count of $\geq5\times10^6$/kg are
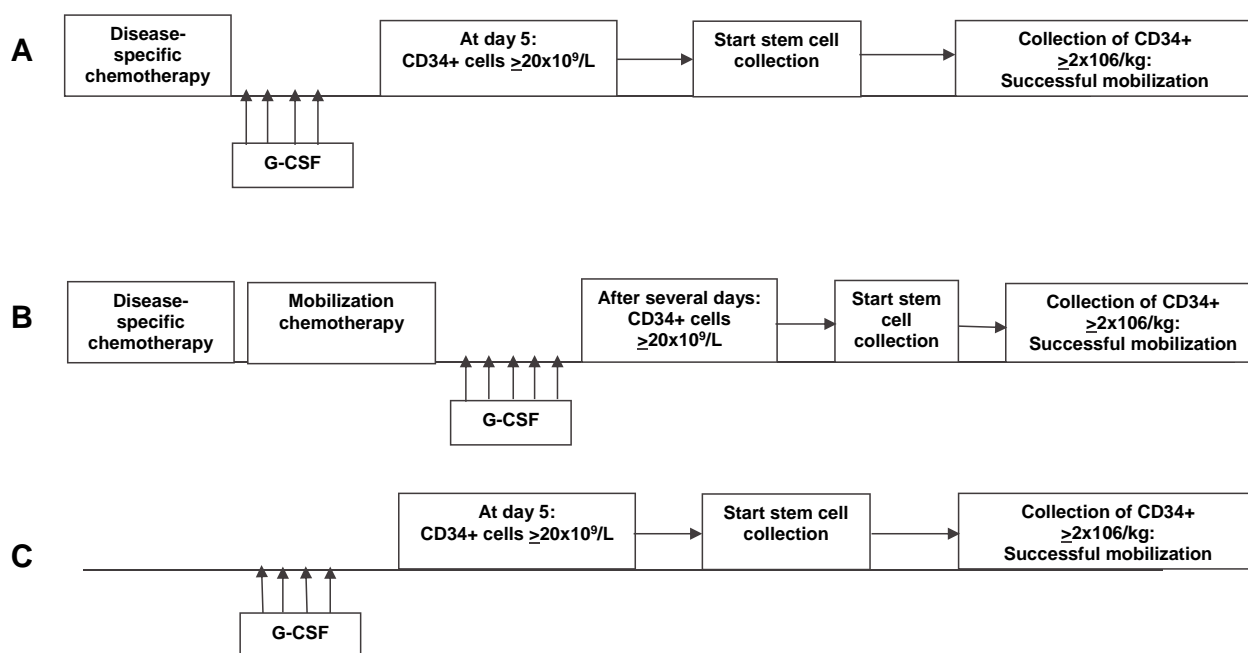
Figure 1 Strategies for stem cell mobilization in practice. Possible strategies in stem cell mobilization: with G-CSF only (**A**), with chemotherapy (**B**), in healthy donors (**C**). **Abbreviation:** G-CSF, granulocyte colony-stimulating factor.

associated with faster hematopoietic recovery, thereby resulting in a lower incidence of blood transfusions and shorter hospital stay.[10] Further, a recent study showed a better 5-year overall survival when the patients are transplanted with higher number of CD34+ cells ($>2.65 \times 10^6$/kg).[11]

Mobilization Failure Among Patients and Donors

Mobilization failure is defined as not able to collect 2×10^6/kg CD34+ cells.[12] Mobilization failure is an important issue and has significant consequences among patients. The incidence of mobilization failure among patients is not fully documented, however the rate varies between 6% and 23%.[8,13] These patients cannot receive an autologous SCT and, subsequently, cannot be treated adequately. This has a significant impact on outcome with a 3-year survival rate of 33% in patients with mobilization failure and 71% in those with a successful mobilization.[14] A second attempt to remobilize with G-CSF is not effective and has a high failure rate. In a previous study only 23% of patients collected sufficient CD34+ cells with remobilization with G-CSF.[15] Mobilization failure is associated with several factors including age, advanced disease, premobilization platelet count $<100 \times 10^9$/L, multiple chemotherapy lines, previous radiotherapy, pretreatment with alkylating agents, purine analogs or immune-modulators, diabetes and smoking.[4,7]

The Italian Group for Stem Cell Transplantation GITMO (Gruppo Italiano Trapianto di Midollo Osseo) developed definitions for patients with mobilization failure.[16] Patients with peripheral blood CD34+ cells $<20 \times 10^9$/L after adequate mobilization with G-CSF and patients who are not able to collect 2×10^6/kg after ≤ 3 apheresis are characterized as proven poor mobilizers. Further, patients are defined as predicted poor mobilizers if they had a previous mobilization failure, they previously received extensive radiotherapy or they met two of the following criteria: advanced disease (≥ 2 lines of chemotherapy), refractory disease, extensive bone marrow involvement or cellularity $<30\%$ at time of mobilization or age ≥ 65 years.

In contrast, in healthy donors mobilization failure with G-CSF is uncommon, with an estimated incidence rate between 5% and 10%.[17] Female gender, older age, low weight and premobilization low leukocyte count were associated with mobilization failure among donors.[18]

Most donors experienced side effects after administration of G-CSF ($>80\%$); which commonly include bone pain, headache, fatigue and nausea/vomiting.[19] Further, transient splenomegaly and even spleen rupture are observed.[20]

Use of Plerixafor for Autologous SCT

Plerixafor (AMD3100) is a bicyclam molecule, which reversibly blocks chemokine receptor-4 (CXCR-4), thereby inhibiting binding with its ligand stromal-cell-derived factor-1 (SDF-1). This mechanism results in the release of hematopoietic progenitor cells in the blood circulation.[21] Randomized (phase III) trials including patients with MM and NHL have shown that addition of plerixafor to upfront mobilization was associated with significantly higher CD34 + cells.[22,23] Patients with MM have a higher CD34+ cell count than those with NHL (71% vs 59%). The two trials were followed by compassionate use programs in patients with mobilization failure with G-CSF. These patients received G-CSF in combination with plerixafor in a remobilization attempt; the success rates varied from 60% to 80%.[24] Based on these studies the use of plerixafor in patients with mobilization failure with G-CSF was approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Several approaches with the use of plerixafor were investigated in patients with (predicted) mobilization failure: pre-emptive and risk-adapted approaches and in a second attempt to mobilize. The possible approaches are depicted in Figure 1. Plerixafor is added in the pre-emptive approach for patients with predicted mobilization failure based on pre-apheresis CD34+ cell count. Plerixafor is recommended in the mobilization scheme when the targeted CD34+ cell count in the peripheral blood is $<10 \times 10^9$/L on days 4–5 of mobilization with G-CSF alone. If the CD34+ cell count is $<10 \times 10^9$/L in patients with chemomobilization after 12–14 days, the incidence rate of predicted mobilization failure is high.[25] Therefore, in these patients plerixafor is recommended if the CD34+ cell count in the peripheral blood is $<10 \times 10^9$/L and the leukocyte count is increasing. In patients with CD34+ cell count between $10–20 \times 10^9$/L a dynamic approach is suggested based on patient's characteristics and treatment history.[6,7] In these patients apheresis can be started and plerixafor is mandatory if insufficient CD34+ is collected ($<2 \times 10^6$/kg). In addition, plerixafor is recommended when $<25\%$ of the targeted CD34+ cells is collected on the first day of apheresis (Figure 2A).[6,7]
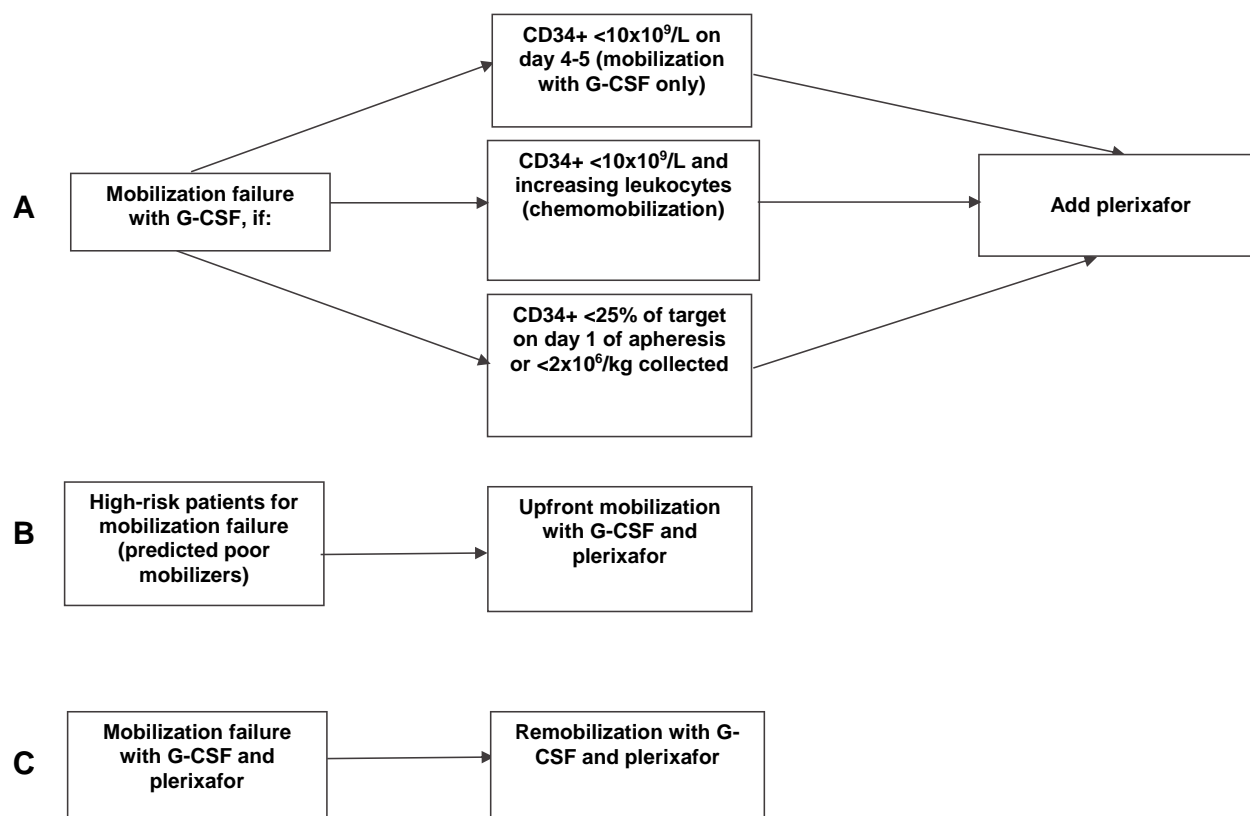
Figure 2 Approaches for use of plerixafor in patients with (predicted) mobilization failure. Approaches for the use of plerixafor: after mobilization failure with G-CSF (**A**), in patients with high-risk for mobilization failure with G-CSF (**B**), in patients with mobilization failure with G-CSF and plerixafor (**C**).
Abbreviation: G-CSF, granulocyte colony-stimulating factor.

Several studies showed that this pre-emptive approach resulted in almost 4-fold increase in CD34+ cells in the peripheral blood after administration of plerixafor, with mobilization rates of >90%.[26–28] In another approach plerixafor is administered to patients who are at high-risk for mobilization failure (predicted poor mobilizers) based on baseline characteristics (Figure 2B). A low failure rate (~4%) was observed with this approach as well.[28] Until now, it is not clear which approach is superior. Both approaches were compared in two nation-wide surveys (in France and Canada). In both studies the success rates of both approaches were comparable (>70%).[29,30] However, more studies should be performed to investigate the optimal use of plerixafor in patients with (predicted) mobilization failure.

Some studies investigated the effect of plerixafor in a second remobilization attempt after previous failure with plerixafor (Figure 2C). Also, in these studies high success rates (66–83%) were achieved.[31,32] In one analysis pretreatment with fludarabine, low premobilization platelet count ($<140 \times 10^9$/L), age >65 years and radiotherapy were

significant predictors of mobilization failure with plerixafor.[33] Other studies revealed that patients receiving fludarabine- and lenalidomide-based induction regimens required higher number of plerixafor administrations to achieve a successful mobilization.[34,35] To date novel drugs are used in induction therapy among patients with hematologic malignancies. Daratumumab, a novel monoclonal CD38 antibody, is increasingly administered to transplant-eligible newly diagnosed myeloma patients. In one study patients who received daratumumab during induction therapy required significant more administrations of plerixafor for a successful mobilization than in those who did not received it (21.7% versus 7.9%).[36] Further, the effects of the novel drugs in hematologic diseases should be elucidated in the future.

Patients with a very low pre-apheresis CD34+ cell count ($<5 \times 10^9$/L) have a significantly low probability for a successful mobilization. Even in these patients the addition of plerixafor was beneficial. The success rate was >70% in patients with a pre-apheresis CD34+ count of $<5 \times 10^9$/L.[37] In another analysis, mobilization was

successful in about 50% of patients with pre-plerixafor CD34+ cell count of 0–1×10^9/L and >70% of those with a CD34+ cell count >2×10^9/L.[34] Plerixafor is recommended to be injected subcutaneously 9–11 hours before the planned apheresis. Some studies have shown that the peak of CD34+ cells with plerixafor was observed at an earlier time (3 and 8 hours) in patients with poor mobilization.[38,39] Therefore, in these patients the peak of CD34+ cells can be missed and consequently sufficient CD34+ cells cannot be collected. One recent study has shown that the efficacy is higher if apheresis was performed on the same day as the administration of plerixafor.[40] However, there are limited studies regarding the effect of plerixafor on the kinetics of stem cell mobilization.

To date plerixafor is widely used in patients with MM and NHL with mobilization failure with G-CSF. Further, plerixafor improved mobilization rates in patients with other diagnoses. In patients with Hodgkin's lymphoma the success rate was 74%.[41] This was even higher in patients with nonhematologic malignancies (85%) and in children with malignant diseases (87%).[42,43] In addition, plerixafor was successful for the purpose of gene therapy in sickle cell disease.[44] Due to the possible mobilization of leukemic cells plerixafor is not recommended for acute myeloid leukemia (AML). Although a recent small study showed a successful mobilization with plerixafor in 5 (minimal residual disease negative) AML patients with mobilization failure.[45]

With plerixafor-induced mobilization more immature CD34+/CD38-cells and T- and NK-cells are collected, which can lead to faster hematopoietic recovery after transplantation.[46,47] Few studies have shown that the grafts of MM patients had a higher number of NK-cells and CD19+ cells than those of NHL patients. However, no difference was observed in recovery.[48–50] Further, the long-term effects of mobilization with plerixafor compared with G-CSF did no differ in progression-free survival or 5-year survival.[51] However, one study showed that 5 of 43 patients developed secondary MDS or AML 29 months after an autologous SCT.[52]

The administration of plerixafor is well tolerated and <2% of patients reported adverse events. Of which the most common were nausea/vomiting, diarrhea, fatigue, and headache. All adverse events resolved immediately and there were no grade 3 or 4 events.[53] Plerixafor is costly, this might be an important factor in the use among patients with mobilization failure. However, some studies have shown that patients who received plerixafor required less apheresis sessions, which can equalize the costs of plerixafor.[54,55] Moreover, lower cost was mentioned in cost-simulation analyses in patients with upfront stem cell mobilization with plerixafor compared to G-CSF.[56,57] More studies are warranted to evaluate the cost-effectiveness of plerixafor.

Plerixafor for Allogeneic SCT

The use of plerixafor is not approved for allogeneic SCT. Since 2011 several case reports mentioned for the first that donors had a successful mobilization using plerixafor after mobilization failure with G-CSF.[58,59] In the last years few studies have investigated the role of plerixafor among healthy donors.

In human leukocyte antigen (HLA)-identical and haplo-identical donors plerixafor was administered using several approaches: upfront,[60,64,66,67] pre-emptive after failure with G-CSF,[61,62,65,68,69] or remobilization after mobilization failure with G-CSF.[63] Moreover plerixafor was used successfully for haplo-identical SCT requiring a higher number of CD34+ cells.[61,62,65] The characteristics of these studies are depicted in Table 1. In studies with upfront approach the median collected CD34+ after single injection with plerixafor was 2.9 x 10^6/kg to 4.7×10^6/kg. Although the failure rate with a single injection with plerixafor was high (33% to 48%) and subsequently a second gift of plerixafor was necessary to achieve a successful stem cell collection. In two studies published in 2021 plerixafor was added pre-emptively to the mobilization with G-CSF.[68,69] The peripheral CD34+ cell count showed 2.9-fold and 3.4-fold increases after adding a single injection of plerixafor. In these two studies, the collected CD34+ cells was 1.1×10^6/kg and 1.6×10^6/kg with G-CSF alone and it was raised to 2.8×10^6/kg and 4.9 x 10^6/kg with the combination G-CSF and plerixafor. Therefore, these studies suggest that if the collected CD34 + cells are not sufficient for an allogeneic SCT, addition of a single injection of plerixafor to the mobilization with G-CSF can be effective. Further, in some studies with healthy donors plerixafor was administered at different doses (0.24 mg/kg or 0.32 mg/kg). In one study plerixafor was administered at a higher dose (0.48 mg/kg) resulting in a higher peak of CD34+ cells, indicating that this might improve harvesting.[70] In contrast, another study showed no difference in failure rates in patients who were injected intravenously with higher doses of plerixafor (0.32 mg/kg) compared with those who were subcutaneously injected

Table 1 Studies with Plerixafor for Allogeneic Stem Cell Transplantation

Author/ Year	No. Donors	Transplantation Setting	Mobilization Setting	Plerixafor Dose	CD34+ in PB After 1 Gift Plerixafor (Median; x10⁹/L)	Failure After 1 Gift Plerixafor	Total CD34+ Collected (Median; x10⁶/kg)
Devine/ 2008[60]	25	HLA-identical siblings	Upfront	0.24 mg/kg sc	16	33%	2.9
Hauge/ 2014[61]	6	Haplo-identical (n=4)/ HLA-identical (n=2) donors	Pre-emptive	0.24 mg/kg sc	Too small sample size	None	Too small sample size
Gattilo/ 2015[62]	10	Haplo-identical (n=8)/ HLA-identical (n=2) donors	Pre-emptive (n=8)/ contraindication to G-CSF (n=2)	0.35 mg/kg sc	41	Not documented	2.8
Fiala/ 2016[63]	32	Not documented	Remobilization	Not documented	30	None	7.1
Schroeder/ 2017[64]	21	HLA-identical siblings	Upfront, Phase 1	0.24 mg/kg sc	Not documented	33%	3.5
Schroeder/ 2017[64]	29	HLA-identical siblings	Upfront, Phase 2	0.32 mg/kg iv	Not documented	34%	2.9
Jaiswal/ 2017[65]	26	Haplo-identical donors	Pre-emptive	0.24 mg/kg sc	136	0%	2.7
de Greef/ 2019[66]	23	HLA-identical siblings	Upfront	0.32 mg/kg sc	26	48%	3.3
Chen/ 2019[67]	64	HLA-identical siblings	Upfront	0.24 mg/kg sc	19	30%	4.7
Holig/ 2021[68]	37	HLA-identical donors	Pre-emptive	0.24 mg/kg sc	44	43%	3.7
Cid/ 2021[69]	30	HLA-identical donors	Pre-emptive	0.24 mg/kg sc	55	17%	4.2

Abbreviations: HLA, human leukocyte antigen; G-CSF, granulocyte colony-stimulating factor; sc, subcutaneous; iv, intravenous; PB, peripheral blood.

with plerixafor (0.24 mg/kg).[64] More studies should be conducted to investigate the use of plerixafor for an allogeneic SCT.

In addition, among donors the use of plerixafor is well tolerated. About 60–70% presented grade 1–2 adverse events and no donor experienced grade 4 events. The most common events were tingling, pain, fatigue, nausea, diarrhea, abdominal bloating and injection site reactions.[66,68] All events resolved immediately, in contrast with G-CSF no bone pain was observed after administration of plerixafor.[67] This suggests that the adverse events of administration of G-CSF for multiple days can overcome with one or two injections of plerixafor. Further, it has been suggested that viral infections can negatively influence stem cell mobilization with G-CSF. In one case-report sufficient CD34+ cells was collected with single gift of plerixafor in a donor with influenza A and mobilization failure with G-CSF.[71] Nevertheless, the possible effects of the

COVID-19 pandemic on mobilization failure should be evaluated in the future.

Immunologic studies have shown higher CD3+ and CD4+ cell counts in grafts mobilized with plerixafor, however there was no increase in the incidence of graft-versus-host disease.[72] Some studies have shown that in allogeneic SCT engraftment of neutrophils and platelets after stem cell mobilization with plerixafor was faster than that with mobilization failure with G-CSF.[67,73]

Future Directions

Plerixafor was the first drug that has been approved for patients with mobilization failure. In recent years, early phase or preclinical trials were conducted to assess the efficacy of novel agents. The use of POL6326, a CXCR-4 antagonist, was effective based on a Phase II trial conducted on myeloma patients,[74] and another CXCR-4

antagonist, BKT140, was found to be associated with a 78-fold increase in the number of stem cells.[75] Moreover, it was even more effective than the combination of G-CSF and plerixafor. Parathormone (PTH), SEW2871, a sphingosine-1 phosphate agonist, and BIO5192, an inhibitor of VLA-4/VCAM, increased the number of stem cells in mobilization in animal studies.[76] However, the use of more novel agents with promising results among healthy donors and animals are still investigated.

Conclusions

SCT is a well-established for patients with hematologic malignancies. Mobilization failure is an important issue as it is associated with poor survival. Plerixafor is the first agent approved for patients with mobilization failure with G-CSF. The success rates are high. Therefore, more patients can be treated adequately with autologous SCT. In patients with pre-apheresis very low CD34+ cell count plerixafor is effective. Plerixafor is well tolerated with mild adverse events which resolve immediately. However, there is no consensus on the optimal approach with plerixafor in patients with mobilization failure with G-CSF. The use of plerixafor for allogeneic SCT plerixafor has not been approved yet. Only few studies have shown that plerixafor is efficient among donors. Moreover, the adverse events of G-CSF can be overcome with the use of one or two injections of plerixafor among donors. To assess the optimal use of plerixafor for autologous and allogeneic SCTs more clinical, immunologic and cost-effectiveness studies should be conducted. Further, the effects of the COVID-19 pandemic in stem cell mobilization must be elucidated in the future.

Disclosure

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Passweg JR, Baldomero H, Chabannon C, et al. Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. *Bone Marrow Transplant*. 2021. doi:10.1038/s41409-021-01227-8.
2. To LB, Dyson PG, Juttner CA. Cell-dose effect in circulating stem-cell autografting. *Lancet*. 1986;2(8503):404–405. doi:10.1016/s0140-6736(86)90096-6.
3. Weaver CH, Hazelton B, Birch R, et al. An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. *Blood*. 1995;86(10):3961–3969. doi:10.1182/blood.V86.10.3961.bloodjournal86103961

4. Namdaroglu S, Korkmaz S, Altuntas F. Management of mobilization failure in 2017. *Transfus Apher Sci*. 2017;56(6):836–844. doi:10.1016/j.transci.2017.11.017.
5. Armitage S, Hargreaves R, Samson D, Brennan M, Kanfer E, Navarrete C. CD34 counts to predict the adequate collection of peripheral blood progenitor cells. *Bone Marrow Transplant*. 1997;20(7):587–591. doi:10.1038/sj.bmt.1700938.
6. Mohty M, Hubel K, Kroger N, et al. Autologous haematopoietic stem cell mobilisation in multiple myeloma and lymphoma patients: a position statement from the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant*. 2014;49:865–872. doi:10.1038/bmt.2014.39.
7. Goker H, Etgul S, Buyukasik Y. Optimizing mobilization strategies in difficult-to-mobilize patients: the role of plerixafor. *Transfus Apher Sci*. 2015;53(1):23–29. doi:10.1016/j.transci.2015.05.011.
8. Wuchter P, Hubel K. How to find the optimal mobilisation strategy – impact, challenges and solutions. *Eur Oncol Haematol*. 2016;12(2):87–92. doi:10.17925/EOH.2016.12.02.87.
9. Zannetti BA, Saraceni F, Cellini C, et al. Low-dose cyclophosphamide versus intermediate-high-dose cyclophosphamide versus granulocyte colony-stimulating factor alone for stem cell mobilization in multiple myeloma in the era of novel agents: a multicenter retrospective analysis. *Transplant Cell Ther*. 2021;27(3):244.e1–244.e8. doi:10.1016/j.jtct.2020.12.009.
10. Siena S, Schiavo R, Pedrazzoli P, Carlo-Stella C. Therapeutic relevance of CD34 cell dose in blood cell transplantation for cancer therapy. *J Clin Oncol*. 2000;18(6):1360–1377. doi:10.1200/JCO.2000.18.6.1360.
11. Partanen A, Turunen A, Valtola J, et al. Mobilization characteristics, blood graft composition, and outcome in diffuse large B-cell lymphoma after autologous stem cell transplantation: results from the prospective multicenter GOA study. *Transfusion*. 2021;61(2):516–525. doi:10.1111/trf.16198.
12. Ataca Atilla P, Bakanay Ozturk SM, Demirer T. How to manage poor mobilizers for high dose chemotherapy and autologous stem cell transplantation? *Transfus Apher Sci*. 2017;56(2):190–198. doi:10.1016/j.transci.2016.11.005.
13. Hopman RK, DiPersio JF. Advances in stem cell mobilization. *Blood Rev*. 2014;28(1):31–40. doi:10.1016/j.blre.2014.01.001.
14. Pavone V, Gaudio F, Console G, et al. Poor mobilization is an independent prognostic factor in patients with malignant lymphomas treated by peripheral blood stem cell transplantation. *Bone Marrow Transplant*. 2006;37(8):719–724. doi:10.1038/sj.bmt.1705298.
15. Pusic I, Jiang SY, Landua S, et al. Impact of mobilization and remobilization strategies on achieving sufficient stem cell yields for autologous transplantation. *Biol Blood Marrow Transplant*. 2008;14(9):1045–1056. doi:10.1016/j.bbmt.2008.07.004.
16. Olivieri A, Marchetti M, Lemoli R, et al. Proposed definition of 'poor mobilizer' in lymphoma and multiple myeloma: an analytic hierarchy process by ad hoc working group Gruppo Italiano Trapianto di Midollo Osseo. *Bone Marrow Transplant*. 2012;47(3):342–351. doi:10.1038/bmt.2011.82.
17. Ings SJ, Balsa C, Leverett D, Mackinnon S, Linch DC, Watts MJ. Peripheral blood stem cell yield in 400 normal donors mobilised with granulocyte colony-stimulating factor (G-CSF): impact of age, sex, donor weight and type of G-CSF used. *Br J Haematol*. 2006;134(5):517–525. doi:10.1111/j.1365-2141.2006.06223.x.
18. Wang TF, Wen SH, Chen RL, et al. Factors associated with peripheral blood stem cell yield in volunteer donors mobilized with granulocyte colony-stimulating factors: the impact of donor characteristics and procedural settings. *Biol Blood Marrow Transplant*. 2008;14(11):1305–1311. doi:10.1016/j.bbmt.2008.09.002.
19. D'Souza A, Jaiyesimi I, Trainor L, Venuturumili P. Granulocyte colony-stimulating factor administration: adverse events. *Transfus Med Rev*. 2008;22(4):280–290. doi:10.1016/j.tmrv.2008.05.005.

20. Becker PS, Wagle M, Matous S, et al. Spontaneous splenic rupture following administration of granulocyte colony-stimulating factor (G-CSF): occurrence in an allogeneic donor of peripheral blood stem cells. *Biol Blood Marrow Transplant*. 1997;3:45–49.

21. Uy G, Rettig M, Cashen AF. Plerixafor, a CXCR4 antagonist for the mobilization of hematopoietic stem cells. *Expert Opin Biol Ther*. 2008;8(11):1797–1804. doi:10.1517/14712598.8.11.1797.

22. DiPersio JF, Stadtmauer EA, Nademanee A, et al. Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. *Blood*. 2009;113(23):5720–5726. doi:10.1182/blood-2008-08-174946.

23. DiPersio JF, Micallef IN, Stiff PJ, et al. Phase III prospective rando-mized double-blind placebo-controlled trial of plerixafor plus granu-locyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobi-lization and transplantation for patients with non-Hodgkin's lym-phoma. *J Clin Oncol*. 2009;27(28):4767–4773. doi:10.1200/JCO.2008.20.7209.

24. Hubel K, Fresen MM, Apperley JF, et al. European data on stem cell mobilization with plerixafor in non-Hodgkin's lymphoma, Hodgkin's lymphoma and multiple myeloma patients. A subgroup analysis of the European consortium of stem cell mobilization. *Bone Marrow Transplant*. 2012;47(8):1046–1050. doi:10.1038/bmt.2011.216.

25. Farina L, Spina F, Guidetti A, et al. Peripheral blood CD34+ cell monitoring after cyclophosphamide and granulocyte-colony-stimulating-factor: an algorithm for pre-emptive use of plerixafor. *Leuk Lymphoma*. 2014;55(2):331–336. doi:10.3109/10428194.2013.802783.

26. Worel N, Fritsch G, Agis H, et al. Plerixafor as preemptive strategy results in high success rates in autologous stem cell mobilization failure. *J Clin Apher*. 2017;32(4):224–234. doi:10.1002/jca.21496.

27. Bilgin YM, de Greef GE. Plerixafor for stem cell mobilization: the current status. *Curr Opin Hematol*. 2016;23(1):67–71. doi:10.1097/MOH.0000000000000200.

28. Giralt S, Costa L, Schriber J, et al. Optimizing autologous stem cell mobilization strategies to improve patient outcomes: consensus guidelines and recommendations. *Biol Blood Marrow Transplant*. 2014;20(3):295–308. doi:10.1016/j.bbmt.2013.10.013.

29. Sheppard D, Bredeson C, Huebsch L, Allan D, Tay J. A plerixafor-based strategy allows adequate hematopoietic stem cell collection in poor mobilizers: results from the Canadian Special Access Program. *Bone Marrow Transplant*. 2014;49(6):751–7555. doi:10.1038/bmt.2014.33.

30. Chabannon C, Bijou F, Miclea JM, Milpied N, Grouin JM, Mohty M. A nationwide survey of the use of plerixafor in patients with lym-phoid malignancies who mobilize poorly demonstrates the predomi-nant use of the "on-demand" scheme of administration at French autologous hematopoietic stem cell transplant programs. *Transfusion*. 2015;55(9):2149–2157. doi:10.1111/trf.13141.

31. Veeraputhiran M, Jain T, Cronin S, et al. Successful hematopoietic stem cell collection in patients who fail initial plerixafor mobilization for autologous stem cell transplant. *J Clin Apher*. 2014;29 (6):293–298. doi:10.1002/jca.21321.

32. Yuan S, Nademanee A, Krishnan A, Kogut N, Shayani S, Wang S. Second time a charm? Remobilization of peripheral blood stem cells with plerixafor in patients who previously mobilized poorly despite using plerixafor as a salvage agent. *Transfusion*. 2013;53 (12):3244–3250. doi:10.1111/trf.12198.

33. Lanza F, Lemoli RM, Olivieri A, et al. Factors affecting successful mobilization with plerixafor: an Italian prospective survey in 215 patients with multiple myeloma and lymphoma. *Transfusion*. 2014;54(2):331–339. doi:10.1111/trf.12265.

34. Bilgin YM, Visser O, Beckers EA, et al. Evaluation of Dutch guide-line for just-in-time addition of plerixafor to stem cell mobilization in patients who fail with granulocyte-colony-stimulating factor. *Transfusion*. 2015;55(5):1021–1027. doi:10.1111/trf.12979.

35. Laurent V, Fronteau C, Antier C, et al. Autologous stem-cell collec-tion following VTD or VRD induction therapy in multiple myeloma: a single-center experience. *Bone Marrow Transplant*. 2021;56 (2):395–399. doi:10.1038/s41409-020-01033-8.

36. Hulin C, Offner F, Moreau P, et al. Stem cell yield and transplantation in transplant-eligible newly diagnosed multiple myeloma patients receiving daratumumab+bortezomib/thalidomide/dexamethasone in the Phase 3 CASSIOPEIA study. *Haematologica*. 2021. doi:10.3324/haematol.2020.261842.

37. Maziarz RT, Nademanee AP, Micallef IN, et al. Plerixafor plus granulocyte colony-stimulating factor improves the mobilization of hematopoietic stem cells in patients with non-Hodgkin lymphoma and low circulating peripheral blood CD34+ cells. *Biol Blood Marrow Transplant*. 2013;19(4):670–675. doi:10.1016/j.bbmt.2013.01.005.

38. Lefrere F, Mauge L, Réa D, et al. A specific time course for mobi-lization of peripheral blood CD34+ cells after plerixafor injection in very poor mobilizer patients: impact on the timing of the apheresis procedure. *Transfusion*. 2013;53(3):564–569. doi:10.1111/j.1537-2995.2012.03744.x.

39. Shi PA, Miller LK, Isola LM. Prospective study of mobilization kinetics up to 18 hours after late afternoon dosing of plerixafor. *Transfusion*. 2014;54(5):1263–1268. doi:10.1111/trf.12459.

40. Cid J, Castillo C, Marin P, et al. Increased collection efficiency of CD34+ cells after mobilization with preemptive use of plerixafor followed by leukocytapheresis on the same day. *Transfusion*. 2020;60(4):779–785. doi:10.1111/trf.15711.

41. Yuan S, Nademanee A, Kaniewski M, Palmer J, Shayani S, Wang S. Efficacy of just-in-time plerixafor rescue for Hodgkin's lymphoma patients with poor peripheral blood stem cell mobilization. *Transfusion*. 2014;54(3):2015–2021. doi:10.3109/10428194.2012.713480.

42. Worel N, Apperley JF, Basak GW, et al. European data on stem cell mobilization with plerixafor in patients with nonhematologic dis-eases: an analysis of the European consortium of stem cell mobilization. *Transfusion*. 2012;52(11):2395–2400. doi:10.1111/j.1537-2995.2012.03603.x.

43. Bhunia N, Abu-Arja R, Stanek JR, et al. A multicenter report on the safety and efficacy of plerixafor based stem cell mobilization in children with malignant disorders. *Transfusion*. 2021;61 (3):894–902. doi:10.1111/trf.16260.

44. Esrick EB, Manis JP, Daley H, et al. Successful hematopoietic stem cell mobilization and apheresis collection using plerixafor alone in sickle cell patients. *Blood Rev*. 2018;2(19):2505–2512. doi:10.1182/bloodadvances.2018016725.

45. Shumilov E, Novak U, Jeker B, Taleghani BM, Bacher U, Pabst T. Hematopoietic stem cell mobilization with plerixafor is safe and effective in poorly mobilizing acute myeloid leukemia patients. *HemaSphere*. 2019;3(2):e176. doi:10.1097/HS9.0000000000000176.

46. Porrata LF. Autograft immune effector cells and survival in autolo-gous peripheral blood hematopoietic stem cell transplantation. *J Clin Apher*. 2018;33(3):324–330. doi:10.1002/jca.21611.

47. Saraceni F, Shem-Tov N, Olivieri A, Nagler A. Mobilized peripheral blood grafts include more than hematopoietic stem cells: the immu-nological perspective. *Bone Marrow Transplant*. 2015;50 (7):886–891. doi:10.1038/bmt.2014.330.

48. Valtola J, Silvennoinen R, Ropponen A, et al. Preemptive plerixafor injection added to pegfilgrastim after chemotherapy in non-Hodgkin lymphoma patients mobilizing poorly. *Ann Hematol*. 2017;96 (11):1897–1906. doi:10.1007/s00277-017-3123-6.

49. Turunen A, Partanen A, Valtola J, et al. CD34+cell mobilization, blood graft composition and posttransplant recovery in myeloma patients compared to non-Hodgkin's lymphoma patients: results of the prospective multicenter GOA study. *Transfusion*. 2020;60 (7):1519–1528. doi:10.1111/trf.15820.

50. Valtola J, Silvennoinen R, Ropponen A, et al. Blood graft composition and post-transplant recovery in myeloma patients mobilized with plerixafor: a prospective multicenter study. *Leuk Lymphoma*. 2019;60 (2):453–461. doi:10.1080/10428194.2018.1485911.

51. Micallef IN, Stiff PJ, Nademanee AP, et al. Plerixafor plus granulocyte colony-stimulating factor for patients with non-Hodgkin lymphoma and multiple myeloma: long-term follow-up report. *Biol Blood Marrow Transplant*. 2018;24(6):1187–1195. doi:10.1016/j. bbmt.2018.01.039.

52. Deol A, Abrams J, Masood A, et al. Long-term follow up of patients proceeding to transplant using plerixafor mobilized stem cells and incidence of secondary myelodysplastic syndrome/AML. *Bone Marrow Transplant*. 2013;48(8):1112–1116. doi:10.1038/ bmt.2013.10.

53. Fruehauf S. Current clinical indications for plerixafor. *Transfus Med Hemother*. 2013;40(4):246–250. doi:10.1159/000354229.

54. Milone G, Martino M, Spadaro A, et al. Plerixafor on-demand combined with chemotherapy and granulocyte colony-stimulating factor: significant improvement in peripheral blood stem cells mobilization and harvest with no increase in costs. *Br J Haematol*. 2014;164 (1):113–123. doi:10.1111/bjh.12606.

55. Hundemer M, Engelhardt M, Bruckner T, et al. Rescue stem cell mobilization with plerixafor economizes leukapheresis in patients with multiple myeloma. *J Clin Apher*. 2014;29(6):299–304. doi:10.1002/jca.21323.

56. Watts NL, Marques MB, Peavey DB, et al. Mobilization of hematopoietic progenitor cells for autologous transplantation using pegfilgrastim and plerixafor: efficacy and cost implications. *Biol Blood Marrow Transplant*. 2019;25(2):233–238. doi:10.1016/j. bbmt.2018.09.005.

57. Mohty M, Azar N, Chabannon C, et al. Plerixafor in poor mobilizers with non-Hodgkin's lymphoma: a multi-center time-motion analysis. *Bone Marrow Transplant*. 2018;53(3):246–254. doi:10.1038/s41409-017-0033-0.

58. Neumann T, Kruger WH, Busermann C, Kiefer T, Dolken G. Successful mobilization of PBSCs in a healthy volunteer donor by addition of plerixafor after failure of mobilization with G-CSF alone. *Bone Marrow Transplant*. 2011;46(5):762–763. doi:10.1038/ bmt.2010.178.

59. Schriber J, Fauble V, Sproat LO, Briggs A. Plerixafor 'just in time' for stem cell mobilization in a normal donor. *Bone Marrow Transplant*. 2011;46(7):1026–1027. doi:10.1038/bmt.2010.226.

60. Devine SM, Vij R, Rettig M, et al. Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. *Blood*. 2008;112 (4):990–998. doi:10.1182/blood-2007-12-130179.

61. Hauge AW, Haastrup EK, Sengeløv H, Minulescu L, Dickmeiss E, Fischer-Nielsen A. Addition of plerixafor for CD34+ cell mobilization in six healthy stem cell donors ensured satisfactory grafts for transplantation. *Transfusion*. 2014;54(4):1055–1058. doi:10.1111/ trf.12383.

62. Gattillo S, Marktel S, Rizzo L, et al. Plerixafor on demand in ten healthy family donors as a rescue strategy to achieve an adequate graft for stem cell transplantation. *Transfusion*. 2015;55 (8):1993–2000. doi:10.1111/trf.13059.

63. Fiala MA, Park S, Slade M, DiPersio JF, Stockerl-Goldstein KE. Remobilization of hematopoietic stem cells in healthy donors for allogeneic transplantation. *Transfusion*. 2016;56(9):2331–2335. doi:10.1111/trf.13688.

64. Schroeder MA, Rettig MP, Lopez S, et al. Mobilization of allogeneic peripheral blood stem cell donors with intravenous plerixafor mobilizes a unique graft. *Blood*. 2017;129(19):2680–2692. doi:10.1182/ blood-2016-09-739722.

65. Jaiswal SR, Bhakuni P, Joy A, et al. Impact of single-dose plerixafor as an adjunct to granulocyte colony-stimulating factor-based peripheral blood stem cell mobilization on the graft composition and outcome for T cell-replete haploidentical peripheral blood stem cell transplantation with post-transplantation cyclophosphamide: a comparative study. *Biol Blood Marrow Transplant*. 2018;24 (3):542–548. doi:10.1016/j.bbmt.2017.11.014.

66. de Greef GE, Braakman E, van der Holt B, et al. The feasibility and efficacy of subcutaneous plerixafor for mobilization of peripheral blood stem cells in allogeneic HLA-identical sibling donors: results of the HOVON-107 study. *Transfusion*. 2019;59(1):316–324. doi:10.1111/trf.15037.

67. Chen YB, Le-rademacher J, Brazauskas R, et al. Plerixafor alone for the mobilization and transplantation of HLA-matched sibling donor hematopoietic stem cells. *Blood Adv*. 2019;3(6):875–883. doi:10.1182/bloodadvances.2018027599.

68. Holig K, Schmidt H, Hutter G, et al. Salvage treatment with plerixafor in poor mobilizing allogeneic stem cell donors: results of a prospective Phase II-trial. *Bone Marrow Transplant*. 2021;56 (3):635–645. doi:10.1038/s41409-020-01053-4.

69. Cid J, Monsalvo S, Castillo C, et al. Addition of plerixafor to G-CSF in poor mobilizing healthy related donors overcame mobilization failure: an observational case series on behalf of the Grupo Espanol de Trasplante Hematopoyetico (GETH). *Transfus Apher Sci*. 2021;60 (2):103052. doi:10.1016/j.transci.2021.103052.

70. Pantin J, Purev E, Tian X, et al. Effect of high-dose plerixafor on CD34$_+$ cell mobilization in healthy stem cell donors: results of a randomized crossover trial. *Haematologica*. 2017;102(3):600–609. doi:10.3324/haematol.2016.147132.

71. Yeral M, Aytan P, Boga C. Use of plerixafor to mobilize a healthy donor infected with influenza A. *Turk J Haematol*. 2018;35 (2):138–139. doi:10.4274/tjh.2017.0304.

72. Couban S, Wong PC, Schultz KR. The case for plerixafor to replace filgrastim as the optimal agent to mobilize peripheral blood donors for allogeneic hematopoietic cell transplantation. *Exp Hematol*. 2019;70:1–9. doi:10.1016/j.exphem.2018.11.003.

73. Green MM, Chao N, Chhabra S, et al. Plerixafor (a CXCR4 antagonist) following myeloablative allogeneic hematopoietic stem cell transplantation enhances hematopoietic recovery. *J Hematol Oncol*. 2016;9(1):71. doi:10.1186/s13045-016-0301-2.

74. Schmitt S, Weinhold N, Dembrowsky K, et al. First results of a phase-II study with the new CXCR4 antagonist POL6326 to mobilize hematopoietic stem cells (HSC) in multiple myeloma (MM). *Blood*. 2010;116:824. doi:10.1182/blood.V116.21.824.824

75. Abraham M, Biyder K, Begin M, et al. Enhanced unique pattern of hematopoietic cell mobilization induced by the CXCR4 antagonist 4F-benzoyl-TN14003. *Stem Cells*. 2007;25(9):2158–2166. doi:10.1634/stemcells.2007-0161.

76. Domingues MJ, Nilsson SK, Cao B. New agents in HSC mobilization. *Int J Hematol*. 2017;105(2):141–152. doi:10.1007/ s12185-016-2156-2.

Non Vitamin K Antagonist Oral Anticoagulants in Atrial Fibrillation Patients Scheduled for Electrical Cardioversion: A Real-Life Propensity Score Matched Study

Anna Rago, Enrica Pezzullo, Marco Malvezzi Caracciolo d'Aquino, Gabriella Scognamiglio, Valentina Maria Caso, Francesco Martone, Emilio Attena, Valentina Parisi, Antonio D'Onofrio, Paolo Golino, Gerardo Nigro & Vincenzo Russo

ORIGINAL RESEARCH

Non Vitamin K Antagonist Oral Anticoagulants in Atrial Fibrillation Patients Scheduled for Electrical Cardioversion: A Real-Life Propensity Score Matched Study

Anna Rago[1]
Enrica Pezzullo[1]
Marco Malvezzi Caracciolo d'Aquino[1]
Gabriella Scognamiglio[1]
Valentina Maria Caso [2]
Francesco Martone[3]
Emilio Attena[4]
Valentina Parisi[5]
Antonio D'Onofrio[4]
Paolo Golino[1]
Gerardo Nigro [1]
Vincenzo Russo[1]

[1]Department of Cardiology, Monaldi Hospital, Naples, Italy; [2]Department of Medical Translational Sciences, University of the Study of Campania "Luigi Vanvitelli", Naples, Italy; [3]Clinical Biochemistry Unit, Monaldi Hospital, Naples, Italy; [4]Departmental Unit of Electrophysiology, Monaldi Hospital, Naples, Italy; [5]Department of Translational Medical Sciences, University of Naples Federico II, Naples, Italy

Correspondence: Vincenzo Russo
Department of Medical Transalational Sciences, University of Campania - Monaldi Hospital, Via Leonardo Bianchi, Naples, 80131, Italy
Fax +39-0817587482
Email v.p.russo@libero.it

Aim: The aim of the present study was to assess the safety and effectiveness of non-vitamin K antagonist oral anticoagulants (NOACs) versus vitamin K antagonists (VKAs) in atrial fibrillation (AF) patients undergoing electrical cardioversion (EC).

Methods: A propensity score-matched analysis was performed in order to identify two homogeneous groups including AF patients on NOACs and VKAs treatment scheduled for EC. The primary safety endpoint was major bleeding. The composite of stroke, transient ischemic attack (TIA) and systemic embolism (SE) was the primary effectiveness endpoint. The discontinuation rate of anticoagulant therapy was assessed.

Results: A total of 495 AF patients on NOACs therapy and scheduled for EC were compared to 495 VKAs recipients. No statistically significant differences in the incidence of both major bleeding (1.01% versus 1.4%; $P= 0.5$) and thromboembolic events (0.6% versus 0.8%; $P= 0.7$) were observed during a mean follow-up of 15 ± 3 months. The discontinuation rate of NOACs was significantly lower compared to VKAs (1.6% versus 3.6%, $P=0.04$).

Conclusion: We showed a safe and effective clinical profile of NOACs among AF patients scheduled for electrical cardioversion in real-life setting. Patients on NOACs therapy showed a lower discontinuation rate compared to those on VKAs.

Keywords: atrial fibrillation, electrical cardioversion, transesophageal echocardiogram, non-vitamin K antagonist oral anticoagulants, discontinuation rate, vitamin K antagonists

Introduction

An increased risk of peri-procedural thromboembolic events was shown among atrial fibrillation (AF) patients undergoing electrical cardioversion;[1] in particular it is about 7% in those who underwent cardioversion without adequate anticoagulation. The safety and efficacy of non-vitamin K antagonist oral anticoagulants (NOACs) in AF patients have been demonstrated in large randomized clinical trials (RCTs)[2–5] and real-world observational studies,[6–10] resulting in a rapid increase of their use across different clinical scenarios.[11–19] Both RCTs post-hoc analyses[20–23] and observational studies[24–26] evaluating the periprocedural use of NOACs in AF patients undergoing EC showed a similar risk of thromboembolic and bleeding events compared to vitamin K antagonists (VKAs). The current guidelines recommend the early use of NOACs before every AF cardioversion. For patients with AF

lasting more than 48 hours the oral anticoagulation is recommended at least 3 weeks before cardioversion and for at least 4 weeks afterwards.[27,28] Actually, little is still known about the clinical outcomes of AF patients on NOACs therapy undergoing EC.[29–33] The aim of our study was to assess the safety and effectiveness of NOACs compared to VKAs treatment in AF patients scheduled for transesophageal echocardiography (TEE)-guided EC.

Materials and Methods

All consecutive AF patients followed at our Institution were prospectively included in the multicenter Atrial Fibrillation Research Database, which actually includes 7230 AF patients. Among this population, we retrospectively analyzed a cohort of 1510 patients (ambulatory setting) scheduled for TEE-guided EC at our Institution-University of Campania "Luigi Vanvitelli", Naples from September 2017 to September 2019. All AF patients included in the Database were on oral anticoagulant treatment, both NOACs and VKAs. The indication to NOACs or VKAs was based on treating physician's decision. All AF patients were OAC treatment naïve at inclusion and were treated on oral anticoagulants at least 3 weeks before cardioversion.

At each 6 months follow-up visit, during a mean follow up of was 15± 3 months, the clinical status, occurrence of stroke, transient ischemic attack (TIA), systemic embolism (SE), myocardial infarction, major bleeding (MB), clinically relevant non-major bleedings (CRNMB) or other side effects were assessed. Patients with a diagnosis of persistent non-valvular AF (duration>48 h), aged≥18 years and without contraindications to oral anticoagulant therapy were eligible for inclusion in the study. Patients with a follow-up less than one year (n:11), patients on VKAs treatment with time in therapeutic range (TTR) lower than 70% (n:29) and patients with previous left atrial thrombosis or gastrointestinal major bleeding history were excluded from the study.

The electrical cardioversions were electively performed in electrophysiology operating room through biphasic DC shock from 150 to 200 Joules, depending on the patients weight, in deep sedation with propofol administered with the anesthetist support. Adherence to treatment was defined by using the cutoff point for proportion of days covered ≥ 80% from the first anticoagulants prescription; in presence of proportion of days on anticoagulant treatment ≤ 80% we considered a therapy

discontinuation. The adherence to treatments was checked before and after cardioversion through an accurate anamnesis. The most frequent causes of anticoagulant treatment discontinuation were dyspepsia, gastrointestinal disorders and poor compliance.

Two groups (NOACs versus VKAs) without significant differences in baseline characteristics were generated by propensity score matching analysis (Table 1). The occurrence of major bleedings (MBs), defined according to the International Society of Hemostasis and Thrombosis (ISTH) criteria,[34] and of thromboembolic events, defined as composite of ischemic stroke, systemic embolism and transient ischemic attack, were considered the primary safety and effectiveness endpoints, respectively. Ischemic stroke was defined as an event of neurologic deficit in the absence of an intracranial hemorrhage, diagnosed by cerebral computerized tomography and lasting more than 24 hours. TIA was considered as a temporary neurologic deficit lasting more than 24 hours. SE was defined as an extremity or organ acute vascular occlusion.[34] The secondary safety and effectiveness endpoints included all-cause mortality and the clinically relevant non-major bleeding (CRNMB) events, respectively. CRNMB was defined as a clinically evident bleeding not meeting the criteria for MB but requiring medical intervention, pain, unscheduled contact (visit or telephone) with a physician, temporary interruption of study drug (ie, delayed or missed dosing) or impairment of daily activities according to ISTH criteria.[34] The institutional review committee of University of Campania – Monaldi Hospital approved the database and the present analysis.

Statistical Analysis

Categorical data were expressed as number and percentage, while continuous variables either as median and interquartile range [IQR] or mean and standard deviation (SD), based on their distribution assessed both by the Kolmogorov–Smirnov and the Shapiro–Wilk tests. Between group differences, for categorical variables, were assessed by the Chi-square test, as the sample size was >50 subjects, with the application of Yates correction where appropriate. Either parametric Student t test or non-parametric Mann–Whitney U-Test and Wilcoxon test were instead used to compare continuous variables, according to their distribution. The nearest neighbor propensity score matching with 1:1 ratio was used to minimize bias between NOACs and VKAs groups. The variables included in the propensity score were: age, female sex,

Table I Baseline Clinical Characteristics of the Study Population Before and After Propensity Score Matching

Variable	Before Propensity Score Matching			After Propensity Score Matching		
	NOACs (n=825)	VKAs (n=625)	P-value	NOACs (n=495)	VKAs (n=495)	P-value
Age (years)	64.1±10.4	73.9±6.4	<0.001	63.3±10.1	64.1±9.9.	0.73
Female (%)	45.4	43.1	0.58	43.5	42.8	0.72
BMI (kg/m^2),	26.8±5.9	28.8±7.1	0.82	25.9±8.3	26.4±7.1	0.77
Hypertension (%)	48.6	58.2	0.001	50.2	53.1	0.57
CHA2DS2VASc score	2.6± 1.4	3.2± 1.5	0.02	2.3±0.4	2.2 ± 0.5	0.56
HAS-BLED score	2.5 ±1.1	3.3 ±1.0	0.001	2.4 ±1.1	2.1 ±1.5	0.55
Diabetes Mellitus (%)	16	25	0.06	13	14	0.4
Heart failure (%)	17.8	27.2	0.002	22.5	23.3	0.8
Previous stroke/TIA (%)	27.8	37.4	0.001	28.8	29.3	0.6
Previous MI (%)	8.2	13.1	0.01	5.6	6.2	0.7
CrCl (milliliter/minute)	71.3±12.1	60.5±12.9	0.001	70.3±21.1	71.2±21.2	0.7
Left atrial diameter (millimeters)	45.3±5.7	46.7±5.6	0.8	46.2±4.3	47.1±5.4	0.9
LAVI (milliliter/meter^2)	32.2±1.1	33.4±1.5	0.42	32.4±2.4	33.1±1.2	0.7
LV EF (%)	53.2±6.4	44.3±6.1	0.002	55.3±5.1	54.4±3.8	0.8
TEE performed (%)	100	100		100	100	
Antiplatelets (%)	21	18	0.8	20	19	0.8
PPI (%)	88	85	0.5	86	84	0.5
Beta-blockers (%)	90	92	0.5	89	87	0.5
ACE-I or ARBs (%)	75	77	0.5	74	73	0.5

Notes: Values are expressed as mean ± SD unless otherwise stated.
Abbreviations: NOACs, non-vitamin K antagonist oral anticoagulants; SD, standard deviation; BMI, body mass index; LAVI, Indexed left atrial volume; MI, myocardial infarction; LV EF, left ventricular ejection fraction; TEE, transesophageal echocardiogram; TIA, transient ischemic attack; CrCl, creatinine clearance; PPI, proton pump inhibitors; ACE-I, angiotensin converting enzyme inhibitors; ARBs, angiotensin II receptors blockers.

BMI, hypertension, CHA2DS2-VASc score, HAS-BLED score, diabetes mellitus, heart failure, prior stroke/TIA, prior myocardial infarction (MI), glomerular filtration rate, left atrial diameter, indexed left atrial volume and left ventricular ejection fraction. All collected covariates were defined at inclusion according to clinical criteria. Persisting significant differences in baseline characteristics after matching were checked. For all test, a p value <0.05 was considered statistically significant. All statistical analyses were performed using RStudio (RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc, Boston, MA URL http://www.rstudio.com/.)

Results

1510 consecutive AF patients scheduled for TEE-guided EC and who received NOACs (n: 825) or VKAs therapy (n: 625) have been analyzed. 495 NOACs [24% in dabigatran (DAB),26% in apixaban (API), 28% in edoxaban (EDO), 22% in rivaroxaban (RIVA) treatment]and 495 VKAs recipients with similar demographic and clinical characteristics were identified by propensity score matching. Table 1 shows the baseline characteristics of study

population before and after propensity score matching. The mean follow-up was 15± 3 months for both NOACs and VKA groups. 11 patients with a follow up of < 1 year were excluded to perform an accurate analysis of the long-term safety and effectiveness outcomes of AF patients on anticoagulant therapy. None of them experienced thromboembolic or bleeding events. 29 VKAs patients with time in therapeutic range lower than 70% were excluded in order to eliminate the possible bias due to a not well controlled VKAs treatment. After PSM, all AF patients were taking only full dosages of NOACs.

A left atrial appendage thrombus was revealed by TEE in four patients (0.4%) in whom the EC was not performed and they were excluded from the analysis. After PSM, all AF patients were taking only full dosages of NOACs. Time in therapeutic range did not vary between VKA users and did not affect results.

These patients presented high thromboembolic risk and moderate renal impairment: two patients (0.4%) in the NOACs group [CHA$_2$DS$_2$VASc score: 5 and creatinine clearance (CC; calculated by Cockcroft-Gault Equation): 36 milliliter/minute in one patient in RIVA therapy;CHA$_2$
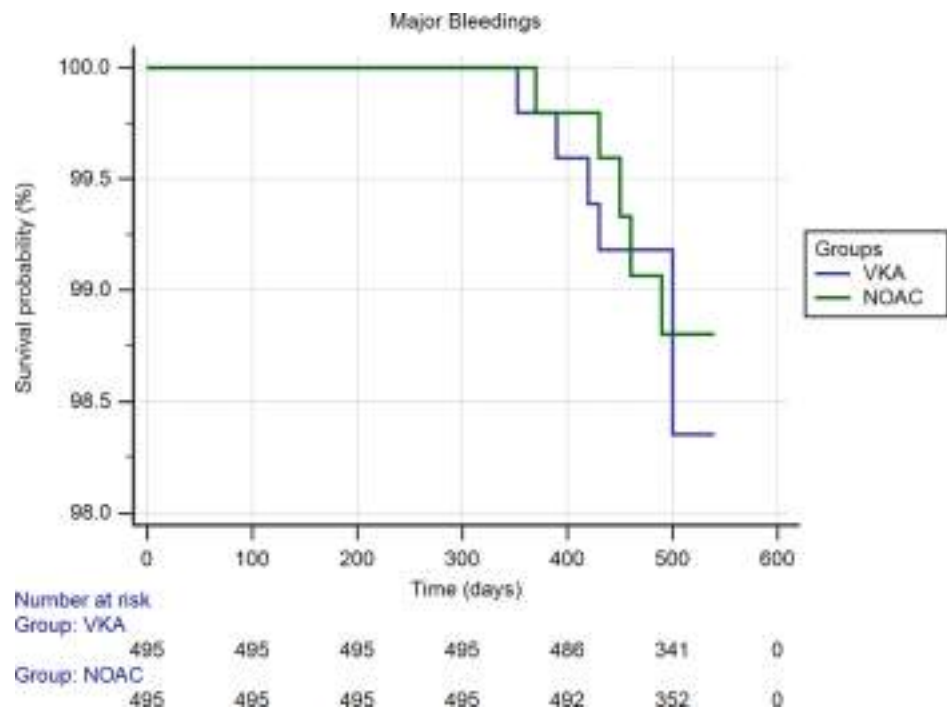
Figure 1 Kaplan Meier survival curve analysis estimating the risk of major bleedings in VKA and NOAC groups.

DS_2VASc score: 6 and CC: 34 milliliter/minute in one patient in DAB therapy]; and two patients (0.4%) in the VKAs group (INR: 2.2; CHA_2DS_2VASc c score: 4; CC: 32milliliter/minute in one patient and INR 2.1; CHA_2DS_2 VASc score: 3; CC: 31 milliliter/minute in another patient). The EC was performed within 23±2 days. There
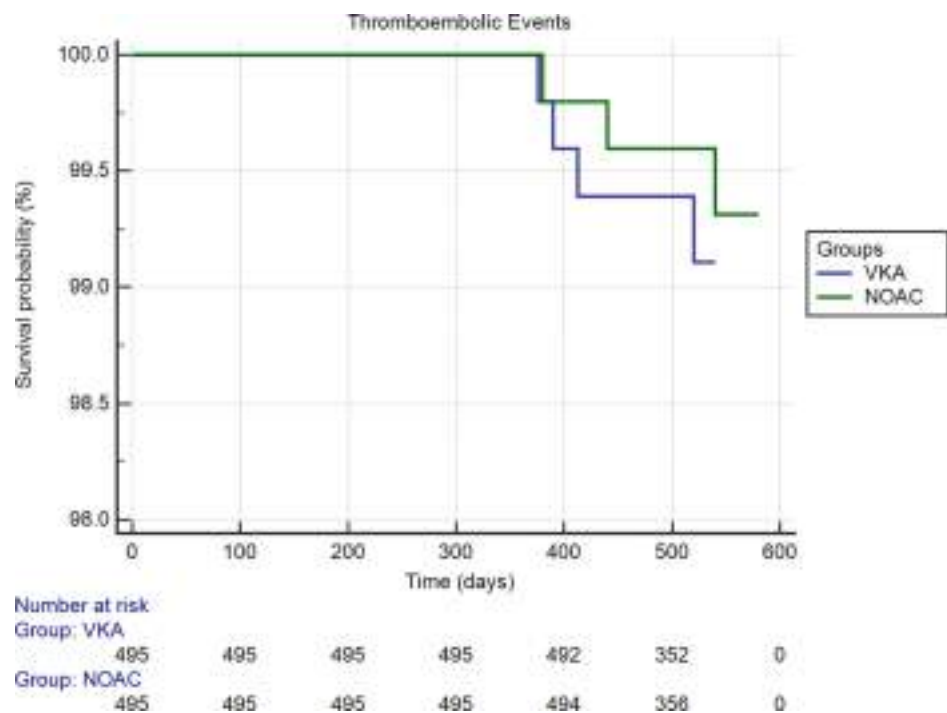
Figure 2 Kaplan Meier survival curve analysis estimating the risk of thromboembolic events in VKA and NOAC groups.
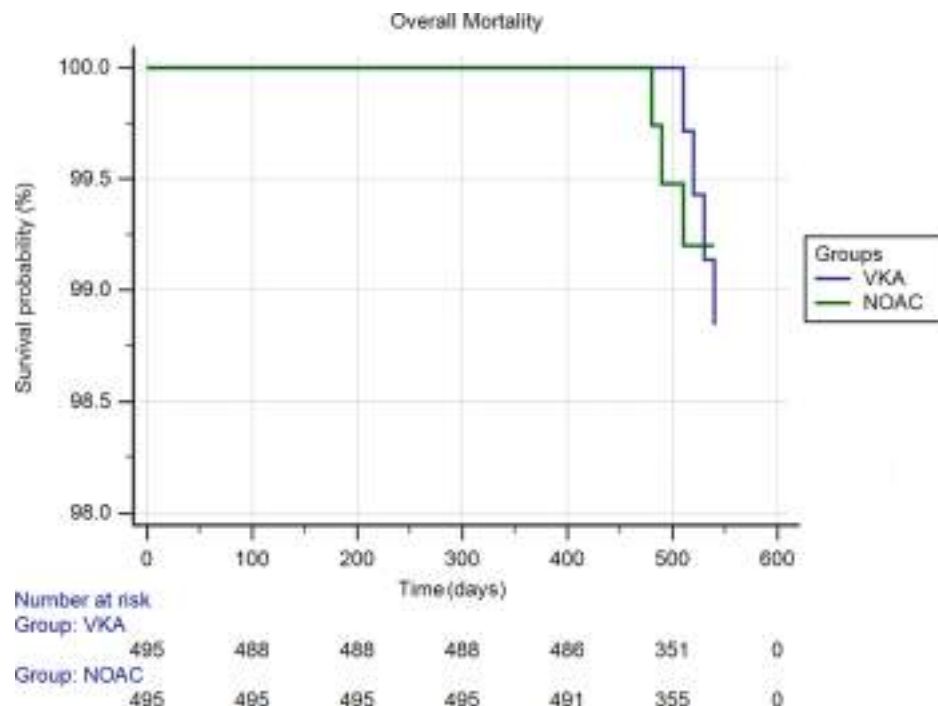
Figure 3 Kaplan Meier survival curve analysis estimating the risk of overall mortality in VKA and NOAC groups.

was an acute cardioversion success rate of 87.6% (n: 434/495) in the NOACs group and of 85.6% (n: 424/495) in the VKAs group (*P*=0.35). All patients continued the anticoagulation treatment four weeks after EC, having a CHA$_2$DS$_2$VASc score greater than 2. The primary safety endpoint was experienced by 12 patients (5 in NOACs and 7 in VKAs). The cumulative MBs incidence was 1.01% in the NOACs group and 1.4% in the VKAs group (*P*= 0.5) (Figure 1). The intracranial hemorrhages (ICH) percentage was 0.2% in NOACs group (1/495) and 0.4% in VKAs group (*P*=0.6). The gastrointestinal bleedings (GIB) percentage was 0.8% in NOACs group (4/495) and 1.01% in VKAs group (*P*=0.7). The hemorrhagic event rates were 1.6% in Dabigatran group (n 2/119), 0.9% in rivaroxaban group (1/109), 0.8% in apixaban group (n 1/129), 0.7% in edoxaban group (n 1/138). The primary effectiveness endpoint was experienced by 7 patients (3 in NOACs and 4 in VKAs). The cumulative thromboembolic events incidence was 0.6% in NOACs group and 0.8% in VKAs group (*P*= 0.7) (Figure 2). The thromboembolic event rate was 0.8% in dabigatran group (n 1/119), 0.9% in rivaroxaban group (n 1/129), 0.7% in apixaban group (n 1/129). There were not thromboembolic events in edoxaban group. We found no significant differences in terms of thromboembolic and hemorrhagic event rates between NOACs. The cumulative incidence of all-causes mortality was 0.6% (n: 3) in the

NOACs group and of 0.8% (n: 4) in VKAs group (P= 0.7) (Figure 3). The cumulative incidence of CRNMB events was 1.2% (n: 6) in the NOACs group and 2.2% (n: 11) in the VKAs group (*P*= 0.2). The anticoagulant treatment discontinuation rate was 1.6% (N: 8) in the NOACs group and 3.6% (n: 18) in the VKAs group (*P*= 0.04) (Table 2).

Discussion

The clinical performance of NOACs among AF patients scheduled for EC in real life setting represents an unmet medical need. Previous meta-analyses showed NOACs effectiveness and safety versus VKAs in the cardioversion setting including AF patients from randomized clinical trials (X–Vert, Ensure-AF and Emanate study).[35–37] Our study assessed the NOACs safety and effectiveness versus VKAs treatment including only real-world patients with AF, scheduled for TEE-guided electrical cardioversion and followed for at least twelve months. Our experience did not show significant differences in the cumulative incidence of major bleedings, thromboembolic events, CRNMB and all-cause mortality between AF patients on NOACs compared to those on VKAs. Our real-world data confirmed the results of three previous RCTs (X–VeRT, ENSURE, EMANATE) which prospectively compared NOACs (rivaroxaban, edoxaban

Table 2 Safety and Effectiveness Endpoints and Anticoagulant Treatment Discontinuation Rate in the NOACs and VKAs Groups

Variable	NOACs	VKAs	P value
	(n = 495)	(n = 495)	
Stroke/SE/TIA	0.6%	0.8%	0.7
Major Bleedings	1.01%	1.4%	0.5
All-cause mortality	0.6%	0.8%	0.7
CRNMB	1.2%	2.2%	0.2
ICH	0.2%	0.4%	0.6
GIB	0.8%	1.01%	0.7
Anticoagulant therapy discontinuation rate	1.6%	3.6%	0.04

Abbreviations: SE, systemic embolism; TIA, transient ischemic attack; CRNMB, clinically non relevant major bleedings; ICH, intracranial hemorrhages; GIB, gastrointestinal bleedings.

and apixaban) versus VKAs in patients undergoing AF cardioversion,[24–26] supporting the evidence of their use in the real-world setting.

The optimal and persistent use of NOACs is of pivotal importance for the thromboembolic risk reduction in AF patients. Among our study population, the NOACs treatment was characterized by a lower discontinuation rate compared to VKAs therapy, confirming previous real-world studies data.[38] Furthermore, low and similar incidences of left atrial appendage thrombus revealed by TEE were reported in NOACs (0.4%) and VKAs (0.4%) therapy groups, lower than those showed by previous prospective randomized studies in which the left atrial appendage thrombus percentage ranged from 3.5 to 8%.[25–27] These differences could be related to the different clinical features between our study cohort and those of randomized clinical trials. In particular, our patients showed a mean CHA_2DS_2VASc score (2.3±0.4 in NOACs group versus 2.2 ± 0.5 in VKAs group) lower than those of patients included in the RCTs[2.3 (1.6) in X–Vert study (24); 2–6 (SD 1·4) in Ensure-AF study;[25] 2.4±1.7 in Emanate study.[26]

The high thromboembolic risk and the moderate renal impairment might be clinical predictors of left atrial thrombus in real-world setting. Further studies on larger populations are necessary to confirm this hypothesis.

Limitations

The data collection performed in a single hospital represents a limitation of this study. The side effects detection is usually more accurate in RCTs than in real life mono-center observational studies. Since our study included mostly AF patients with low CHA2DS2VAsc score, conserved glomerular filtration rate and left ventricular ejection, our results cannot be generalized to the overall AF population undergoing electrical cardioversion. Moreover, the exclusion of VKA patients with low TTR could cause analysis bias in favour of VKA treatment. Therefore, further studies with larger sizes are needed to detect any eventual differences in haemorrhagic and thromboembolic events between patients on NOACs and those on VKAs treatment undergoing AF electrical cardioversion, given the low incidence of haemorrhagic and thromboembolic events with each of the two strategies.

Conclusions

The present propensity score-matched study showed a safe and effective clinical profile of NOACs in AF patients scheduled for electrical cardioversion with no statistically significant differences in the both bleeding and thromboembolic events incidences compared to VKAs. NOACs therapy was associated with lower discontinuation rate, supporting the hypothesis of a better compliance among AF patients undergoing EC.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Airaksinen KE, Grönberg T, Nuotio I, et al. Thromboembolic complications after cardioversion of acute atrial fibrillation: the FinCV (Finnish CardioVersion) study. *J Am Coll Cardiol*. 2013;62 (13):1187–1192. doi:10.1016/j.jacc.2013.04.089
2. Connolly SJ, Ezekowitz MD, Yusuf S, et al. Dabigatran versus Warfarin in Patients with Atrial Fibrillation. *N Eng J Med*. 2009;361 (12):1139–1151. doi:10.1056/NEJMoa0905561
3. Giugliano RP, Ruff CT, Braunwald E, et al. Edoxaban versus warfarin in patients with atrial fibrillation. *N Engl J Med*. 2013;369 (22):2093–2104. doi:10.1056/NEJMoa1310907
4. Granger CB, Alexander JH, McMurray JJV, et al. Apixaban versus Warfarin in Patients with Atrial Fibrillation. *N Eng J Med*. 2011;365 (11):981–992. doi:10.1056/NEJMoa1107039
5. Patel MR, Mahaffey KW, Garg J, et al. Rivaroxaban versus Warfarin in Nonvalvular Atrial Fibrillation. *N Eng J Med*. 2011;365 (10):883–891. doi:10.1056/NEJMoa1009638
6. Russo V, Bianchi V, Cavallaro C, et al. Efficacy and safety of dabigatran in a "real-life" population at high thromboembolic and hemorrhagic risk: data from MonaldiCare registry. *Eur Rev Med Pharmacol Sci*. 2015;19(20):3961–3967.
7. Russo V, Rago A, Proietti R, et al. Efficacy and safety of the target-specific oral anticoagulants for stroke prevention in atrial fibrillation: the real-life evidence. *Ther Adv Drug Saf*. 2017;8(2):67–75. doi:10.1177/2042098616673990

8. Russo V, Rago A, D'Onofrio A, et al. The clinical performance of dabigatran in the Italian real-life experience. *J Cardiovasc Med*. 2017;18(11):922–923. doi:10.2459/JCM.0000000000000548

9. Proietti R, Joza J, Arensi A, et al. Novel nonpharmacologic approaches for stroke prevention in atrial fibrillation: results from clinical trials. *Med Devices*. 2015;8:103–114. doi:10.2147/MDER.S70672

10. Melillo E, Carbone A, Rago A, et al. Update on Direct Oral Anticoagulants in Atrial Fibrillation Patients Undergoing Cardiac Interventional Procedures: from Clinical Trials to Real-World Evidence. *J Cardiovasc Pharmacol*. 2020;75(3):185–199. doi:10.1097/FJC.0000000000000786

11. Russo V, Carbone A, Rago A, et al. Direct Oral Anticoagulants in Octogenarians With Atrial Fibrillation: it Is Never Too Late. *J Cardiovasc Pharmacol*. 2019;73(4):207–214. doi:10.1097/FJC.0000000000000661

12. Russo V, Bottino R, Rago A, et al. Atrial Fibrillation and Malignancy: the Clinical Performance of Non-Vitamin K Oral Anticoagulants-A Systematic Review. *Semin Thromb Hemost*. 2019;45(2):205–214. doi:10.1055/s-0038-1661386

13. Russo V, Bottino R, Rago A, et al. Clinical Performance of Nonvitamin K Antagonist Oral Anticoagulants in Real-World Obese Patients with Atrial Fibrillation. *Semin Thromb Hemost*. 2020;46(8):970–976. doi:10.1055/s-0040-1715792

14. Russo V, Paccone A, Rago A, et al. Apixaban in a Morbid Obese Patient with Atrial Fibrillation: a Clinical Experience Using the Plasmatic Drug Evaluation. *J Blood Med*. 2020;11:77–81. doi:10.2147/JBM.S229526

15. Russo V, Rago A, Papa A, et al. Use of Non-Vitamin K Antagonist Oral Anticoagulants in Atrial Fibrillation Patients with Malignancy: clinical Practice Experience in a Single Institution and Literature Review. *Semin Thromb Hemost*. 2018;44(4):370–376. doi:10.1055/s-0037-1607436

16. Russo V, Attena E, Mazzone C, et al. Nonvitamin K Antagonist Oral Anticoagulants Use in Patients with Atrial Fibrillation and Bioprosthetic Heart Valves/Prior Surgical Valve Repair: a Multicenter Clinical Practice Experience. *Semin Thromb Hemost*. 2018;44(4):364–369. doi:10.1055/s-0037-1615261

17. Russo V, Carbone A, Attena E, et al. Clinical Benefit of Direct Oral Anticoagulants Versus Vitamin K Antagonists in Patients with Atrial Fibrillation and Bioprosthetic Heart Valves. *Clin Ther*. 2019;41(12):2549–2557. doi:10.1016/j.clinthera.2019.10.008

18. Russo V, Attena E, Mazzone C, et al. Real-life Performance of Edoxaban in Elderly Patients With Atrial Fibrillation: a Multicenter Propensity Score-Matched Cohort Study. *Clin Ther*. 2019;41(8):1598–1604. doi:10.1016/j.clinthera.2019.04.041

19. Russo V, Attena E, Rago A, et al. Clinical Outcome of Edoxaban vs. Vitamin K Antagonists in Patients with Atrial Fibrillation and Diabetes Mellitus: results from a Multicenter, Propensity-Matched, Real-World Cohort Study. *J Clin Med*. 2020;9(6):1621. doi:10.3390/jcm9061621

20. Nagarakanti R, Ezekowitz MD, Oldgren J, et al. Dabigatran versus warfarin in patients with atrial fibrillation: an analysis of patients undergoing cardioversion. *Circulation*. 2011;123(2):131–136. doi:10.1161/CIRCULATIONAHA.110.977546

21. Piccini JP, Stevens SR, Lokhnygina Y, et al. Outcomes after cardioversion and atrial fibrillation ablation in patients treated with rivaroxaban and warfarin in the ROCKET AF trial. *J Am Coll Cardiol*. 2013;61(19):1998–2006. doi:10.1016/j.jacc.2013.02.025

22. Flaker G, Lopes RD, Al-Khatib SM, et al. Efficacy and safety of apixaban in patients after cardioversion for atrial fibrillation: insights from the ARISTOTLE Trial (Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation). *J Am Coll Cardiol*. 2014;63(11):1082–1087. doi:10.1016/j.jacc.2013.09.062

23. Plitt A, Ezekowitz MD, De Caterina R, et al. Cardioversion of Atrial Fibrillation in ENGAGE AF-TIMI 48. *Clin Cardiol*. 2016;39(6):345–346. doi:10.1002/clc.22537

24. Cappato R, Ezekowitz MD, Klein AL, et al. Rivaroxaban vs. vitamin K antagonists for cardioversion in atrial fibrillation. *Eur Heart J*. 2014;35(47):3346–3355. doi:10.1093/eurheartj/ehu367

25. Goette A, Merino JL, Ezekowitz MD, et al. Edoxaban versus enoxaparin-warfarin in patients undergoing cardioversion of atrial fibrillation (ENSURE-AF): a randomised, open-label, phase 3b trial. *Lancet*. 2016;388(10055):1995–2003. doi:10.1016/S0140-6736(16)31474-X

26. Ezekowitz MD, Pollack CV, Sanders P, et al. Apixaban compared with parenteral heparin and/or vitamin K antagonist in patients with nonvalvular atrial fibrillation undergoing cardioversion: rationale and design of the EMANATE trial. *Am Heart J*. 2016;179:59–68. doi:10.1016/j.ahj.2016.06.008

27. Steffel J, et al. The 2018 European Heart Rhythm Association Practical Guide on the use of non-vitamin K antagonist oral anticoagulants in patients with atrial fibrillation. *Eur Heart J*. 2018;39(16):1330–1393.

28. Hindricks G, et al. ESC Guidelines for the diagnosis and management of atrial fibrillation developed in collaboration with the European Association for Cardio-Thoracic Surgery (EACTS). *Eur Heart J*. 2020;42(5):373–498.

29. Russo V, Rago A, Papa AA, et al. Efficacy and safety of dabigatran in patients with atrial fibrillation scheduled for transoesophageal echocardiogram-guided direct electrical current cardioversion: a prospective propensity score-matched cohort study. *J Thromb Thrombolysis*. 2018;45(2):206–212. doi:10.1007/s11239-017-1599-5

30. Rago A, Papa AA, Cassese A, et al. Clinical Performance of Apixaban vs. Vitamin K Antagonists in Patients with Atrial Fibrillation Undergoing Direct Electrical Current Cardioversion: a Prospective Propensity Score-Matched Cohort Study. *Am J Cardiovasc Drugs*. 2019;19(4):421–427. doi:10.1007/s40256-019-00341-9

31. Rago A, Papa AA, Attena E, et al. Direct Current Cardioversion in Atrial Fibrillation Patients on Edoxaban Therapy Versus Vitamin K Antagonists: a Real-world Propensity Score-Matched Study. *Cardiovasc Drugs Ther*. 2020. doi:10.1007/s10557-020-07078-7

32. Stabile G, Russo V, Rapacciuolo A, et al. Transesophageal echocardiography in patients with persistent atrial fibrillation undergoing electrical cardioversion on new oral anticoagulants: a multi center registry. *Int J Cardiol*. 2015;184:283–284. doi:10.1016/j.ijcard.2015.02.075

33. Bertaglia E, Anselmino M, Zorzi A, et al. NOACs and atrial fibrillation: incidence and predictors of left atrial thrombus in the real world. *Int J Cardiol*. 2017;249:179–183. doi:10.1016/j.ijcard.2017.07.048

34. Kaatz S, Ahmad D, Spyropoulos AC, et al. Definition of clinically relevant non-major bleeding in studies of anticoagulants in atrial fibrillation and venous thromboembolic disease in non-surgical patients: communication from the SSC of the ISTH. *J Thromb Haemost*. 2015;13(11):2119–2126. doi:10.1111/jth.13140

35. Telles-Garcia N, Dahal K, Kocherla C, et al. Non-vitamin K antagonists oral anticoagulants are as safe and effective as warfarin for cardioversion of atrial fibrillation: a systematic review and meta-analysis. *Int J Cardiol*. 2018;268:143–148. doi:10.1016/j.ijcard.2018.04.034

36. Brunetti ND, Tarantino N, De Gennaro L, et al. Direct oral anti-coagulants compared to vitamin-K antagonists in cardioversion of atrial fibrillation: an updated meta-analysis. *J Thromb Thrombolysis*. 2018;45(4):550–556. doi:10.1007/s11239-018-1622-5

37. Kotecha D, Pollack CV, De Caterina R, et al. Direct Oral Anticoagulants Halve Thromboembolic Events After Cardioversion of AF Compared With Warfarin. *J Am Coll Cardiol*. 2018;72(16):1984–1986. doi:10.1016/j.jacc.2018.07.083

38. Verdecchia P, D'Onofrio A, Russo V, et al. Persistence on apixaban in atrial fibrillation patients: a retrospective multicentre study. *J Cardiovasc Med*. 2019;20(2):66–73. doi:10.2459/JCM.0000000000000744

Effect of Cytokine Gene Polymorphisms on Eltrombopag Reactivity in Japanese Patients with Immune Thrombocytopenia

Shosaku Nomura, Misao Abe, Manabu Yamaoka & Tomoki Ito

Published online: 04 Jun 2021.

Submit your article to this journal 🗗

Article views: 155

View related articles 🗗

View Crossmark data 🗗

Citing articles: 2 View citing articles 🗗

Dovepress

open access to scientific and medical research

ORIGINAL RESEARCH

Effect of Cytokine Gene Polymorphisms on Eltrombopag Reactivity in Japanese Patients with Immune Thrombocytopenia

Shosaku Nomura[1]
Misao Abe[2]
Manabu Yamaoka[2]
Tomoki Ito [ID][1]

[1]First Department of Internal Medicine, Kansai Medical University, Hirakata, Osaka, Japan; [2]Division of Blood Transfusion, Kansai Medical University, Hirakata, Osaka, Japan

Background: Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by low platelet counts resulting from antiplatelet autoantibodies. Analysis of polymorphisms in cytokine-encoding genes is important for understanding the pathophysiology of ITP and selecting appropriate treatments. We investigated associations between polymorphisms in cytokine-encoding genes and responses to therapy in Japanese patients with ITP.

Methods: The participants in this study comprised 153 patients with ITP and 70 healthy controls. We extracted data on sex, age, platelet counts, bleeding symptoms, and therapeutic responses, including those to prednisolone (PSL) and eltrombopag. Genomic DNA was isolated from peripheral blood and polymorphisms in TNF-α, IL-10, TGF-β_1, and IFN-γ genes were analyzed using the PCR-SSP method.

Results: Our results showed that the TGF-β_1 +869 C/C genotype might be related to ITP in Japanese patients. The IL-10 −592 C/C and A/A, −819 C/C and T/T, and −1082, −819, −592 ATA/ATA genotypes might be associated with reactivity to PSL. Furthermore, the IL-10 −592 C/A −819 C/T genotypes, IL-10 ACC/ATA genotype, and TGF-β_1 +869 T/T and T/C genotypes might be linked to the response to eltrombopag.

Conclusion: Our results indicate that analysis of polymorphisms in cytokine-encoding genes could aid in understanding PSL and eltrombopag responsiveness in Japanese patients with ITP.

Keywords: ITP, cytokine gene polymorphism, SNP, prednisolone, eltrombopag

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by low platelet counts resulting from antiplatelet autoantibodies.[1,2] The target of these antibodies is glycoprotein (GP), and anti-GP antibody is produced through actions of autoreactive B and T cells.[3] In particular, GPIIb/IIIa proteins serve as major target antigens recognized by platelet-reactive CD4^{+} T cells (Figure 1).[4–7] Helper T (Th) cells play an important role in the production of this antibody, and Th cells are classified into Th1, Th2, Th3 or Th17 populations based on their cytokine production pattern.[7–9] The imbalance of Th1/Th2 participates in the production of antibody in ITP, similar to several other autoimmune diseases.[3,7] Furthermore, regulatory T cells that regulate Th3 and Th17 are also important. Platelets that bind antiplatelet antibodies are destroyed by reticuloendothelial cells such as monocytes/macrophages in the spleen (Figure 1).[1–9] Additionally, antiplatelet

Correspondence: Shosaku Nomura
First Department of Internal Medicine, Kansai Medical University, 2-5-1 Shin-Machi, Hirakata, Osaka, 573-1010, Japan
Tel + 81 72 804 2754
Fax + 81 72 804 2041
Email shosaku-n@mbp.ocn.ne.jp
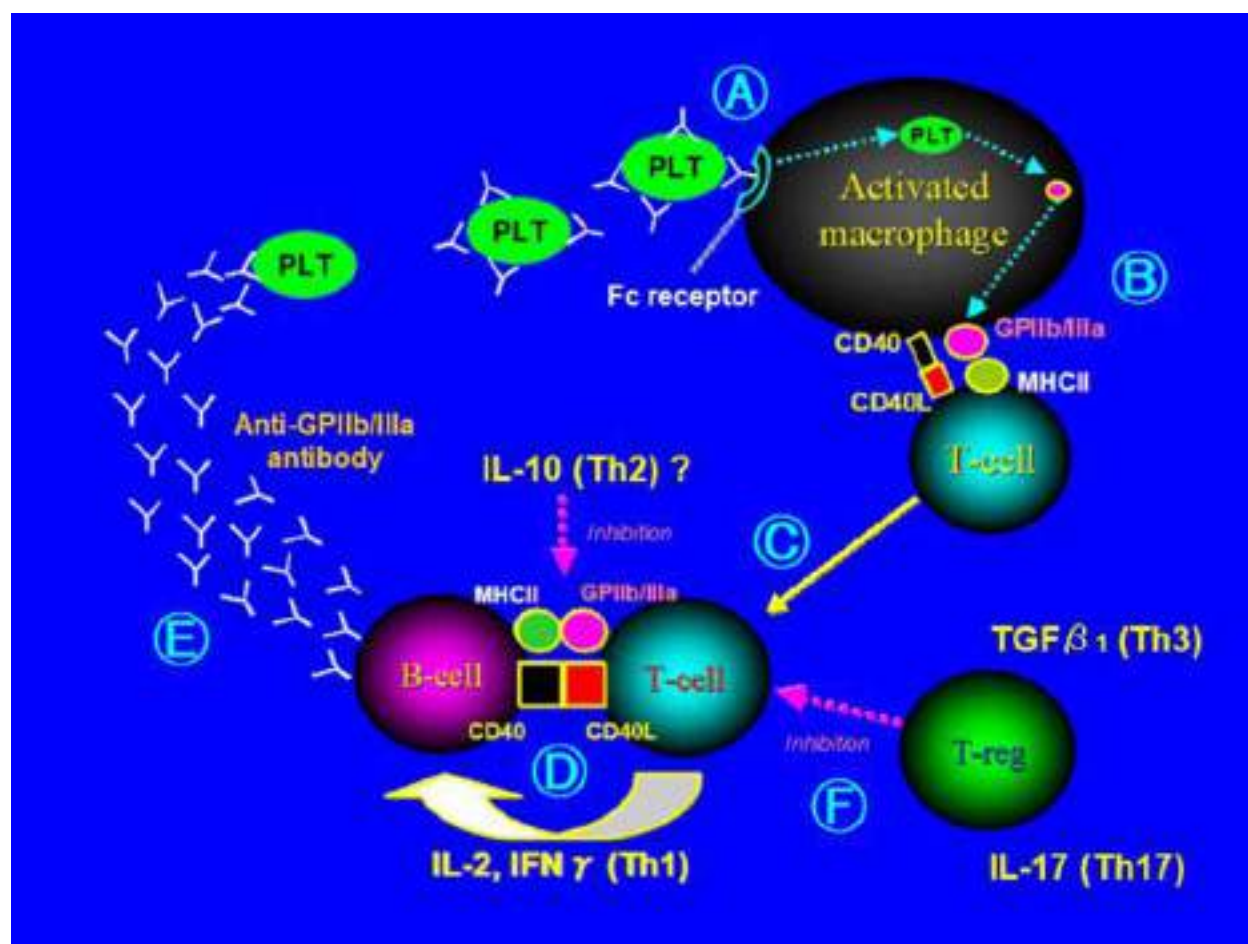
Figure 1 Presumed mechanism of anti-platelet antibody and platelet destruction in ITP. A. Anti-GPIIb/IIIa antibody-coated platelets are trapped by the Fc receptors of activated macrophages, following which the platelets are destroyed. B. Activated macrophages present GPIIb/IIIa-derived antigen to T cells. C. These T cells interact with autoreactive B cells and present the GPIIb/III-derived antigen. D. Th1 cytokines such as IL-2 and IFNγ promote the activation of B cells. E. Anti-GPIIb/IIIa antibodies are produced by activated B cells. F. T-reg's control is weakened.
Abbreviations: PLT, platelet; CD40L, CD40 ligand; MHCII, major histocompatibility complex II.

autoantibodies induce megakaryocytic maturity disorder and apoptosis, which affects platelet production.[9]

The management of ITP has changed substantially over the last 50 years as understanding of the disease has increased.[10,11] The standard therapy for ITP comprises corticosteroids.[1,2] Recently, physiologic therapeutics have been developed as viable alternatives to immunosuppressive therapy.[11,12] These drugs include thrombopoietin receptor agonists (TRAs) such as romiplostim and eltrombopag.[11,13–16] TRAs are largely regarded as a third-line therapy, a position which has remained unchanged in recent years, because they are effective for the improvement of maturation disorders in megakaryocytes.[11,12] Trials are underway to test several new drugs for their ability to treat ITP.[10] These drugs contribute to improvements in the prognosis of refractory ITP.[17–19]

It is known that autoimmune diseases have genetic factors.[20] As previously mentioned, several cytokines (interleukin [IL]-1, IL-2, IL-3 and IL-17) are very important in the pathophysiology of ITP.[7–9] Therefore, the analysis of polymorphisms in cytokine-encoding genes is important for clinical setting of ITP.[21–23] The distribution of single-nucleotide polymorphisms varies among different ethnic groups, which is also true specifically for polymorphisms in the cytokine-encoding genes related to ITP.[24–38] However, there are few reports on the association of cytokine-related polymorphisms with responsiveness to TRAs in Japanese patients with ITP. Therefore, the associations between polymorphisms in cytokine-encoding genes and therapeutic responses to steroids and TRA in Japanese patients with ITP were investigated in the present study.

Materials and Methods
Study Design and Participants
This study was approved by the ethics committee of Kansai Medical University (Osaka, Japan). All participants, or a parent or legal guardian for those under 20 years of age, provided informed consent to participate in this study, which was conducted in accordance with the Declaration of Helsinki. The participants in this study included 153 patients with ITP and 70 healthy controls. Because there are no disease-specific tests for ITP, the diagnosis of ITP was based on excluding other diagnoses.[1,2,39] We diagnosed ITP according to several international reports.[40,41]

Disease Grade by Assessment of Platelet Count and Bleeding Symptoms
Disease grade was established according to platelet count (PC) and bleeding symptoms (BS), as follows: grade 0 = PC of 81×10^9/L to 100×10^9/L and/or no BS; grade 1 = PC of 51×10^9/L to 80×10^9/L, and/or no BS or purpura 0–2; grade 2 = PC of 31×10^9/L to 50×10^9/L, and/or purpura 3–10 or mucosal bleeding; grade 3 = PC of 11×10^9/L to 30×10^9/L, and/or many purpura or moderate bleeding, with hemoglobin of 7.1–10.0 g/dL; grade 4 = PC, 0 to 10×10^9/L, and/or severe or organ bleeding, with hemoglobin of <7.0 g/dL.

Therapeutic Responses
Therapeutic responses to steroid (prednisolone; PSL) and TRA (eltrombopag) were determined using the following criteria after 3 to 12 months of therapy.

PSL start: grade 0, observation grade 1, PSL 5–10 mg/d grade 2, 3, and 4, PSL 0.5–1 mg/kg of body weight per day.

Eltrombopag start: at the time PSL ineffective. Eltrombopag 12.5–37.5 mg/d.

Responders and nonresponders to PSL or eltrombopag were defined according to the following criteria: (1) No response: PC <30×10^9/L or the absence of a twofold increase in baseline PC or hemorrhage; (2) partial response: PC between 30×10^9/L and 100×10^9/L, a minimum twofold increase in baseline PC, and the absence of hemorrhage; and (3) complete response: PC >100×10^9/L and the absence of hemorrhage. Patients who met (2) or (3) criteria were considered responders, and those meeting (1) were considered nonresponders.

The effectiveness of PSL was assessed 3 to 6 months after the start of treatment, and effectiveness of eltrombopag was assessed at 12 months. We extracted data on sex, age, PC, BS, and therapeutic responses to PSL and eltrombopag.

Genotyping
The details have already been reported.[42] Polymorphisms in the TNF-α, TGF-β$_1$, IL-10, and IFN-γ genes were analyzed using a PCR-SSP method using the Cytokine Genotyping Tray (One Lambda Inc., Los Angeles, CA, USA). For the cytokines under investigation, the following polymorphisms were analyzed. For TNF-α, the polymorphism in the promoter region at position −308 (G/A; rs1800629); for TGF-β$_1$, a polymorphism in the coding region, that is, codon 10 +869 (T/C; rs1982073); for IL-10, three polymorphisms in the promoter region, −1082 (G/A; rs1800896), −819 (C/T; rs1800871), and −592 (C/A; rs1800872); for IFN-γ, a polymorphism in the coding region at position +874 (T/A; rs2430561). DNA fragments corresponding to each cytokine were amplified in accordance with the manufacturer's instructions.

Statistical Analysis
All statistical analyses were performed using StatFlex v7 software (Artech, Osaka, Japan). Comparison of age between patient and control groups was done using an unpaired t-test. Analyses for the studied polymorphisms were done using Pearson's chi-squared test and Fisher's exact probability test. In addition, genotype distribution was analyzed for the studied polymorphisms in two groups of patients with ITP divided by whether treatment (with PSL and/or eltrombopag) had been undertaken. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for disease susceptibility and whether PSL or eltrombopag had been administered (discriminated analysis).

Results
Patient Characteristics
Table 1 exhibits the characteristics of ITP and controls. The mean age of the ITP patients was 49.2 ± 13.1 (range 19–86) years, which was significantly higher than the age of the control groups (49.2 ± 13.1, p=0.00054). In terms of sex, the frequency of females was not significantly different between patient and control groups (64.1% vs 55.7%, p=0.23517). Platelet counts ranged from 3×10^9/L to 91×10^9/L, with a mean of 26×10^9/L at the initial diagnosis. Additionally, 54 patients (35.3%) had severe thrombocytopenia. Steroid

Table 1 Baseline Characteristics of the Patients with ITP and Healthy Controls

	ITP	Control
Number	153	70
Age, years, mean±SD	**49.2±13.1**	**41.3±7.8**
Age range	19–86	24–62
Gender, F/M	98/55	39/31
First platelet count ×10^9/L (range)	26 (3–91)	
Severe thrombocytopenia*[a] No. of cases (%)	54 (35.3)	
Bleeding symptoms No. of cases (%), Yes No	94 (61.4) 59 (38.6)	
Treatment, No. of cases (%) Steroid, prednisolone TRA, eltrombopag	135 (88.2) 62 (40.5)	
Eradication of *H. pylori* Splenectomy IVIG Other, rituximab etc. Observation	19 (12.4) 5 (3.3) 18 (11.8) 22 (14.4) 18 (11.8)	
Response to steroid treatment Responsive, PR & CR (%) Unresponsive (%)	73/135 (54.1) 62/135 (45.9)	
Complications Cardiovascular, no. of cases (%) Diabetes, no. of cases (%) Malignancy, no. of cases (%)	14 (9.2) 8 (5.2) 3 (2.0)	

Notes: *[a]Platelet count <30 ×10^9/L. Bold letters/numbers represent statistical significance ($p < 0.05$).
Abbreviations: ITP, immune thrombocytopenia; TRA, thrombopoietin receptor agonist; IVIG, intravenous-immunoglobulin therapy; PR, partial response; CR, complete response.

treatment was given to 135 patients (88.2%); 73 (54.1%) were responsive and 62 (45.9%) were unresponsive. Therefore, TRA (eltrombopag) was administered in 62 patients (40.5%). Eighteen patients (11.8%) were untreated.

Association of ITP Susceptibility with Polymorphisms in Cytokine-Encoding Genes

Three of the 153 ITP patients had malignancies. Therefore, we removed these three malignancy cases from the analysis, resulting in 150 ITP patients. We detected significantly lower frequencies of the TGF-β_1 +869 C/C genotype in ITP patients compared with controls (9.3% vs 22.9%; χ^2 =

5.4154; $p = 0.01996$; OR = 0.408) (Table 2). However, no differences in IFN-γ, TNF-α, or IL-10 genotype frequencies were observed between patients with ITP and controls (Table 2).

Association of Therapeutic Responses in ITP Patients with Polymorphisms in Cytokine-Encoding Genes
PSL

A total of 135 patients with ITP began PSL treatment; 73 patients responded to PSL, whereas 62 did not. Responders had higher frequencies of the IL-10 −819 CC and −592 CC genotypes than did nonresponders (26.0% vs 3.2%; $\chi^2 = 9.9532$, $p = 0.00161$, OR = 0.124) (Table 3). In contrast, responders had a lower frequency of the IL-10 −819 TT and −592 AA genotypes than did nonresponders (31.5% vs 59.7%; $\chi^2 = 4.1178$, $p = 0.04243$, OR = 1.894) (Table 3).

Eltrombopag

A total of 62 patients were treated with eltrombopag; 32 responded to eltrombopag, whereas 30 did not. The responders had a higher frequency of the IL-10 −819 C/T and −592 C/A genotypes than did nonresponders (56.3% vs 16.7%; $\chi^2 = 4.9185$, $p = 0.02657$, OR = 0.296) (Table 4). Additionally, responders had a higher frequency of the TGF-β_1 T/C genotype than did nonresponders (68.7% vs 13.3%; $\chi^2 = 8.4153$, $p = 0.00372$, OR = 0.194) (Table 4). In contrast, eltrombopag responders had a lower frequency of the TGF-β_1 T/T genotype than nonresponders (18.8% vs 70.0%; $\chi^2 = 6.6407$, $p = 0.00997$, OR = 3.733) (Table 4).

IL10 Haplotype Frequencies and Genotypes in Drug Responders and Nonresponders

The frequency of the IL-10 ACC (−1082, −819, −592) haplotype was significantly increased in PSL and eltrombopag responders (PSL: 44.5% vs 20.2%; eltrombopag: 31.2% vs 8.3%) (Table 5). In contrast, the IL-10 ATA haplotype was decreased in PSL responders (52.8% vs 78.2%). The frequency of the ACC/ACC genotype was higher (26.0% vs 3.2%), whereas the frequency of the ATA/ATA genotype was lower in PSL responders (31.5% vs 59.7%) (Table 5). In contrast, the frequency of the ACC/ATA genotype was higher in eltrombopag responders than in nonresponders (56.3% vs 10.0%) (Table 5).

Table 2 Frequencies of Cytokine Gene Polymorphisms in ITP and Normal Controls

Polymorphism	Genotype	150 Patients N (%)	70 Controls N (%)	χ^2 Value	P value	OR	95% CI
IL-10-592	C/C	24 (16.0)	17 (24.3)	1.4468	0.22904	1.518	0.767–3.005
	A/A	66 (44.0)	28 (40.0)	0.1265	0.72206	0.909	0.538–1.537
	C/A	60 (40.0)	25 (35.7)	0.1656	0.68409	0.893	0.517–1.541
IL-10-819	C/C	24 (16.0)	17 (24.3)	1.4468	0.22904	1.518	0.767–3.005
	T/T	66 (44.0)	28 (40.0)	0.1265	0.72206	0.909	0.538–1.537
	C/T	60 (40.0)	25 (35.7)	0.1656	0.68409	0.893	0.517–1.541
IL-10-1082	G/G	0 (0)	0 (0)	–	–	–	–
	A/A	143 (95.3)	66 (94.3)	0.0028	0.95755	0.989	0.658–1.486
	G/A	7 (4.7)	4 (5.7)	0.0994	0.75253	1.224	0.347–4.320
TNF-α-308	G/G	149 (99.3)	70 (100)	0.0011	0.97395	1.007	0.674–1.504
	A/A	0 (0)	0 (0)	–	–	–	–
	G/A	1 (0.7)	0 (0)	0.4657	0.49498	–	–
TGF-β1 +869	T/T	56 (37.3)	18 (25.7)	1.4827	0.22335	0.689	0.377–1.258
	C/C	**14 (9.3)**	**16 (22.9)**	**5.4154**	**0.01996**	**0.408**	**0.189–0.883**
	T/C	80 (53.4)	36 (51.4)	0.0216	0.88315	0.964	0.594–1.566
IFN-γ +874	T/T	7 (4.7)	4 (5.7)	0.0994	0.75253	1.224	0.347–4.320
	A/A	121 (80.6)	55 (78.6)	0.0146	0.90378	0.974	0.636–1.492
	T/A	22 (14.7)	11 (15.7)	0.0303	0.86189	1.071	0.492–2.331

Note: Bold letters/numbers represent statistical significance ($p < 0.05$).
Abbreviations: N, absolute number; OR, odds ratio; CI, confidence interval; TNF, tumor necrosis factor; TGF, transforming growth factor; IL-10, interleukin-10; IFN, interferon.

Table 3 Distribution of Cytokine Genotypes in Prednisolone (PSL) Responder and Nonresponder Patients with ITP

Polymorphism	Genotype	PSL-res. 73 Patients N (%)	PSL-non. 62 Patients N (%)	χ^2 Value	P value	OR	95% CI
IL-10-592	**C/C**	**19 (26.0)**	**2 (3.2)**	**9.9532**	**0.00161**	**0.124**	**0.028–0.553**
	A/A	**23 (31.5)**	**37 (59.7)**	**4.1178**	**0.04243**	**1.894**	**1.018–3.524**
	C/A	31 (42.5)	23 (37.1)	0.1732	0.67730	0.874	0.462–1.651
IL-10-819	**C/C**	**19 (26.0)**	**2 (3.2)**	**9.9532**	**0.00161**	**0.124**	**0.028–0.553**
	T/T	**23 (31.5)**	**10 (59.7)**	**4.1178**	**0.04243**	**1.894**	**1.018–3.524**
	C/T	31 (42.5)	11 (37.1)	0.1732	0.67730	0.874	0.462–1.651
IL-10-1082	G/G	0 (0)	0 (0)	–	–	–	–
	A/A	69 (94.5)	60 (96.8)	0.00091	0.92399	1.024	0.631–1.661
	G/A	4 (5.5)	2 (3.2)	0.3675	0.54437	0.589	0.104–3.323
TNF-α-308	G/G	73 (100)	61 (98.4)	0.0044	0.94704	0.984	0.609–1.590
	A/A	0 (0)	0 (0)	–	–	–	–
	G/A	0 (0)	1 (1.6)	1.1673	0.28000	–	–
TGF-β1 +869	T/T	23 (31.5)	27 (43.6)	0.9528	0.32901	1.382	0.721–2.650
	C/C	5 (6.8)	9 (14.5)	1.7141	0.19051	2.119	0.688–6.524
	T/C	45 (61.7)	26 (41.9)	1.6468	0.19942	0.680	0.378–1.225
IFN-γ +874	T/T	4 (5.5)	1 (1.6)	1.3094	0.25251	0.294	0.036–2.391
	A/A	58 (79.5)	52 (83.9)	0.0442	0.83354	1.056	0.637–1.749
	T/A	11 (15.0)	9 (14.5)	0.0060	0.93820	0.963	0.375–2.475

Note: Bold letters/numbers represent statistical significance ($p < 0.05$).
Abbreviations: PSL-res., prednisolone responder; PSL-non., prednisolone nonresponder; other abbreviations: see Table 2.

Table 4 Distribution of Cytokine Genotypes in Eltrombopag Responder and Nonresponder Patients with ITP

Polymorphism	Genotype	Elt-res. 32 Patients N (%)	Elt-non. 30 Patients N (%)	χ^2 Value	P value	OR	95% CI
IL-10-592	C/C	1 (3.1)	1 (3.3)	0.7381	0.96421	1.067	0.064–17.820
	A/A	13 (40.6)	24 (80.0)	2.5376	0.11122	1.970	0.855–4.533
	C/A	**18 (56.3)**	**5 (16.7)**	**4.9185**	**0.02657**	**0.296**	**0.098–0.898**
IL-10-819	C/C	1 (3.1)	1 (3.3)	0.7381	0.96421	1.067	0.064–17.820
	T/T	13 (40.6)	24 (80.0)	2.5376	0.11122	1.970	0.855–4.533
	C/T	**18 (56.3)**	**5 (16.7)**	**4.9185**	**0.02657**	**0.296**	**0.098–0.898**
IL-10-1082	G/G	0 (0)	0 (0)	–	–	–	–
	A/A	32 (100)	28 (93.3)	0.0362	0.84913	0.933	0.458–1.900
	G/A	0 (0)	2 (6.7)	2.0645	0.15080	–	–
TNF-α-308	G/G	32 (100)	29 (96.7)	0.0088	0.92521	0.967	0.476–1.961
	A/A	0 (0)	0 (0)	–	–	–	–
	G/A	0 (0)	1 (3.3)	1.0489	0.49212	–	–
TGF-β1 +869	**T/T**	**6 (18.8)**	**21 (70.0)**	**6.6407**	**0.00997**	**3.733**	**1.326–10.510**
	C/C	4 (12.5)	5 (16.7)	0.1616	0.68772	1.333	0.328–5.422
	T/C	**22 (68.7)**	**4 (13.3)**	**8.4153**	**0.00372**	**0.194**	**0.060–0.629**
IFN-γ +874	T/T	0 (0)	1 (3.3)	1.0489	0.49212	–	–
	A/A	28 (87.5)	24 (80.0)	0.0566	0.81203	0.914	0.437–1.913
	T/A	4 (12.5)	5 (16.7)	0.1616	0.68772	1.333	0.328–5.422

Note: Bold letters/numbers represent statistical significance ($p < 0.05$).
Abbreviations: Elt-res., eltrombopag responder; Elt-non., eltrombopag nonresponder; other abbreviations: see Table 2.

Discussion

Cellular immunity and cytokine-related responses are very important in the pathophysiology of ITP.[1,3,8,12] Cytokine gene polymorphisms have been associated with various autoimmune diseases.[22,27] In the present study, we analyzed the frequencies of some cytokine polymorphisms of

Table 5 Individual IL-10 Haplotype Frequencies and Genotypes in Prednisolone (PSL) or Eltrombopag (Elt) Responders and Nonresponder Patients with ITP

IL-10 HT & GT	PSL-res. 73 Patients N (%)	PSL-non. 62 Patients N (%)	χ^2, p value	Elt-res. 32 Patients N (%)	Elt-non. 30 Patients N (%)	χ^2, p value
Haplotype						
ACC	**65 (44.5)**	**25 (20.2)**	**0.00247**	**20 (31.2)**	**5 (8.3)**	**0.00903**
ATA	**77 (52.8)**	**97 (78.2)**	**0.04333**	44 (68.8)	53 (88.4)	0.35616
GCC	4 (2.7)	2 (1.6)	0.54022	0 (0)	2 (3.3)	0.14751
Genotype						
ACC/ACC	**19 (26.0)**	**2 (3.2)**	**0.00161**	1 (3.1)	1 (3.3)	0.96417
ACC/ATA	27 (37.0)	21 (33.9)	0.79480	**18 (56.3)**	**3 (10.0)**	**0.00578**
ATA/ATA	**23 (31.5)**	**37 (59.7)**	**0.04243**	13 (40.6)	24 (80.0)	0.11117
GCC/ACC	0 (0)	0 (0)	–	0 (0)	0 (0)	–
GCC/ATA	4 (5.5)	2 (2.2)	0.54437	0 (0)	2 (6.7)	0.15076
GCC/GCC	0 (0)	0 (0)	–	0 (0)	0 (0)	–

Note: Bold letters/numbers represent statistical significance ($p < 0.05$).
Abbreviations: PSL-res., prednisolone responder; PSL-non., prednisolone nonresponders; Elt-res., eltrombopag responder; Elt-non., eltrombopag nonresponder; other abbreviations: see Table 2.

patients with ITP and healthy controls. The frequency of the TGF-β_1 +869 C/C genotype was significantly lower in patients with ITP than in healthy controls. TGF-β_1 is produced by dendritic cells and T-cells, and it plays important roles in regulating immune function.[43] Serum TGF-β_1 concentration is decreased in systemic lupus erythematosus (SLE).[44,45] Moreover, mice deficient in TGF-β_1 exhibit enhanced production of autoantibodies, which could be related to the pathology of SLE.[46] However, the relationship between ITP and polymorphisms in TGF-β_1 is not well defined, because there are few studies in the literature assessing TGF-β_1 in ITP patients.[24,28,30] The TGF-β_1 polymorphism might be useful as a predictor of radiographic progression but further investigation is needed into the feasibility of this.

IL-10 is an anti-inflammatory cytokine produced mainly by Th2 cells and a variety of other cells, including Th1 and Th17 cells, B cells, and macrophages.[47,48] Serum concentrations of IL-10 are elevated in the serum of patients with ITP compared with those in healthy individuals.[32] Furthermore, IL-10 has been reported to affect immunological modulation in ITP[49,50] and might, therefore, be involved in the pathophysiology of ITP.[26,32,38] In this study, however, we observed no significant differences in IL-10 genotypes in ITP patients and healthy controls. This discrepancy between studies may be related to ethnic factors. Saitoh et al[31] reported that Japanese patients with ITP did not differ significantly from healthy controls with respect to polymorphisms in IL-10.

In the present study, we investigated the relationship between such polymorphisms and therapeutic responses to PSL and eltrombopag in patients with ITP. Patients who responded to PSL had higher frequencies of IL-10 −592 C/C and −819 C/C genotypes compared with nonresponders. Similarly, PSL responders had a lower frequency of the IL-10 −592 A/A and −819 T/T genotypes than responders. Other reports have described a relationship between IL-10 −592 A/A genotype and ITP severity.[31,35] Our results partly support these previous reports. In the present study, we analyzed the IL-10 promoter polymorphism (−1082, −819, −592) haplotypes ACC, ATA, and GCC (Table 5). PSL nonresponders had a lower frequency of the ACC/ATA genotype and a higher frequency of the ATA/ATA genotype. These results suggested that presence of the ATA haplotype and absence of the ACC haplotype were associated with response to PSL or ITP severity, which is similar to some previously reported

studies.[31,32,35] It is reported that IL-10 itself participates in reactivity to steroid in a murine model of ITP.[47]

Eltrombopag is a well-established treatment for ITP and is increasingly used in second-line management.[17,19,51,52] However, the relationship between eltrombopag and cytokine gene polymorphisms has yet been reported, which we addressed in the present study. A total of 62 patients were treated with eltrombopag; 32 patients responded and 30 did not. Eltrombopag responders had higher frequencies of the IL-10 −819 C/T and −592 C/A genotypes than the nonresponders. Furthermore, the IL-10 ACC/ATA genotype was significantly more common in eltrombopag responders. Thus, the results of IL-10 haplotype and genotype analysis did not always match in terms of responses to PSL and eltrombopag therapy. Eltrombopag is a second-line therapy used in patients who do not respond to PSL.[17–19] However, the differences in IL-10 haplotypes and genotypes between eltrombopag responders and nonresponders are very interesting. It will be useful to determine the eltrombopag effect ITP patients. In addition, eltrombopag responders exhibited a higher frequency of the TGF-β_1 T/C genotype than nonresponders and a lower frequency of the T/T genotype. TGF-β_1 plays important roles in regulating immune function.[43] We previously reported that TGF-β_1 and soluble cytotoxic T-lymphocyte-associated antigen 4 (sCTLA-4) levels are increased in eltrombopag-exposed patients with ITP.[53] Our previous results suggested that eltrombopag partly modulates some immune responses through TGF-β_1 and sCTLA-4. TGF-β_1 plays an important role in the induction of tolerance by regulatory T cells in ITP.[43,53,54] Further studies are needed to determine the clinical significance of polymorphisms in TGF-β_1 and immune modulation of eltrombopag.

This study had some limitations. First, the samples were obtained from a single facility and thus our data are probably not representative of the Japanese ITP population. Second, eltrombopag is only a second-line treatment. Finally, we did not define the relationship between polymorphisms in cytokine-encoding genes and TRAs other than eltrombopag in this study. Therefore, we do not know whether all TRAs are directly linked to such polymorphisms in patients with ITP. Prospective studies are needed to confirm the observations from this study.

Conclusions

In this study, we showed that the TGF-β_1 +869 C/C genotype might be related to ITP in Japanese patients with ITP. The IL-10 −592 C/C and A/A, −819 C/C and T/T, and (−1082, −819, −592) ATA/ATA genotypes may be

associated with the response to PSL. Furthermore, the IL-10 −592 C/A, −819 C/T, and −1082, −819, −592 ACC/ATA genotypes and the TGF-β$_1$ +869 T/T and T/C genotypes might be linked to the response to eltrombopag. These results indicate that polymorphism analysis of the cytokine-encoding genes could be useful in understanding PSL and eltrombopag responsiveness in Japanese patients with ITP.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors do not have any conflicts of interest to report in this work.

References

1. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. *N Engl J Med*. 2002;346(13):995–1008. doi:10.1056/NEJMra010501

2. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113(11):2386–2393. doi:10.1182/blood-2008-07-162503

3. Semple JW, Milev Y, Cosgrave D, et al. Differences in serum cytokines levels in acute and chronic autoimmune thrombocytopenic purpura: relationhip to platelet phenotype and antiplatelet T-cell reactivity. *Blood*. 1996;87(10):4245–4254. doi:10.1182/blood.V87.10.4245. bloodjournal87104245

4. Kuwana M, Kaburaki J, Ikeda Y. Autoreactive T cells to platelet GPIIb-IIIa in immune thrombocytopenic purpura: role in production of antiplatelet autoantibody. *J Clin Invest*. 1998;102(7):1393–1402. doi:10.1172/JCI4238

5. Nomura S, Matsuzaki T, Ozaki Y, et al. Clincal significance of HLA-DRB1*0410 in Japanese patients with idiopathic thrombocytopenic purpura. *Blood*. 1998;91(10):3616–3622. doi:10.1182/blood. V91.10.3616

6. Kuwana M, Kaburaki J, Kitasato H, et al. Immunodominant epitopes on glycoprotein IIb-IIIa recognized by autoreactive T cells in patients with immune thrombocytopenic purpura. *Blood*. 2001;98(1):130–139. doi:10.1182/blood.V98.1.130

7. Zufferey A, Kapur R, Semple JW. Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). *J Clin Med*. 2017;6(2):16. doi:10.3390/jcm6020016

8. Li J, Sullivan JA, Ni H. Pathophysiology of immune thrombocytopenia. *Curr Opin Hematol*. 2018;25(5):373–381. doi:10.1097/MOH.0000000000000447

9. Marini I, Backhoul T. Pathophysiol of autoimmune thrombocytopenia: current insight with a focus on thrombopoiesis. *Hamostaseologie*. 2019;39(3):227–237. doi:10.1055/s-0039-1678732

10. Cooper N. State of the art – how I manage immune thrombocytopenia. *Br J Haematol*. 2017;177(1):39–54. doi:10.1111/bjh.14515

11. Miltiadous O, Hou M, Bussel JB. Identifying and treating refractory ITP: difficulty in diagnosis and role of combination treatment. *Blood*. 2020;135(7):472–490. doi:10.1182/blood.2019003599

12. Nomura S. Advances in diagnosis and treatment for immune thrombocytopenia. *Clin Med Insight Blood Disord*. 2016;9:15–22.

13. Bussel JB, Cheng G, Saleh MN, et al. Eltrombopag for the treatment of chronic idiopathic thrombocytopenic purpura. *N Engl J Med*. 2007;357(22):2237–2247. doi:10.1056/NEJMoa073275

14. Bussel JB, Kuter DJ, Pullarkat V, et al. Safty and efficacy of long-term treatment with romiplostim in thrombocytopenic patients with chronic ITP. *Blood*. 2009;113(10):2161–2171. doi:10.1182/blood-2008-04-150078

15. Kuter DJ, Rummel M, Roccia R, et al. Romiplostim or standard of care in patients with immune thrombocytopenia. *N Engl J Med*. 2010;363(20):1889–1899. doi:10.1056/NEJMoa1002625

16. Cheng G, Saleh MN, Marcher C, et al. Eltrombopag for management of chronic immune thrombocytopenia (RAISE): a 6-month, randomized, Phase 3 study. *Lancet*. 2011;377(9763):393–402. doi:10.1016/S0140-6736(10)60959-2

17. Taylor A, Westwood JP, Laskou F, McGuckin S, Scully M. Thrombopoietin receptor agonist therapy in thrombocytopenia: ITP and beyond. *Br J Haematol*. 2017;177(3):475–480. doi:10.1111/bjh.14564

18. Wong RSM, Saleh MN, Khelif A, et al. Safety and efficacy of long-term treatment of chronic/persistent ITP with eltrombopag: final results of the EXTEND study. *Blood*. 2017;130 (23):2527–2536. doi:10.1182/blood-2017-04-748707

19. Ghanima W, Cooper N, Rodeghiero F, Godeau B, Bussel JB. Thrombopoietin receptor agonists: tenyears later. *Haematologica*. 2019;104(6):1112–1123. doi:10.3324/haematol.2018.212845

20. Bax M, van Heemst J, Huizinga TWJ, Toes REM. Genetics of rheumatoid arthritis: what have we learned? *Immunogenetics*. 2011;63(8):459–466. doi:10.1007/s00251-011-0528-6

21. Bettinotti MP, Hartung K, Deicher H, et al. Polymorphism of the tumor necrosis factor beta gene in systemic lupus erythematosus; TNFB-MHC haplotypes. *Immunogenetics*. 1993;37(6):449–454. doi:10.1007/BF00222469

22. Vandevyver C, Raus P, Stinisen P, et al. Polymorphism of the tumor necrosis factor beta gene in multiple sclerosis and rheumatoid arthritis. *Eur J Immunogenet*. 1994;21(5):377–382. doi:10.1111/j.1744-313X.1994.tb00208.x

23. Bidwell J, Keen L, Gallagher G, et al. Cytokine gene polymorphism in human disease: online databases. *Gene Immunol*. 1999;1(1):3–19. doi:10.1038/sj.gene.6363645

24. Atabay B, Oren H, Irken G, et al. Role of transforming growth factor-beta 1 gene polymorphisms in childhood idiopathic thrombocytopenic purpura. *J Pediatr Hematol Oncol*. 2003;25(11):885–889. doi:10.1097/00043426-200311000-00012

25. Satoh T, Pandey JP, Okazaki Y, et al. Single nucleotide polymorphisms of the inflammatory cytokine genes in adults with chronic immune thrombocytopenic purpura. *Br J Haematol*. 2004;124 (6):796–801. doi:10.1111/j.1365-2141.2004.04843.x

26. Wu KH, Peng CT, Li TC, et al. Interleukin 4, interleukin 6 and interleukin 10 polymorphisms in children with acute and chronic immune thrombocytopenic purpura. *Br J Haematol*. 2005;128 (6):849–852. doi:10.1111/j.1365-2141.2005.05385.x

27. Emmerich F, Bal G, Barakat A, et al. High-level serum B-cell activating factor and promorter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br J Haematol.* 2007;136 (2):309–314. doi:10.1111/j.1365-2141.2006.06431.x

28. Ma D, Zhu X, Zhao P, et al. Profile of Th17 cytokines (IL-17, TGF-β, IL-6) and Th1 cytokine (IFN-γ) in patients with immune thrombocytopenic purpura. *Ann Hematol.* 2008;87(11):899–904. doi:10.1007/s00277-008-0535-3

29. Okulu E, Ileri T, Kosan Culha V, et al. The role of tumor necrosis factor-alpha −308 G/A and transforming growth factor-beta 1-915 G/C polymorphisms in childhood idiopathic thrombocytopenic purpura. *Turk J Haematol.* 2011;28(3):170–175. doi:10.5152/tjh.2011.50

30. Pehlivan M, Okan V, Sever T, et al. Investigation of TNF-alpha, TGF-beta 1, IL-10, IFN-gamma, MBL, GPIA, and IL1A gene polymorphisms in patients with idiopathic thrombocytopenic purpura. *Platelets.* 2011;22(8):588–595. doi:10.3109/09537104.2011.577255

31. Saitoh T, Kasamatsu T, Inoue M, et al. Interkeukin-10 gene polymorphism reflects the severity of chronic immune thrombocytopenia in Japanese patients. *Int J Lab Hematol.* 2011;33(5):526–532. doi:10.1111/j.1751-553X.2011.01320.x

32. Tesse R, Del Vecchio GC, Demattia D, et al. Association of interleukin-(IL)10 haplotypes and serum IL-10 levels in the progression of childhood immune thrombocytopenic purpura. *Gene.* 2012;505(1):53–56. doi:10.1016/j.gene.2012.05.050

33. Zhao H, Zhang Y, Xiao G, et al. Interleukin-18 gene promoter—607 A/C polymorphism and the risk of immune thrombocytopenia. *Autoimmunity.* 2014;47(7):478–481. doi:10.3109/08916934.2014.921812

34. Pavkovic M, Angelovic R, Popova-Simjanovska M, et al. Single nucleotide polymorphisms of the inflammatory xcytokine genes: interleukin-1B, tumor necrosis factor-A and tumor necrosis factor-B in adult patients with immune thrombocytopenia. *Pril.* 2015;36(1):109–115. doi:10.1515/prilozi-2015-0035

35. Ghannam DEI, Fawzy IM, Azmy E, Hakim H, Eid I. Relation of interleukin-10 promorter polymorphisms to ault chronic immune thrombocytopenic purpura in a cohort of Egyptian population. *Immunol Invest.* 2015;44(7):616–626. doi:10.3109/08820139.2015.1064948

36. Yadav DK, Tripathi AK, Kumar A, et al. Association of TNF-α −308 G>A and TNF-β +252 A.G genes polymorphisms with primary immune thrombocytopenia: a North Indian study. *Blood Coagul Fibrinolysis.* 2016;27(7):791–796. doi:10.1097/MBC.0000000000000492

37. Morgan DS, Afifi RA, EI-Hoseiny SM, Amin DG, Ibrahim SYG. The potential association of tumor necrosis factor-β (252 G/A) cytokine gene polymorphism with immune thrombocytopenic purpura among Egyptian children. *Hematology.* 2018;23(5):299–303. doi:10.1080/10245332.2017.1386429

38. Ou Y, Yang Y, Xiang X, Wu Y. Relationship between the IL-10 (−1082 A/G) polymorphism and the risk of immune/idiopathic thrombocytopenic purpura: a meta-analysis. *Cytokine.* 2020;125:154820. doi:10.1016/j.cyto.2019.154820

39. AI-Samkari H, Kuter DJ. Immune thrombocytopenia in adults: modern approaches to diagnosis and treatment. *Semin Thromb Hemost.* 2020;46(3):275–288. doi:10.1055/s-0039-1700512

40. Provan D, Stasi R, Newland AC, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood.* 2010;115(2):168–186. doi:10.1182/blood-2009-06-225565

41. Neunert C, Noroozi N, Norman G, et al. Severe bleeding events in adults and children with primary immune thrombocytopenia: a systematic review. *J Thromb Haemost.* 2015;13(3):457–464. doi:10.1111/jth.12813

42. Tsujimoto S, Ozaki Y, Ito T, Nomura S. Usefukness of cytokine gene polymorphisms for the therapeutic choice in Japanese patients with rheumatoid arthritis. *Int J Gene Med.* 2021;14:131–139. doi:10.2147/IJGM.S287505

43. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-β. *Annu Rev Immunol.* 1998;16:137–161. doi:10.1146/annurev.immunol.16.1.137

44. Gualtierotti R, Biggioggero M, Penatti AE, Meroni PL. Updating on the pathogenesis of systemic lupus erythematosus. *Autoimmun Rev.* 2010;10(1):3–7. doi:10.1016/j.autrev.2010.09.007

45. Su DL, Lu ZM, Shen MN, Li X, Sun LY. Roles of pro- and anti-inflammatory cytokines in the pathogenesis of SLE. *J Biomed Biotechnol.* 2012;2012:347141. doi:10.1155/2012/347141

46. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature.* 1992;359(6397):693–699. doi:10.1038/359693a0

47. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev.* 2008;226:205–218. doi:10.1111/j.1600-065X.2008.00706.x

48. Tian G, Li JL, Wang DG, Zhou D. Targeting IL-10 in auto-immune diseases. *Cell Biochem Biophys.* 2014;70(1):37–49. doi:10.1007/s12013-014-9903-x

49. Ma L, Liang Y, Fang M, et al. The cytokines (IFN-gamma, IL-2, IL-4, IL-10, IL-17) and Treg cytokine (TGF-beta1) kevels in adult with immune thrombocytopenia. *Pharmazie.* 2014;69(9):694–697.

50. Goelz N, Bosch AMS, Rand ML, et al. Increased levels of IL-10 and IL-1Ra counterbalance the proinflammatory cytokine pattern in acute pediatric immune thrombocytopenia. *Cytokine.* 2020;130:155078. doi:10.1016/j.cyto.2020.155078

51. Ma L, Simpson E, Li J, et al. CD8$^+$ T cells are predominantly protective and required for effective steroid therapy in murine models of immune thrombocytopenia. *Blood.* 2015;126(2):247–256. doi:10.1182/blood-2015-03-635417

52. Gonzalez-Porras JR, Bastida JM. Eltrombopag in immune thrombocytopenia: efficacy review and update on drug safety. *The Adv Drug Saf.* 2018;9(6):263–285. doi:10.1177/2042098618769587

53. Fujita S, Nakanishi T, Yoshimura H, et al. TGFβ1 and sCTLA-4 levels are increased in eltrombopag-exposed patients with ITP. *Thromb Res.* 2012;130(3):415–419. doi:10.1016/j.thromres.2012.06.002

54. Bao W, Bussel JB, Heck S, et al. Improved regulatory T-cellactivity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents. *Blood.* 2010;116(22):4639–4645. doi:10.1182/blood-2010-04-281717

Emergency Splenectomy in a Patient with Splenic Marginal Zone Lymphoma, Acute Portal Vein Thrombosis, and Chronic Viral Hepatitis B

Radmila Karpova, Andrey Gorbunov, Marina Mnatsakanyan, Aleksandr Pogromov, Irina Sokolova, Yuliya Shumskaya, Ksenia Russkova, Kirill Chernousov & Daria Momatyuk

Published online: 09 Jun 2021.

Submit your article to this journal

Article views: 113

View related articles

View Crossmark data

CASE REPORT

Emergency Splenectomy in a Patient with Splenic Marginal Zone Lymphoma, Acute Portal Vein Thrombosis, and Chronic Viral Hepatitis B

Radmila Karpova [1]
Andrey Gorbunov [1]
Marina Mnatsakanyan [2]
Aleksandr Pogromov [2]
Irina Sokolova [2]
Yuliya Shumskaya [2]
Ksenia Russkova [3]
Kirill Chernousov [1]
Daria Momatyuk [3]

[1]Department of Faculty Surgery No.1, University Clinical Hospital No.1, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia; [2]Department of Hospital Therapy No.1, University Clinical Hospital No.1, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia; [3]Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia

Correspondence: Ksenia Russkova
Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University (Sechenov University), 8-2 Trubetskaya st, Moscow, 119991, Russia
Tel +79050164054
Email russkova.ksy@gmail.com

Abstract: Splenic marginal zone lymphoma (SMZL) is a type of non-Hodgkin's lymphoma (NL) that occurs in 2 out of 100 cases and is more common in women aged >60 years. A sluggish, asymptomatic course of the disease does not exclude transformation into a malignant form that occurs in 25% of patients with SMZL. Another equally important sign of an NL is thrombosis that occurs in 3.6% to 17.1% of the cases. In this report, we present a case of emergency splenectomy in a patient owing to difficulties in the diagnosis of SMZL, rapid onset of acute portal vein thrombosis, and the fulminant enlargement of the spleen accompanied by an increased risk of its rupture. Chronic hepatitis B was likely the trigger for transformation of the disease to an aggressive course. Portal vein thrombosis and the aggressive course of SMZL with rapid enlargement of the spleen and threat of its rupture in the background of viral hepatitis B required emergency splenectomy followed by anticoagulant, antiviral, and antitumor therapy.

Keywords: splenic marginal zone lymphoma, SMZL, portal vein thrombosis, emergency splenectomy, chronic viral hepatitis B

Introduction

Splenic marginal zone lymphoma (SMZL) is a type of non-Hodgkin's lymphoma (NL) that occurs in 2 out of 100 cases and is more common in women aged >60 years.[1] A sluggish, asymptomatic course of the disease does not exclude transformation into a malignant form that occurs in 25% of patients with SMZL.[2]

Most frequently, SMZL develops in the background of viral hepatitis C, which causes antigenic stimulation of B-cells and triggers mutations in immunoglobulin coding genes.[3] A similar pathogenesis has been described in studies on SMZL among patients with viral hepatitis B.[4]

Another equally important sign of an NL is thrombosis that occurs in 3.6% to 17.1% of the cases. According to these cases, arguments for the desirability of anticoagulant administration remain controversial and require additional research.[5–7] The literature describes a clinical case report about the onset of iliac vein thrombosis in a patient with SMZL that had been caused by the antiphospholipid activity of monoclonal IgM.[8] Administration of anticoagulants in this case led to recanalization of the iliac vein.

In this report, we present a case of emergency splenectomy in a patient owing to difficulties in the diagnosis of SMZL, rapid onset of acute portal vein thrombosis,

431

and the fulminant enlargement of the spleen accompanied by an increased risk of its rupture.

Case Report

A 57-year-old female was admitted to the Clinical Hospital No. 1 of Sechenov University on August 16, 2019 because of weakness, recurrent extended abdominal pain, and an increase in body temperature up to 39°C. The patient had a medical history of untreated viral hepatitis B (inactive carriage) for over 20 years. The first symptoms presented 2 weeks before admission.

Physical examination of the patient revealed pallor of the skin and moist tongue that was furred with a white coating. The lungs were clear to auscultation bilaterally. The heart sounds were muffled at auscultation with a pulse rate of 72 beats/min. The abdomen was soft and non-tender, and the liver size was within normal limits. Splenomegaly was found during percussive estimation of the spleen, with dimensions of 13×10 cm. The spleen protruded from the costal arch within 7 cm, the surface of which was elastic and smooth during palpation.

The patient had no family history of cancer, addiction to alcohol, tobacco, or drugs, or known allergies. Signed consent of the patient for the examination and treatment was obtained.

Complete blood count and metabolic panel included: hemoglobin 98 g/L, red blood cells $3.95×10^{12}$ cells/L, platelet count $110×10^9$ cells/L, white blood cells $2.8×10^9$ cells/L, ferritin 1080 ng/mL, ferrum 5 mmol/L, total protein 71.6 g/L, albumin 37.1 g/L, total bilirubin 13.2 mmol/L, AST 27 IU/L, ALT 40 IU/L, and alkaline phosphatase 269 IU/L.

Coagulogram data included: plasma fibrinogen 5.51 g/L, prothrombin index (PTI), 74%, international normalised ratio (INR) 1.17, and partial thromboplastin time (PTT) 0.97.

Ultrasonography and computed tomography (CT) of the abdominal organs revealed normal size of the liver and intra-hepatic bile ducts. The hepatic parenchyma included multiple calcifications of up to 1 cm in diameter. The portal vein was 11 mm in diameter, and its linear blood flow velocity was estimated to be 12 cm/s. The gallbladder was normal. The spleen was enlarged to 135 mm × 75 mm × 155 mm. Its parenchyma included calcifications up to 0.5 cm in diameter and active specific tissue. The splenic vein diameter was 11 mm, and its linear blood flow velocity was 12 cm/s. No free liquids were detected in the abdominal cavity. These observations were indicative of splenomegaly, portal hypertension, and no lymphadenopathy.

During EGD, atrophic gastritis of the antrum was observed. Esophageal or gastric varicose veins were not detected. Liver fibrosis was estimated by FibroScan indicating 1st degree fibrosis (stage F1 on the METAVIR scale). The serological study showed the reactivation of chronic hepatitis (titer of HBsAg 6819 IU/mL DNA HBV [+]); therefore, antiviral therapy, including entecavir 1 mg ×1 time per day, was prescribed.

The etiology of splenomegaly remained unclear. Symptoms, fever, laboratory changes (pancytopenia), and enlargement of the spleen during reactivation of viral replication indicated the presence of a lymphoproliferative disease. Based on this fact, during the follow-up examination, bone marrow biopsy was performed. Cytological examination revealed insignificant lymphoproliferation. There were no pathological changes or lymphocytosis shown in the myelogram. There were no mutations in the JAK2 V617F, MPL 515, and 12ex genes detected by polymerase chain reaction assays.

Antibodies to platelet levels were 260% (reference range up to 200), soluble glycocalicin 70%, immunochemical examination of blood and urine demonstrated decreased IgM concentration up to the low end of the normal range, and inflammatory dysproteinemia was found.

On August 22, 2019, the patient's condition significantly deteriorated due to the onset of acute pain in the abdomen, weakness, and an increase in body temperature up to 38.5°C. The patient was urgently transferred to the Surgery Department.

On physical examination, the patient's condition was found to be severe, accompanied by skin dryness and a coated tongue. Vesicular respiration was defined throughout the lung fields, and no wheezing was detected. The respiratory rate was 20 breaths/min. Heart sounds were rhythmic with a pulse rate of 122 beats/min and blood pressure of 110/75 mmHg. The abdomen was distended and painful on palpation. There was muscle tension over the entire surface of the abdominal cavity. The patient had no stool, passage of flatus was normal. The entire left half of the abdominal cavity was occupied by the spleen, which was painful and found to have solid consistency during palpation. The spleen had increased in size by 2 cm in 3 h during dynamic monitoring. Ultrasonography and CT of the abdominal organs revealed that the portal vein was 16 mm in diameter and contained an occlusive thrombus. The dimensions of the spleen were 260×111 cm. The splenic vein diameter was 18 mm, and its linear blood flow velocity was 3 cm/s. Due to the threat of rupture of the spleen due to acute thrombosis of the portal vein, an emergency splenectomy

was performed. The removed spleen was approximately 380×210×170 mm in size (Figure 1). Histological and immunohistochemical examination of the operating material enabled the identification of splenic marginal zone lymphoma.

The postoperative period was uneventful, lab-based indicators normalized, and the patient's condition improved. The patient was prescribed antitumor therapy (rituximab 375 mg/m^2 of body surface once a week for 4 weeks) and anticoagulant therapy (rivaroxaban 20 mg once a day for 6 months). The patient was discharged on the 8th day after surgery. Blood test indicators were as follows: RDW, 16.9%; neutrophils, 63.7%; and platelet count, 99 × 10^9/L. During anticoagulant medication, thrombocytopenia and hypocoagulation persisted. Ultrasonography and CT of the abdominal organs confirmed that there were no free liquids in the abdominal cavity, portal vein diameter was estimated at 18 mm, and partial recanalization with parietal thrombi was determined.

Discussion

A long-term and asymptomatic clinical course of SMZL is an indication for dynamic observation every 3–6 months.[1] The

Figure 1 Removed spleen with thin capsule showing threat of rupture.

combination of this type of lymphoma with viral hepatitis B (HBs+) requires antiviral therapy to treat lymphoma.[9] Absence of antiviral therapy leads to mutations and errors in the DNA repair pathway with increased synthesis of pathological immunoglobulins as well as stimulation of lymphogenesis.[10,11] As a result, the benign course of SMZL is transformed into an aggressive form with a mortality rate of 10%–20%.[11] The risk of thrombotic complications is 9.8% among patients with an aggressive form of the disease that requires anticoagulant therapy.[12]

Malignant transformation of SMZL is a strict indication for splenectomy, which enables the establishment of a diagnosis and prescription of appropriate therapy. The survival rate after splenectomy is 50%–80%.[1,13] However, splenectomy is not always effective in SMZL treatment, and most patients require chemotherapy.[1]

In the present clinical case, accurate diagnosis could not be made initially owing to difficulties during the examination. The disease was long-term and asymptomatic. Chronic hepatitis B was likely the trigger for transformation of the disease into an aggressive course and the development of acute thrombosis, and fulminant enlargement of the spleen accompanied by an increased risk of its rupture required an emergency splenectomy.

After splenectomy, patients with SMZL are traditionally treated with antitumor chemotherapy (rituximab) and antiviral therapy for viral liver damage (hepatitis B or C). Antiviral therapy is added to the treatment for 1 year because the virus can be reactivated due to the effects of chemotherapy drugs and tumor-associated immunodeficiency.[1,10]

In the present case, treatment included antiviral (entecavir 1 mg once a day), antitumor (rituximab 375 mg/m^2 of patient's body surface once a week for 4 weeks), and anticoagulant therapy (rivaroxaban 20 mg once a day for 6 months), which stabilized the patient's condition.

Conclusion

Portal vein thrombosis and the aggressive course of SMZL with rapid enlargement of the spleen with the threat of its rupture in the background of viral hepatitis B requires emergency splenectomy followed by anticoagulant, antiviral, and antitumor therapy.

Consent

Written informed consent was provided by the patient to have the case details and any accompanying images published. The Institutional consilium of the Clinical Hospital

No. 1 of Sechenov University gave approval for this case report.

Author Contributions

All authors made an equal, significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Zucca E, Arcaini L, Buske C, et al. Marginal zone lymphomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2020;31(1):17–29. doi:10.1016/j.annonc.2019.10.010
2. Arcaini L, Rossi D, Paulli M. Splenic marginal zone lymphoma: from genetics to management. *Blood*. 2016;127(17):2072–2081. doi:10.1182/blood-2015-11-624312
3. Xiong W, Lv R, Li H, et al. Prevalence of hepatitis B and hepatitis C viral infections in various subtypes of B-cell non-Hodgkin lymphoma: confirmation of the association with splenic marginal zone lymphoma. *Blood Cancer J*. 2017;7(3):e548. doi:10.1038/bcj.2017.28
4. Ulcickas Yood M, Quesenberry CP, Guo D, et al. Incidence of non-Hodgkin's lymphoma among individuals with chronic hepatitis B virus infection. *Hepatology*. 2007;46(1):107–112. doi:10.1002/hep.21642
5. Mahajan A, Wun T, Chew H, White RH. Lymphoma and venous thromboembolism: influence on mortality. *Thromb Res*. 2014;133 (SUPPL. 2):S23–S28. doi:10.1016/S0049-3848(14)50004-7
6. Gangaraju R, Chen Y, Hageman L, et al. Risk of venous thromboembolism in patients with non-Hodgkin lymphoma surviving blood or marrow transplantation. *Cancer*. 2019;125(24):4498–4508. doi:10.1002/cncr.32488
7. Fedorova AS, Dmitriev VV, Lipay N, Markovets AF, Begun I, Dmitriev E. Venous thrombosis in children, adolescents and young adults with lymphomas: incidence, characteristics, risk factors and prognostic value. *Oncogematologiya*. 2018;13(2):9–20. doi:10.17650/1818-8346-2018-13-2-9-20
8. Martin SE, Abel RF. Splenic marginal zone lymphoma, iliac vein thrombosis, and monoclonal immunoglobulin Mκ antiphospholipid antibody with Annexin A5 interaction [16]. *Leuk Lymphoma*. 2006;47(9):1994–1996. doi:10.1080/10428190600731972
9. Yi S, Yan Y, Xiong W, et al. Distinct clinical characteristics draw a new prognostic model for splenic marginal zone lymphoma in HBV high prevalent region. *Oncotarget*. 2017;8(58):98757–98770. doi:10.18632/oncotarget.21931
10. Dalia S, Suleiman Y, Croy DW, Sokol L. Association of lymphoma-genesis and the reactivation of hepatitis B virus in non-hodgkin lymphoma. *Cancer Control*. 2015;22(3):360–365. doi:10.1177/107327481502200315
11. Sehn LH. Introduction to a review series: the paradox of indolent B-cell lymphoma. *Blood*. 2016;127(17):2045–2046. doi:10.1182/blood-2016-03-692442
12. Yıldız A, Albayrak M, Pala Ç, et al. The incidence and risk factors of thrombosis and the need for thromboprophylaxis in lymphoma and leukemia patients: a 9-year single-center experience. *J Oncol Pharm Pract*. 2020;26(2):386–396. doi:10.1177/1078155219851540
13. Lenglet J, Traullé C, Mounier N, et al. Long-term follow-up analysis of 100 patients with splenic marginal zone lymphoma treated with splenectomy as first-line treatment. *Leuk Lymphoma*. 2014;55 (8):1854–1860. doi:10.3109/10428194.2013.861067

Hematopoietic Stem Cell Therapy for Wiskott–Aldrich Syndrome: Improved Outcome and Quality of Life

Kanwaldeep K Mallhi, Aleksandra Petrovic & Hans D Ochs

Published online: 11 Jun 2021.

Submit your article to this journal

Article views: 404

View related articles

View Crossmark data

Citing articles: 14 View citing articles

REVIEW

Hematopoietic Stem Cell Therapy for Wiskott–Aldrich Syndrome: Improved Outcome and Quality of Life

Kanwaldeep K Mallhi[1–3]
Aleksandra Petrovic[1,2,4,*]
Hans D Ochs[2,5,*]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA, USA; [2]Department of Pediatrics, University of Washington School of Medicine, Seattle, WA, USA; [3]Division of Hematology and Oncology, Seattle Children's Hospital, Seattle, WA, USA; [4]Division of Immunology and Division of Hematology and Oncology, Seattle Children's Hospital, Seattle, WA, USA; [5]Seattle Children's Research Institute, Seattle, WA, USA

*These authors contributed equally to this work

Abstract: The Wiskott–Aldrich syndrome (WAS) is an X-linked disorder caused by mutations in the *WAS* gene resulting in congenital thrombocytopenia, eczema, recurrent infections and an increased incidence of autoimmune diseases and malignancies. Without curative therapies, affected patients have diminished life expectancy and reduced quality of life. Since WAS protein (WASP) is constitutively expressed only in hematopoietic stem cell-derived lineages, hematopoietic stem cell transplantation (HSCT) and gene therapy (GT) are well suited to correct the hematologic and immunologic defects. Advances in high-resolution HLA typing, new techniques to prevent GvHD allowing the use of haploidentical donors, and the introduction of reduced intensity conditioning regimens with myeloablative features have increased overall survival (OS) to over 90%. The development of GT for WAS has provided basic knowledge into vector selection and random integration of various viral vectors into the genome, with the possibility of inducing leukemogenesis. After trials and errors, inactivating lentiviral vectors carrying the WAS gene were successfully evaluated in clinical trials, demonstrating cure of the disease except for insufficient resolution of the platelet defect. Thus, 50 years of clinical evaluation, genetic exploration and extensive clinical trials, a lethal syndrome has turned into a curable disorder.

Keywords: Wiskott–Aldrich syndrome, WAS, X-linked thrombocytopenia, XLT, X-linked neutropenia, XLN, hematopoietic stem cell transplantation, HSCT, reduced intensity conditioning, gene therapy, GT, lentiviral vectors

Introduction

In 1937, Wiskott reported 3 brothers who died in early infancy with a history of early onset thrombocytopenia, frequent infections and severe eczema.[1] Wiskott, examining blood smears of his patients, noticed that the platelets were tiny, in contrast to the large platelets characteristic for "Morbus Werlhofii", the term used at that time for idiopathic thrombocytopenia (ITP). Seventeen years later, Aldrich observed a family with multiple generations where boys died during infancy of similar complications, demonstrating X-linked inheritance.[2] Subsequently, the Wiskott–Aldrich Syndrome (WAS) was expanded to include adaptive and innate immune deficiency, autoimmunity and lymphoid malignancies.[3–9] The identification of the molecular defect, mutations in the *WAS* gene,[10] has broadened the clinical spectrum of the syndrome.[11–15]

Correspondence: Hans D Ochs
Department of Pediatrics, University of Washington School of Medicine, Seattle Children's Research Institute, 1900 Ninth Avenue, Seattle, WA, 98101-1304, USA
Email allgau@uw.edu

Pathogenesis

The incidence of WAS has been estimated to be 1:100,000 live birth.[16] With awareness of the different clinical phenotypes and the availability of reliable diagnostic tools, the incidence of pathologic entities caused by mutations in the *WAS* gene may be higher. The WAS protein (WASP) is constitutively expressed in all hematopoietic stem cell-derived lineages and is located predominantly in the cytoplasm with the highest protein expression toward the cell membrane. WASP is a member of a distinct family of proteins that link signaling pathways to actin cytoskeleton reorganization by inducing actin polymerization mediated by actin-related protein (Arp) 2/3. WASP is organized into several functional domains.[17] The pleckstrin homology domain, located at the N-terminal region of WASP, interacts with lipids and may play a role in the localization of WASP in the immunologic synapse, the site of the interaction between various leukocyte subpopulations. A functional synapse facilitates the physical contact between antigen presenting cells, T and B cells required for a strong and lasting adaptive immune response, including class switch recombination, somatic hypermutation and cytokine production.[3–6,18,19] While circulating NK cells are normal in number, the cytotoxicity of WAS-deficient NK cells is impaired as a result of defective synapse formation.[20] For similar reasons, WASP-deficient patients as well as was-/- mice have regulatory T cells (Tregs) that fail to suppress effector cells in vitro and are incapable of controlling autoimmunity when studied in mouse models.[21] While not required for the generation of Tregs in the thymus, WASP plays a crucial role in the homeostasis and function of these cells.[22] The increased incidence of autoimmunity in WAS has been associated with deficient Treg cell function,[21,22] B cell-intrinsic loss of tolerance by positive selection of self-reactive B cells[23] and the production of a broad spectrum of autoantibodies.[24] WASP-deficient neutrophils and monocytes exhibit impaired phagocytosis and chemotaxis,[4,8,17,25,26] and monocytes, macrophages and dendritic cells from WAS patients lack the formation of podosomes and exhibit abnormal polarization and aberrant lamellipodia formation.[9] Thrombocytopenia has been explained by ineffective thrombocytopoiesis,[4] and reduced survival due to intrinsic platelet abnormalities or immune-mediated consumption.[27,28]

Clinical Spectrum of Mutations in the *WAS* Gene

As originally described by Wiskott, the syndrome is characterized by the triad of frequent infections caused by immunodeficiency, eczema and hemorrhagic diathesis due to micro-thrombocytopenia.[1] This "classic" WAS phenotype was subsequently expanded to include autoimmunity initiated by immune dysregulation, and an increased incidence of hematologic malignancies. The generation of a large data base of patients with *WAS* mutations revealed variable clinical phenotypes and allowed a reasonably accurate genotype/phenotype correlation.[29–31] The introduction of a scoring system based on disease severity and clinical phenotype (Table 1) has facilitated the categorization of patients, prognostic predictions and

Table 1 Scoring System to Define Clinical Phenotypes Associated with Mutations in the *WAS* Gene

Clinical Phenotype	XLN	iXLT	XLT.		Classic WAS		
Score	**0**	**<1**	**1**	**2**	**3**	**4**	**5**
Clinical/laboratory findings							
Thrombocytopenia	–	–/+	+	+	+	+	+
Small platelets	–	+	+	+	+	+	+
Eczema	–	–	–	(+)	+	++	–/(+)/+/++
Immunodeficiency	–/(+)	–	–/(+)	(+)	+	+	(+)/+
Infections	–/(+)	–	–	(+)	+	+/++	–/(+)/+/++
Autoimmunity and/or malignancy	–	–	–	–	–	–	+
Congenital neutropenia	+	–	–	–	–	–	–
Myelodysplasia	–/+	–	–	–	–	–	–

Notes: Scoring system: –/(+), absent or mild; –/+, intermittent thrombocytopenia, possible myelodysplasia; (+), mild, transient eczema or mild, infrequent infections not resulting in sequelae; +, thrombocytopenia, persistent but therapy-responsive eczema, and recurrent infections requiring antibiotics and often intravenous immunoglobulin prophylaxis; ++, eczema that is difficult to control and severe, life-threatening infections. Reproduced with permission from Albert MH, Notarangelo LD, Ochs HD. Clinical spectrum, pathophysiology and treatment of the Wiskott–Aldrich syndrome. *Curr Opin Hematol*. 2011;18(1):42–48.[30] Copyright © 2011, Wolters Kluwer Health.
Abbreviations: WAS, Wiskott–Aldrich syndrome; XLN, X-linked neutropenia; XLT, X-linked thrombocytopenia; iXLT, intermittent XLT.

individualized therapeutic decisions.[30] A score of 1 or 2 defines patients with XLT, while a score of 3 and 4 is reserved for those with a classic WAS phenotype; a score of 5 identifies patients who have developed autoimmunity and/or malignancies. Patients with intermittent XLT are given a score of <1 and those with XLN a score of "0". The WAS score merely reflects severity of the clinical phenotype, without considering the type of mutation.[30] Of note, the most consistent genotype/phenotype correlation was obtained when the patients were divided into two categories: WASP positive if peripheral blood mononuclear cells (PBMC) express the mutated protein at normal size, and WASP negative if the protein is absent or truncated.[29–31] The clinical phenotype may evolve over time and is often incomplete in children younger than 2 years of age. For this reason, scoring disease severity at a very young age is of little value.[30]

WAS patients with the severe phenotype and a score of 3 or 4 present typically in early childhood with bloody diarrhea, petechiae, severe eczema and recurrent, often life-threatening bacterial, viral and fungal infections. They suffer from combined immune deficiency with abnormal antibody responses, declining T cell numbers and reduced lymphocyte proliferation to anti-CD3 and to specific antigens. IgA and IgE are elevated, while IgG and IgM are generally within normal range.[17] Unless full hematologic and immunologic reconstitution by hematopoietic stem cell transplantation (HSCT) or gene therapy (GT) is achieved, quality of life is severely impacted and life expectancy reduced.[3,6]

The discovery of the WAS gene confirmed that "familial sex-linked thrombocytopenia"[32] was in fact a mild variant of WAS[11,12] with normal life expectancy, but severely reduced event-free survival probability due to life-threatening infections, cerebral hemorrhage, autoimmunity or cancer.[14] Recognizing these risks in therapeutic decision-making is important, especially in view of the frequent misdiagnosis of XLT patients as ITP. The high success rate of HSCT in WAS patients makes this option more attractive for this group of patients.[33,34]

While loss of function mutations in the WAS gene cause WAS/XLT, unique gain of function (GOF) mutations in the GTPase-binding domain interfere with the autoinhibitory confirmation of the WAS protein, leading to increased and continuous actin polymerization resulting in congenital XLN. These patients have profound neutropenia, at times associated with lymphopenia, reduced in vitro lymphoproliferation in response to anti-CD3 and

are at risk for myelodysplastic changes in the bone marrow.[15,35] The neutropenia of XLN patients respond to G-CSF, but two patients were reported to have developed a myelodysplastic syndrome and acute myelogenous leukemia, respectively, both with somatic mutations in the G-CSF receptor gene and monosomy 7 in the leukemic cells.[36]

The diagnosis of WAS/XLT should be considered in any male patient presenting with petechiae, bruises and congenital or early-onset micro-thrombocytopenia. To confirm the diagnosis, a deleterious mutation in the WAS gene is required, often associated with absent or reduced WASP expression in PBMCs. Presence of mild or severe eczema, bloody diarrhea and a history of recurrent infections supports the diagnosis. The diagnosis of XLN has to be considered in any male patient presenting with severe congenital neutropenia, and is confirmed by sequencing the WAS gene with focus on the GTPase-binding site.[15,35] WASP-interacting protein (WIP) stabilizes WASP. WIP deficiency, an autosomal recessive disorder, should be suspected in patients with features of WAS but normal sequence of the WAS gene and normal levels of mRNA but absent WAS protein. The diagnosis of WIP deficiency is confirmed by sequencing the WIPF1 gene; the treatment of choice is HSCT.[37,38]

Treatment of WAS/XLT is initiated with conventional measures including prophylactic antibiotics and antivirals for recurrent infections, and IgG replacement (IV or subcutaneous) for patients with demonstrated antibody deficiency. Platelet transfusions are used only to treat major bleeding episodes, such as acute CNS hemorrhage, gastrointestinal bleeding, or during major surgery. Eltrombopag, an oral thrombopoietin receptor agonist,[39] or romiplostim given subcutaneously,[40] have been used in WAS patients while waiting for HSCT but is less effective in raising platelet counts in WAS/XLT than in ITP. Immunosuppressive therapy, such as rituximab or other immunosuppressive therapies for autoimmune complications, may be required. Elective splenectomy has been advocated in selected patients with WAS or XLT to reverse the thrombocytopenia and prevent the bleeding tendency by increasing the number of circulating platelets.[41] However, splenectomy markedly increases the risk of septicemia[14,41] and is not recommended as routine procedure, especially not for those patients who are considered for future HSCT or gene therapy. Those who undergo splenectomy require lifelong antibiotic prophylaxis.

Hematopoietic Stem Cell Transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) is the primary curative approach in WAS patients providing long-term correction of the underlying immuno-deficiency and thrombocytopenia. The first sibling donor bone marrow (BM) HSCT for WAS was performed in 1968. Initially, following minimal conditioning, the patient established donor-derived T-cell immunity while severe thrombocytopenia persisted.[42] A second HSCT following myeloablative conditioning (MAC) regimen, from the same donor, was performed resulting in successful mye-loid engraftment and normal platelet counts.[42] Early experiences indicated reconstitution of lymphoid cells but not always platelet reconstitution, demonstrating the need for both immuno-ablation and myeloablation when transplanting WAS patients.[42–45]

Since these early attempts, HSCT outcomes have improved greatly (Table 2). Transplantation with HLA-identical sibling BM grafts has resulted in event-free survival (EFS) reaching 88% as early as 2001[46] and overall survival (OS) of 90–95% in subsequent years.[33,47,48] Results of unrelated and alternative donor HSCT for treatment of WAS have also improved over time.[49,50] Advances in high-resolution HLA allele typing and increasing availability of international donors and cord blood registries have facilitated optimal selection of unrelated donors for WAS patients. Several studies found comparable survival rates among recipients of HLA-matched sibling and HLA-matched unrelated donors. Earlier reports of long-term OS for recipients of unrelated bone marrow grafts ranged between 70%-78%,[46,51,52] improving more recently to upwards of 90%.[33,49] A collaborative study by Moratto et al, analyzing the outcomes of 194 WAS patients transplanted between 1980 and 2009, found improved transplant survival for all donor types, including matched unrelated donor grafts in those transplanted after 2000.[47]

Age at time of HSCT has been reported to consistently impact survival of WAS patients, likely related to the degree of comorbidities and higher WAS scores with increasing age. A 2001 study facilitated by the International Bone Marrow Transplant Registry (IBMTR) revealed that age greater than 5 years at time of transplant was associated with a strikingly increased risk of mortality[46] and subsequent studies have endorsed this observation.[47,48] In the cohort reported by Moratto et al,

the 5-year OS was 91.9% for patients who received a matched unrelated donor transplant at younger than 2 years of age compared to 73.3% for those older than 5 years.[47] Most recently, the Primary Immune Deficiency Treatment Consortium (PIDTC) reported the outcomes of 129 WAS patients (93 were <2 years old) who underwent HSCT between 2005 and 2015 in North America, and again confirmed that age was the only factor that had a significant impact on OS.[33] Specifically, WAS patients < 5 years of age at time of HSCT had an OS of 94% compared to those ≥ 5 years of age who had a OS of 66%.[33] Indications for early HSCT reflect a trend towards earlier and faster diagnosis of WAS, in addition to broader donor availability. While these factors support the strategy of early transplantation before the onset of disease-related complications, the potential late effects of intense conditioning at a young age remains to be determined.

While earlier studies demonstrated poor outcomes for WAS patients receiving alternative donor umbilical cord blood (UCB) and mismatched related/haploidentical HSCT,[47,48] outcomes with the use of alternative donors have improved greatly over the past decades by overcoming barriers of graft rejection and graft-vs-host disease (GvHD). Shekhovtsova et al reported the use of UCB HSCT for 90 WAS patients following myeloablative conditioning between 1996 and 2013 and demonstrated an OS of 75% for the entire cohort, with an event-free survival (EFS) of 70% and graft failure rate of 11%.[53] Multivariate analysis found age > 2 years was a risk factor for a worse outcome following UCB HSCT, and a trend towards improved overall survival in patients transplanted after 2007.[53] Burroughs et al reported a cohort of 39 WAS patients receiving UCB HSCT between 2005 and 2015 with a 5-year OS of 90%.[33] Overall, there was no significant difference in OS based on donor type comparing HLA-matched sibling, unrelated donor, or UCB grafts. This improvement over the past decade may be related to better UCB selection based on the degree of HLA-match, cell dose and improved supportive care during HSCT.

Historically, the selection of mismatched family donors required the use of ex-vivo T-cell depletion to overcome the risk of GvHD, which has been associated with inferior survival. The main challenge in using an HLA-mismatched related donors (MMRD)/haploidentical graft has been related to poor engraftment and incomplete immune reconstitution with high infectious complications. Earlier experiences with T-cell depleted MMRD HSCT for WAS patients resulted in OS between 37% and

Table 2 Allogeneic HSCT for WAS: Evolution of Long-Term Outcomes

	Donor Sources (n)	Overall Survival	Graft Rejection	Acute GvHD	Chronic GvHD	Post-HSCT Autoimmunity	Mixed Chimerism
Filipovich et al[46] 1968–1996 N=170	MSD (55) MRD (3) MMRD (59) MUD (43) MMUD (19)	70% MSD-87% Other RD- 52% UD-71%	9%	Grade II–IV: MSD- 34% Other RD- 30% UD-56% grade III–IV: NR	21%	NR	NR
Ozsahin et al[48] 1979–2001 N= 96	MSD (45) MMRD (19) MUD (32)	96% EFS 75% <2 yo: 84% ≥2 yo: 68%	NR	NR	7%	20%	18% with mixed chimerism Associated with post-HSCT autoimmunity
Moratto et al[47] 1980–2009 N=194	MSD (39) MRD (1) MMRD (35) MUD (91) UCB (24)	84% <5 yo: 91.9% ≥5 yo: 73.3%	7%	Grade II–IV: NR grade III–IV: 11.3%	14.8%	13.9%	27.9% in at least one cell lineage Associated with post-HSCT autoimmunity
Shin et al[67] 1990–2009 N=47	MSD (6) MRD (1) MMRD (1) MUD (17) MMUD (14) UCB (8)	80%	8.5%	Grade II–IV: 40% grade III–IV: 23%	21%	55%	No association with post-HSCT autoimmunity
Elfeky et al[49] 1996–2016 N=34	MSD (7) MMRD (3) MUD (20) MMUD (1) UCB (3)	100%	6%	Grade II–IV: 26% grade III–IV: NR	3%	20%	No association with post-HSCT autoimmunity
Ngwube et al[50] 2004–2016 N=12	MRD (4) MUD (5) MMUD (3)	92%	25%	Grade II–IV: 16.7% grade III–IV: NR	0%	25%	42% with mixed chimerism

(Continued)

Table 2 (Continued).

	Donor Sources (n)	Overall Survival	Graft Rejection	Acute GvHD	Chronic GvHD	Post-HSCT Autoimmunity	Mixed Chimerism
Burroughs et al[33] 2005–2015 N=129	MSD (22) MMRD (2) MUD (41) MMUD (21) UCB (39)	91% <5 yo: 94% ≥5 yo: 66%	4.7%	Grade II–IV: 27% grade III–IV: 15%	17%	14%	No association with post-HSCT autoimmunity
Shekhovtsova et al[53] 1996–2013 N=90	UCB (90)	75% >2 yo: HR 2.61	11%	Grade II–IV: 38% grade III–IV: 14%	17%	NR	24% with mixed chimerism
Balashov et al[62] 2012–2016 N=34	MUD (18) Haplo (16)	93.8% EFS Historical group: 50% GCSF group: 93.8%	30.3% in historical group 0% in GCSF/plerixafor group	Grade II–IV: 26.8% grade III–IV: 0%	6%	NR	50% with mixed chimerism in historical group No patients with mixed chimerism in the GCSF/plerixafor group
Yue et al[55] 2015–2017 N= 5	Haplo (5)	100%	0%	Grade I: 40% grade II–IV: 0%	20%	NR	No patients with mixed chimerism

Abbreviations: EFS, event-free survival; GvHD, graft-vs-host disease; Haplo, haploidentical; HSCT, hematopoietic stem cell transplantation; HR, hazard ratio; MRD, matched related donor; MMRD, mismatched related donor; MMUD, mismatched unrelated donor; MSD, matched sibling donor; MUD, matched unrelated donor; NR, not reported; RD, related donor; UCB, umbilical cord blood.

440

Journal of Blood Medicine 2021:12

55%.[46,48,54] As has been the case with other graft sources, OS improved to 91.7% for recipients of MMRD-HSCT after 2000, compared to OS of 52.2% for those WAS patients transplanted between 1980 and 1999.[47]

Most recently, the introduction of post-transplant cyclophosphamide (PT-Cy)[55,56] or the use of TCRαβ/CD19-depletion[57–62] have yielded favorable results in alternative donor transplantation for WAS and other PIDs. Both methods reduce the incidence of acute and chronic GvHD and improve immune reconstitution. Laberko et al reported 98 PID patients (including 36 WAS patients) who underwent TCRαβ/CD19-depleted unrelated donors or MMRD HSCT[59] and demonstrated an OS of 86% and 87%, respectively, with secondary graft failure occurring in 17% of patients. Late graft dysfunction noted in some WAS patients, was possibly related to the degree of donor chimerism, though the donor chimerism results were not presented in detail. A significantly lower OS of 52% was observed in patients who had poorly controlled infectious or autoimmune diseases at the time of HSCT. Encouragingly, the rates of grade II–IV acute GvHD and mild limited chronic GvHD were low at 17% and 9%, respectively, in the unrelated donor cohort, and 22% and 13%, respectively, in the MMRD group. Rate of CMV viremia was high with 17% of patients developing invasive disease. A similar rate of graft rejection was noted by Balashov et al in a cohort of 37 PID patients undergoing TCRαβ/CD19-depleted HSCT.[60] To improve donor engraftment, they evaluated the addition of G-CSF and plerixafor to the conditioning regimen of 16 WAS patients undergoing TCRαβ/CD19-depleted HSCT.[62] The OS reached 93.8% and no graft rejection was observed, with all participating patients achieving full donor chimerism in whole blood and in the CD3+ compartment.[63]

While the results are encouraging, TCRαβ/CD19-depletion is costly and resource restrictive. As such, the PT-Cy approach for GvHD prophylaxis with alternative donors is a viable option. Fernandes et al published the outcomes of haploidentical BM HSCT using PT-Cy in 73 patients with primary immunodeficiencies (PID), including 14 WAS patients, performed between 2012 and 2019.[56] The 2-year OS for the WAS patients was 86%, while graft failure was experienced in 2 patients who received a reduced-intensity conditioning regimen. Using a MAC regimen reduced the incidence of graft failure and mixed chimerism. The incidence of grade II–IV aGVHD was slightly higher than the rates reported from studies

with in vitro T cell-depletion techniques and TCRab/CD19 depletion, though comparable to those reported with other alternative sources, such as cord blood and MUDs. Yue et al reported good outcomes of 5 WAS patients receiving a busulfan-based MAC regimen with PT-Cy for GvHD prophylaxis following combined BM and peripheral blood (PB) stem cell haploidentical donor HSCT. All 5 patients engrafted, and are alive at last follow-up, with full donor chimerism and no post-HSCT autoimmune cytopenias.[55]

While the published number of patients is limited, there does not appear to be an increase incidence of post-transplant autoimmunity or immune dysregulation with PT-Cy, though larger studies are needed. Full intensity myeloablative conditioning is needed to overcome graft rejection with these methods, and further assessment of the degree and duration of lineage-specific donor chimerism achieved is being evaluated.

Despite an increase in the National Marrow Donor Program's Registry size in recent years, racial disparities in access to matched unrelated donors have not improved, especially for African Americans.[64] Thus, emphasizing the ongoing need to advance use of alternative graft sources such as cord blood transplant and haploidentical transplant in order to provide donor options in the ethnic groups not well represented in the donor databases is imperative. The results of alternative donor HSCT using UCB donors and TCRαβ/CD19-depletion or PT-Cy for mismatched/haploidentical donors is very promising, and provides new opportunities for successful curative therapy in WAS/XLT patients of minority ethnicities. Additionally, advances in gene therapy for WAS patients will increase treatment options for patients of minority ethnicity without suitable donors, though the limitations of high cost and restrictive resources will need to be addressed to make it widely accessible to all patients.

Regardless of the graft source, both myelo- and immuno-ablation are required to overcome graft rejection, establish myeloid and lymphoid engraftment and to correct the clinical manifestations of WAS. Myeloablative conditioning regimens have achieved excellent results in young WAS patients though short- and long-term toxicities remain a concern. While reduced intensity conditioning (RIC) regimens can significantly reduce early transplantation-related morbidity and mortality, especially in older patients, there is a greater risk for mixed chimerism, transplant associated complications and delayed graft failure.[65,66] A more recent report found no difference in OS between patients receiving a MAC or RIC regimen,

although significantly different donor T-cell, B-cell and myeloid engraftment was observed in patients receiving MAC vs RIC regimens.[33] Stability or reversal of WAS symptoms post HSCT depends on the degree and long-term stability of donor cell engraftment in the hematopoietic lineage of interest. The degree of donor chimerism necessary for WAS symptom correction has been established.[47,48] Lower levels of donor chimerism were observed more often in myeloid than in lymphoid lineages. Persistent thrombocytopenia after HSCT was strongly associated with mixed myeloid chimerism, suggesting that robust and stable engraftment of donor-derived myeloid cells is required to correct the defect.[33,47] Burroughs et al noted that platelet count recovery post-HSCT was related to the degree of donor myeloid chimerism. At 1 year post-HSCT, patients with <50% donor myeloid engraftment were found to have a significantly lower median platelet count (40,000/mm^3) compared to patients who attained ≥50% donor myeloid engraftment and achieved normal platelet counts,[33] highlighting the need for ≥50% donor myeloid engraftment for correction of the thrombocytopenia. T and B-cell donor chimerism has also been shown to correlate with the intensity of the conditioning regimen.[33]

An association between mixed chimerism and post-transplant autoimmunity was reported in some WAS patients by several investigators. Specifically, in a multicenter European study of 96 patients who survived at least 2 years after HSCT, as many as 20% of the long-term survivors developed autoimmunity independent of chronic GvHD. The risk of autoimmunity was significantly higher for patients who developed mixed chimerism after receiving matched unrelated or MMRD HSCT.[48] Cytopenias and endocrinopathies were predominant de-novo autoimmune disease following transplantation with time of onset and resolution being year and a half and four years respectively. Similarly, Moratto et al reported that autoimmune manifestations, predominantly cytopenias and endocrinopathies, occurred in 13.9% of patients following transplantation.[47] These patients were noted to have a lower degree of chimerism in the T, B, and myeloid lineages compared with patients who did not develop autoimmunity, suggesting that mixed chimerism is a risk factor for developing autoimmune disease post-HSCT. However, subsequent studies did not confirm such an association.[33,49,67] A retrospective analysis by the PIDTC evaluated if mixed donor chimerism (<95%) at day 100 or at 6 months following HSCT correlated with an increased risk for the development of de novo autoimmunity.[33] Overall, 14% of patients developed 1 or more de novo autoimmune disease within the first year post-HSCT. However, unlike the results published earlier[47,48] the analysis did not find a persistent association between mixed T-cell or B-cell donor chimerism and the development of de novo autoimmunity in long-term survivors. There was, however, an increased incidence of de novo autoimmunity in those with mixed myeloid donor chimerism at 6 months post-HSCT.[33] Autoimmune cytopenia (AIHA being most prevalent) was the predominant de-novo autoimmunity with complete resolution occurring in most of the patients. Importantly, the presence of autoimmune disease prior to HSCT did not increase the risk of developing de novo autoimmune disorders after HSCT, nor did the WAS score and age at HSCT.[33] Interestingly, none of the patients receiving a matched sibling donor HSCT developed de novo autoimmune disease post-HSCT, whereas 23% of patients receiving matched unrelated grafts and 9% receiving UCB grafts did.[33] A smaller cohort of WAS patients followed prospectively at a single transplant center did not reveal an association between autoimmune cytopenia and mixed chimerism.[67]

Gene Therapy

HSCT outcomes have significantly improved over the last five decades since the first WAS patient was transplanted in 1968. Nevertheless, HSCT remains associated with significant short- and long-term problems, especially when a matched donor is not available. The need for intense conditioning to achieve successful myeloid and lymphoid engraftment, the risk of acute and chronic GvHD and the long-term sequelae associated with myeloablative conditioning have led to development of alternative treatment approaches.

Over the last 25 years, the field of gene therapy (GT) has progressed from basic research to standard of care in patients with certain monogenic PID's.[68,69] WAS is an ideal candidate for gene therapy due to selective expression of WASP in cells of the hematopoietic lineage, which are expected to have proliferative advantage over the host WASP negative cells.[70–72] The initial GT trial for WAS patients was started in 2006 and utilized a gibbon ape leukemia virus (GALV)- γ-retroviral vector.[73] The first 2 patients, both 3 years of age, received autologous WASP-expressing retroviral vector transduced PB stem cells following Busulfan (8mg/kg) conditioning. Both patients showed evidence of successful correction of the myeloid and lymphoid defects, demonstrating a selective advantage

of the vector transduced stem cells.[73] Following these promising initial results, 8 additional patients were enrolled by 2009. In all 10 patients, autologous stem cells were isolated from peripheral blood mobilized with G-CSF (n=2) or G-CSF and Plerixafor (n=8). Participants were 2 to 14 years of age (2 patients were older than 5) and each received a robust stem cell product. Nine of ten patients demonstrated sustained engraftment with correction of WASP expression in myeloid and lymphoid lineages. GT resulted in partial or complete correction of infectious, autoimmune or bleeding manifestations of the disease. Eight patients had pre GT autoimmunity, with two patients having ongoing autoimmunity following treatment, however with milder manifestations. The participant with the lowest stem cell dose showed only partial lymphoid correction without myeloid or stem cell WASP expression noted and without improvement in disease manifestations. Unfortunately, despite early clinical success, 7 patients developed acute leukemia 16 to 60 months following therapy. Six patients developed T-ALL (2 of these patients subsequently developed AML during maintenance therapy for T-ALL) and 1 patient AML.[74] Analysis of the retroviral integration demonstrated a polyclonal pattern during the early phase, followed by development of dominant clones with integration at the LMO2, MDS1 and MN1 loci. Unlike the insertional leukemogenesis observed in the X-SCID trials,[75–77] expansion of clones in the WAS patients treated with GT was more rapid. Five patients have subsequently been successfully treated with HSCT.[74] High vector copy number as well as strong enhancer/promoter elements, although associated with better efficacy, may lead to increased genotoxicity and leukemogenesis.

Additional 31 WAS patients were reported to have undergone GT since 2013 utilizing lentiviral vector mediated gene transfer. Transition to lentiviral vector use (based on human immunodeficiency virus) was driven by the ability of lentiviruses to easily transverse the nuclear membrane and transduce non-mitotic hematopoietic stem cells, as opposed to γ-retroviral vectors which require cell division for integration.[78] To improve vector safety, a self-inactivating design has been adopted to decrease or eliminate activation of enhancer and promoter regions.[79,80] Into these novel lentiviral constructs, a codon optimized human *WAS* cDNA was placed under the control of a fragment of the endogenous promoter.

The first three lentiviral treated patients were reported by the Milan group in 2013,[81,82] followed by a publication in 2019 of their interim results.[81,82] The 8 patients treated with GT had WAS scores of 3–5 and age range 1.1 to 12.4 years. Additionally, 9 patients were treated on an expanded access protocol (age 1.4–35.1 years) with a total of 14 patients having more than 1 year of follow-up.[83] Five patients were older than 5 years of age at the time of GT. Bone marrow was the source of stem cells in the first 6 patients, with transition to mobilized PB stem cells in the remaining patients. Reduced intensity conditioning consisted of Rituximab 375mg/m^2 given on day −22, with Busulfan (AUC 45–60 mg*hr/L) and Fludarabine 60mg/m^2 given day −3 to day −1. Overall survival was 94% in 17 treated patients. Lentiviral transduced colonies were noted in the bone marrow at 3 months and persisted up to 8 years. High levels of multilineage engraftment and sustained WASP expression was noted in lymphocytes and platelets. All evaluable patients from the 2 cohorts were able to discontinue IVIG supplementation at a median time of 0.9 years (range 0.2–5 years) following GT. Median platelet counts improved from baseline of 19,500 to 39,800/mm^3 (14,000–272,000/mm^3) at 12 months following GT, with all patients attaining independence from platelet transfusions by 9 months following therapy. Pre GT autoimmunity resolved in all but 1 patient from the initial cohort who developed transient immune thrombocytopenia post GT. Reduction in severe infections, frequency and severity of bleeding episode as well as improvement or resolution of eczema were noted following GT. Stable polyclonal gene marking with high number of unique insertion sites were observed. No replication competent virus was noted in any subjects. Furthermore, significant clinical benefit was observed in patients over 5 years of age, which historically has been the cohort with poorest HSCT survival.

Hacein-Bey-Albina et al described 7 patients who underwent lentiviral GT following more intense myeloablative conditioning with Busulfan 12mg/kg given over 3 days, Fludarabine 120mg/m^3 with Rituximab or Alemtuzumab serotherapy. All patients had a severe WAS phenotype (WAS scores of 3–5), lacked matched related or unrelated donors and ranged in age between 10 months and 15.5 years (4 patients were over 5 years of age).[84] There was equal distribution of BM and mobilized PB stem cells with a robust median transduced CD34$^+$ dose. One patient died 7 months following GT of refractory herpes infection. The remaining 6 patients demonstrated clinical improvement in eczema, bleeding severity and frequency, infections and autoimmunity. Only one patient continued to experience vasculitis following GT

which was mild compared to severe limb vasculitis leading to impaired ambulation pre GT. Minimal improvement in platelet counts occurred in patients who were not splenectomized (either before or following gene therapy) with no patient reaching platelet counts greater than 50,000/mm^3.

Five additional WAS patients were treated in the Boston based U.S trial.[83] As in the other 2 trials, patients had severe WAS with age range of 1.4 to 8 years. Myeloablative conditioning was used with busulfan of 12–15mg/kg total (target AUC 70–80 mg*hr/L) and Fludarabine of 120mg/m^2. Multilineage vector gene marking was sustained over time, however WASP expression even though increased from the baseline remained below normal. Two patients demonstrated an increase in platelet counts over 50,000/mm^3. The clinical benefits observed were similar to those reported in the other trials, except for 2 patients whose autoimmunity failed to resolve with GT. The use of lentiviral vector was not associated with any events concerning safety, with highly polyclonal pattern of vector integration.

The first adult WAS patient treated with lentiviral GT was reported in 2017.[85] He received the same conditioning described by Hacein-Bey-Albina et al GT led to resolution of autoimmunity and IVIG discontinuation. As this patient had prior splenectomy, GT-related platelet count recovery could not be evaluated.

Lentiviral GT trials utilizing the endogenous WAS promoter have demonstrated clinical efficacy, with improvement in infectious, autoimmune and bleeding complications in treated patients. Overall safety of the lentiviral vectors has been demonstrated, and so far, no adverse events associated with the use of transduced stem cells have occurred, with polyclonal pattern of vector integration and without evidence of replication competent lentivirus. However, reconstitution of the platelet count to normal range has not been achieved in the majority of patients. Platelet reconstitution was further evaluated by Sereni et al analyzing platelet phenotype, activation state, and overall function in WAS patients following GT.[86] Post GT, platelets were found as having normal volume, granule content with greater than 90% WASP expressing platelets noted in patients followed longer than 2 years. De-novo autoimmunity following GT occurred at the comparable rate as seen with HSCT, with 3 patients reported.[81–83] However, mean fluorescence intensity of WASP was low demonstrating suboptimal expression. Platelet activation and aggregation normalized following GT. However, while the dysregulated and activated state of platelets typical for WAS patients improved, it did not normalize, possibly contributing to the persistent thrombocytopenia frequently seen following gene therapy.

Conclusion

When originally recognized as a clinical entity, patients with classical WAS typically died during infancy/early childhood. Better understanding of the underlying hematologic and immunologic abnormalities has led to symptomatic therapies, including corticosteroids, antimicrobials and immunoglobulin replacement that improved modestly the quality of life and extended life expectancy into the early twenties. Since the initial success of HSCT in 1968, significant progress has been made in confirming the diagnosis earlier, establishing genotype phenotype correlation, and designing and improving curative therapies for WAS patients. As is the case in other PIDs, earlier intervention and disease correction has led to better outcomes and improved event-free survival. HSCT and GT have reached an OS above 90%, however some challenges remain. Potential late effects due to intense conditioning regimens, insufficient donor chimerism leading to only partial disease correction, difficulties achieving normal platelet recovery and function, and the potential risk of insertional mutagenesis following GT still need to be evaluated in the decades to come.

Disclosure

The authors reported no conflicts of interest for this work.

References

1. Wiskott A. Familiarer, angeborener Morbus Werlhofii? *Mschr Kinderheilk*. 1937;68:212.
2. Aldrich RA, Steinberg AG, Campbell DC. Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhea. *Pediatrics*. 1954;13(2):133.
3. Cooper MD, Chae HP, Lowman JT, Krivit W, Good RA. Wiskott-Aldrich syndrome. An immunologic deficiency disease involving the afferent limb of immunity. *Am J Med*. 1968;44(4):499–513.
4. Ochs HD, Slichter SJ, Harker LA, Von Behrens WE, Clark RA, Wedgwood RJ. The Wiskott-Aldrich syndrome: studies of lymphocytes, granulocytes, and platelets. *Blood*. 1980;55(2):243–252.
5. Orange JS, Stone KD, Turvey SE, Krzewski K. The Wiskott-Aldrich syndrome. *Cell Mol Life Sci*. 2004;61(18):2361–2385.
6. Sullivan KE, Mullen CA, Blaese RM, Winkelstein JA. A multiinstitutional survey of the Wiskott-Aldrich syndrome. *J Pediatr*. 1994;125(6 Pt 1):876–885.
7. Rawlings SL, Crooks GM, Bockstoce D, Barsky LW, Parkman R, Weinberg KI. Spontaneous apoptosis in lymphocytes from patients with Wiskott-Aldrich syndrome: correlation of accelerated cell death and attenuated bcl-2 expression. *Blood*. 1999;94(11):3872–3882.

8. Rengan R, Ochs HD, Sweet LI, et al. Actin cytoskeletal function is spared, but apoptosis is increased, in WAS patient hematopoietic cells. *Blood*. 2000;95(4):1283–1292.

9. Burns S, Cory GO, Vainchenker W, Thrasher AJ. Mechanisms of WASp-mediated hematologic and immunologic disease. *Blood*. 2004;104(12):3454–3462.

10. Derry JM, Ochs HD, Francke U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell*. 1994;79(5):following 922.

11. Villa A, Notarangelo L, Macchi P, et al. X–linked thrombocytopenia and Wiskott–Aldrich are allelic diseases with mutations in the WASP gene. *Nat Genet*. 1995;9(4):414–417. doi:10.1038/ng0495-414

12. Zhu Q, Zhang M, Blaese RM, et al. The Wiskott-Aldrich syndrome and X-linked congenital thrombocytopenia are caused by mutations of the same gene. *Blood*. 1995;86(10):3797–3804.

13. Notarangelo LD, Mazza C, Giliani S, et al. Missense mutations of the WASP gene cause intermittent X-linked thrombocytopenia. *Blood*. 2002;99(6):2268–2269.

14. Albert MH, Bittner TC, Nonoyama S, et al. X-linked thrombocytopenia (XLT) due to WAS mutations: clinical characteristics, long-term outcome, and treatment options. *Blood*. 2010;115 (16):3231–3238.

15. Ancliff PJ, Blundell MP, Cory GO, et al. Two novel activating mutations in the Wiskott-Aldrich syndrome protein result in congenital neutropenia. *Blood*. 2006;108(7):2182–2189.

16. Stray-Pedersen A, Abrahamsen TG, Froland SS. Primary immunodeficiency diseases in Norway. *J Clin Immunol*. 2000;20(6):477–485.

17. Ochs HD, Notarangelo LD. Wiskott-Aldrich syndrome. In: Ochs HD, Smith CIE, Puck JM, editors. *Primary Immunodeficiency Diseases: A Molecular and Genetic Approach*. 3rd ed. Oxford, New York: Oxford University Press; 2014:395–413.

18. Dupre L, Aiuti A, Trifari S, et al. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity*. 2002;17(2):157–166.

19. Malinova D, Fritzsche M, Nowosad CR, et al. WASp-dependent actin cytoskeleton stability at the dendritic cell immunological synapse is required for extensive, functional T cell contacts. *J Leukoc Biol*. 2016;99(5):699–710.

20. Orange JS, Ramesh N, Remold-O'Donnell E, et al. Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proc Natl Acad Sci U S A*. 2002;99(17):11351–11356.

21. Marangoni F, Trifari S, Scaramuzza S, et al. WASP regulates suppressor activity of human and murine CD4(+)CD25(+)FOXP3(+) natural regulatory T cells. *J Exp Med*. 2007;204(2):369–380.

22. Humblet-Baron S, Sather B, Anover S, et al. Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. *J Clin Invest*. 2007;117(2):407–418.

23. Kolhatkar NS, Brahmandam A, Thouvenel CD, et al. Altered BCR and TLR signals promote enhanced positive selection of autoreactive transitional B cells in Wiskott-Aldrich syndrome. *J Exp Med*. 2015;212(10):1663–1677.

24. Crestani E, Volpi S, Candotti F, et al. Broad spectrum of autoantibodies in patients with Wiskott-Aldrich syndrome and X-linked thrombocytopenia. *J Allergy Clin Immunol*. 2015;136(5):1401–1404e1401-1403.

25. Lorenzi R, Brickell PM, Katz DR, Kinnon C, Thrasher AJ. Wiskott-Aldrich syndrome protein is necessary for efficient IgG-mediated phagocytosis. *Blood*. 2000;95(9):2943–2946.

26. Badolato R, Sozzani S, Malacarne F, et al. Monocytes from Wiskott-Aldrich patients display reduced chemotaxis and lack of cell polarization in response to monocyte chemoattractant protein-1 and formyl-methionyl-leucyl-phenylalanine. *J Immunol*. 1998;161 (2):1026–1033.

27. Shcherbina A, Rosen FS, Remold-O'Donnell E. Pathological events in platelets of Wiskott-Aldrich syndrome patients. *Br J Haematol*. 1999;106(4):875–883.

28. Marathe BM, Prislovsky A, Astrakhan A, Rawlings DJ, Wan JY, Strom TS. Antiplatelet antibodies in WASP(-) mice correlate with evidence of increased in vivo platelet consumption. *Exp Hematol*. 2009;37(11):1353–1363.

29. Jin Y, Mazza C, Christie JR, et al. Mutations of the Wiskott-Aldrich Syndrome Protein (WASP): hotspots, effect on transcription, and translation and phenotype/genotype correlation. *Blood*. 2004;104 (13):4010–4019.

30. Albert MH, Notarangelo LD, Ochs HD. Clinical spectrum, pathophysiology and treatment of the Wiskott-Aldrich syndrome. *Curr Opin Hematol*. 2011;18(1):42–48.

31. Imai K, Morio T, Zhu Y, et al. Clinical course of patients with WASP gene mutations. *Blood*. 2004;103(2):456–464.

32. Vestermark B, Vestermark S. Familial sex-linked thrombocytopenia. *Acta Paediatr*. 1964;53:365–370.

33. Burroughs LM, Petrovic A, Brazauskas R, et al. Excellent outcomes following hematopoietic cell transplantation for Wiskott-Aldrich syndrome: a PIDTC report. *Blood*. 2020;135 (23):2094–2105.

34. Oshima K, Imai K, Albert MH, et al. Hematopoietic stem cell transplantation for X-linked thrombocytopenia with mutations in the WAS gene. *J Clin Immunol*. 2015;35(1):15–21.

35. Beel K, Cotter MM, Blatny J, et al. A large kindred with X-linked neutropenia with an I294T mutation of the Wiskott-Aldrich syndrome gene. *Br J Haematol*. 2009;144(1):120–126.

36. Beel K, Vandenberghe P. G-CSF receptor (CSF3R) mutations in X-linked neutropenia evolving to acute myeloid leukemia or myelodysplasia. *Haematologica*. 2009;94(10):1449–1452.

37. Schwinger W, Urban C, Ulreich R, et al. The phenotype and treatment of WIP deficiency: literature synopsis and review of a patient with pre-transplant serial donor lymphocyte infusions to eliminate CMV. *Front Immunol*. 2018;9:2554.

38. Al-Mousa H, Hawwari A, Al-Ghonaium A, et al. Hematopoietic stem cell transplantation corrects WIP deficiency. *J Allergy Clin Immunol*. 2017;139(3):1039–1040e1034.

39. Gerrits AJ, Leven EA, Frelinger AL 3rd, et al. Effects of eltrombopag on platelet count and platelet activation in Wiskott-Aldrich syndrome/X-linked thrombocytopenia. *Blood*. 2015;126 (11):1367–1378.

40. Khoreva A, Abramova I, Deripapa E, et al. Efficacy of romiplostim in treatment of thrombocytopenia in children with Wiskott-Aldrich syndrome. *Br J Haematol*. 2021;192(2):366–374.

41. Lum LG, Tubergen DG, Corash L, Blaese RM. Splenectomy in the management of the thrombocytopenia of the Wiskott-Aldrich syndrome. *N Engl J Med*. 1980;302(16):892–896.

42. Bach FH, Albertini RJ, Joo P, Anderson JL, Bortin MM. Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. *Lancet*. 1968;2(7583):1364–1366.

43. Pai SY, Notarangelo LD. Hematopoietic cell transplantation for Wiskott-Aldrich syndrome: advances in biology and future directions for treatment. *Immunol Allergy Clin North Am*. 2010;30 (2):179–194.

44. Kapoor N, Kirkpatrick D, Blaese RM, et al. Reconstitution of normal megakaryocytopoiesis and immunologic functions in Wiskott-Aldrich syndrome by marrow transplantation following myeloablation and immunosuppression with busulfan and cyclophosphamide. *Blood*. 1981;57(4):692–696.

45. Ochs HD, Lum LG, Johnson FL, Schiffman G, Wedgwood RJ, Storb R. Bone marrow transplantation in the Wiskott-Aldrich syndrome. Complete hematological and immunological reconstitution. *Transplantation*. 1982;34(5):284–288.

46. Filipovich AH, Stone JV, Tomany SC, et al. Impact of donor type on outcome of bone marrow transplantation for Wiskott-Aldrich syndrome: collaborative study of the International Bone Marrow Transplant Registry and the National Marrow Donor Program. *Blood*. 2001;97(6):1598–1603.

47. Moratto D, Giliani S, Bonfim C, et al. Long-term outcome and lineage-specific chimerism in 194 patients with Wiskott-Aldrich syndrome treated by hematopoietic cell transplantation in the period 1980-2009: an international collaborative study. *Blood*. 2011;118 (6):1675–1684.

48. Ozsahin H, Cavazzana-Calvo M, Notarangelo LD, et al. Long-term outcome following hematopoietic stem-cell transplantation in Wiskott-Aldrich syndrome: collaborative study of the European Society for Immunodeficiencies and European Group for Blood and Marrow Transplantation. *Blood*. 2008;111(1):439–445.

49. Elfeky RA, Furtado-Silva JM, Chiesa R, et al. One hundred percent survival after transplantation of 34 patients with Wiskott-Aldrich syndrome over 20 years. *J Allergy Clin Immunol*. 2018;142 (5):1654–1656e1657.

50. Ngwube A, Hanson IC, Orange J, et al. Outcomes after allogeneic transplant in patients with Wiskott-Aldrich syndrome. *Biol Blood Marrow Transplant*. 2018;24(3):537–541.

51. Ozsahin H, Le Deist F, Benkerrou M, et al. Bone marrow transplantation in 26 patients with Wiskott-Aldrich syndrome from a single center. *J Pediatr*. 1996;129(2):238–244.

52. Pai SY, DeMartiis D, Forino C, et al. Stem cell transplantation for the Wiskott-Aldrich syndrome: a single-center experience confirms efficacy of matched unrelated donor transplantation. *Bone Marrow Transplant*. 2006;38(10):671–679.

53. Shekhovtsova Z, Bonfim C, Ruggeri A, et al. A risk factor analysis of outcomes after unrelated cord blood transplantation for children with Wiskott-Aldrich syndrome. *Haematologica*. 2017;102 (6):1112–1119.

54. Friedrich W, Schutz C, Schulz A, Benninghoff U, Honig M. Results and long-term outcome in 39 patients with Wiskott-Aldrich syndrome transplanted from HLA-matched and -mismatched donors. *Immunol Res*. 2009;44(1–3):18–24.

55. Yue Y, Shi X, Song Z, et al. Posttransplant cyclophosphamide for haploidentical stem cell transplantation in children with Wiskott-Aldrich syndrome. *Pediatr Blood Cancer*. 2018;65(8): e27092.

56. Fernandes JF, Nichele S, Arcuri LJ, et al. Outcomes after Haploidentical Stem Cell Transplantation with Post-Transplantation Cyclophosphamide in Patients with Primary Immunodeficiency Diseases. *Biol Blood Marrow Transplant*. 2020;26(10):1923–1929.

57. Shah RM, Elfeky R, Nademi Z, et al. T-cell receptor alphabeta(+) and CD19(+) cell-depleted haploidentical and mismatched hematopoietic stem cell transplantation in primary immune deficiency. *J Allergy Clin Immunol*. 2018;141(4):1417–1426 e1411.

58. Elfeky R, Shah RM, Unni MNM, et al. New graft manipulation strategies improve the outcome of mismatched stem cell transplantation in children with primary immunodeficiencies. *J Allergy Clin Immunol*. 2019;144(1):280–293.

59. Laberko A, Sultanova E, Gutovskaya E, et al. Mismatched related vs matched unrelated donors in TCRalphabeta/CD19-depleted HSCT for primary immunodeficiencies. *Blood*. 2019;134(20):1755–1763.

60. Balashov D, Shcherbina A, Maschan M, et al. Single-center experience of unrelated and haploidentical stem cell transplantation with TCRalphabeta and CD19 depletion in children with primary immunodeficiency syndromes. *Biol Blood Marrow Transplant*. 2015;21 (11):1955–1962.

61. Kharya G, Nademi Z, Leahy TR, et al. Haploidentical T-cell alpha beta receptor and CD19-depleted stem cell transplant for Wiskott-Aldrich syndrome. *J Allergy Clin Immunol*. 2014;134 (5):1199–1201.

62. Balashov D, Laberko A, Shcherbina A, et al. A conditioning regimen with plerixafor is safe and improves the outcome of TCRalphabeta(+) and CD19(+) cell-depleted stem cell transplantation in patients with wiskott-aldrich syndrome. *Biol Blood Marrow Transplant*. 2018;24 (7):1432–1440.

63. Balashov D, Laberko A, Shcherbina A, et al. A conditioning regimen with plerixafor is safe and improves the outcome of TCRαβ. *Biol Blood Marrow Transplant*. 2018;24(7):1432–1440.

64. Barker JN, Boughan K, Dahi PB, et al. Racial disparities in access to HLA-matched unrelated donor transplants: a prospective 1312-patient analysis. *Blood Adv*. 2019;3(7):939–944.

65. Ochs HD, Filipovich AH, Veys P, Cowan MJ, Kapoor N. Wiskott-Aldrich syndrome: diagnosis, clinical and laboratory manifestations, and treatment. *Biol Blood Marrow Transplant*. 2009;15(1 Suppl):84–90.

66. Rao K, Amrolia PJ, Jones A, et al. Improved survival after unrelated donor bone marrow transplantation in children with primary immunodeficiency using a reduced-intensity conditioning regimen. *Blood*. 2005;105(2):879–885.

67. Shin CR, Kim MO, Li D, et al. Outcomes following hematopoietic cell transplantation for Wiskott-Aldrich syndrome. *Bone Marrow Transplant*. 2012;47(11):1428–1435.

68. Cicalese MP, Aiuti A. Clinical applications of gene therapy for primary immunodeficiencies. *Hum Gene Ther*. 2015;26(4):210–219.

69. Ferrua F, Twenty-Five AA. Years of gene therapy for ADA-SCID: from bubble babies to an approved drug. *Hum Gene Ther*. 2017;28 (11):972–981.

70. Klein C, Nguyen D, Liu CH, et al. Gene therapy for Wiskott-Aldrich syndrome: rescue of T-cell signaling and amelioration of colitis upon transplantation of retrovirally transduced hematopoietic stem cells in mice. *Blood*. 2003;101(6):2159–2166.

71. Strom TS, Turner SJ, Andreansky S, et al. Defects in T-cell-mediated immunity to influenza virus in murine Wiskott-Aldrich syndrome are corrected by oncoretroviral vector-mediated gene transfer into repopulating hematopoietic cells. *Blood*. 2003;102 (9):3108–3116.

72. Boztug K, Dewey RA, Klein C. Development of hematopoietic stem cell gene therapy for Wiskott-Aldrich syndrome. *Curr Opin Mol Ther*. 2006;8(5):390–395.

73. Boztug K, Schmidt M, Schwarzer A, et al. Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med*. 2010;363 (20):1918–1927.

74. Braun CJ, Boztug K, Paruzynski A, et al. Gene therapy for Wiskott-Aldrich syndrome–long-term efficacy and genotoxicity. *Sci Transl Med*. 2014;6(227):227ra233.

75. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302(5644):415–419.

76. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*. 2008;118(9):3132–3142.

77. Gaspar HB, Cooray S, Gilmour KC, et al. Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. *Sci Transl Med*. 2011;3 (97):97ra79.

78. Wiznerowicz M, Harnessing TD. HIV for therapy, basic research and biotechnology. *Trends Biotechnol*. 2005;23(1):42–47.

79. Qasim W, Gennery AR. Gene therapy for primary immunodeficiencies: current status and future prospects. *Drugs*. 2014;74(9):963–969.

80. Galy A, Roncarolo MG, Thrasher AJ. Development of lentiviral gene therapy for Wiskott Aldrich syndrome. *Expert Opin Biol Ther*. 2008;8 (2):181–190.

81. Aiuti A, Biasco L, Scaramuzza S, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science*. 2013;341(6148):1233151.

82. Ferrua F, Cicalese MP, Galimberti S, et al. Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, Phase 1/2 clinical study. *Lancet Haematol*. 2019;6(5):e239–e253.

83. Labrosse R, Chu J, Armant M, et al. Outcome of hematopoietic stem cell gene therapy for Wiskott-Aldrich syndrome. *Blood*. 2019;134 (Supplement_1):4629.

84. Hacein-Bey Abina S, Gaspar HB, Blondeau J, et al. Outcomes following gene therapy in patients with severe Wiskott-Aldrich syndrome. *JAMA*. 2015;313(15):1550–1563.

85. Morris EC, Fox T, Chakraverty R, et al. Gene therapy for Wiskott-Aldrich syndrome in a severely affected adult. *Blood*. 2017;130(11):1327–1335.

86. Sereni L, Castiello MC, Di Silvestre D, et al. Lentiviral gene therapy corrects platelet phenotype and function in patients with Wiskott-Aldrich syndrome. *J Allergy Clin Immunol*. 2019;144 (3):825–838.

Glanzmann Thrombasthenia: Perspectives from Clinical Practice on Accurate Diagnosis and Optimal Treatment Strategies

Natalie Mathews, Georges-Etienne Rivard & Arnaud Bonnefoy

REVIEW

Glanzmann Thrombasthenia: Perspectives from Clinical Practice on Accurate Diagnosis and Optimal Treatment Strategies

Natalie Mathews[1]
Georges-Etienne Rivard[2]
Arnaud Bonnefoy[2]

[1]Division of Haematology/Oncology, Department of Paediatrics, The Hospital for Sick Children, Toronto, Ontario, Canada; [2]Division of Hematology-Oncology, Department of Pediatrics, CHU Sainte-Justine, Université de Montréal, Montréal, Québec, H3T 1C5, Canada

Abstract: Glanzmann thrombasthenia (GT) is a rare autosomal recessive disorder of fibrinogen-mediated platelet aggregation due to a quantitative or qualitative deficit of the $\alpha_{IIb}\beta_3$ integrin at the platelet surface membrane resulting from mutation(s) in *ITGA2B* and/or *ITGB3*. Patients tend to present in early childhood with easy bruising and mucocutaneous bleeding. The diagnostic process requires consideration of more common disorders of haemostasis and coagulation prior to confirming the disorder with platelet light transmission aggregation, flow cytometry of CD41 and CD61 expression, and/or exon sequencing of *ITGA2B* and *ITGB3*. Antifibrinolytic therapy, recombinant activated factor VII, and platelet transfusions are the mainstay of therapy, although the latter may trigger formation of anti-platelet antibodies in GT patients and inadvertent platelet-refractory disease. The management of these patients therefore remains complex, particularly in the context of trauma, labour and delivery, and perioperative care. Bone marrow transplantation remains the sole curative option, although the venue of gene therapy is being increasingly explored as a future alternative for definitive treatment of GT.

Keywords: bleeding disorders, inherited platelet defects, platelet aggregation, *ITGA2B*, *ITGB3*, αIIbβ3

Introduction

Glanzmann Thrombasthenia (GT) is a rare inherited bleeding disorder characterized by dysfunctional fibrinogen-mediated platelet aggregation due to decreased or dysfunctional $\alpha_{IIb}\beta_3$ integrin expression at the platelet surface membrane. This autosomal recessive condition affects approximately 1 in 1,000,000 people,[1] though prevalence reaches up to 1 in 200,000 people[2] in populations of increased consanguinity, including those coming from Pakistan, Iraqi Jewish groups, nomadic tribes of Jordan, South Indian Hindu communities, Roma camps within France, and the Canadian province of Newfoundland and Labrador.[1–6]

Historical Context

In 1918, Dr. Glanzmann, a Swiss pediatrician, coined the term "thrombasthenia," or "weak platelets," when describing a patient exhibiting purpura despite having platelets of normal quantity and appearance on peripheral smear.[1,7–10] Curiously, he also noted absence of platelet clumping, a prolonged bleeding time, and inferior clot retraction.[7] Forty-four years later, in 1962, Drs. Caen and Cousin demonstrated

Correspondence: Natalie Mathews
Division of Haematology/Oncology, Department of Paediatrics, The Hospital for Sick Children, 555 University Ave, Toronto, Ontario, M5G 1X8, Canada
Email natalie.mathews@sickkids.ca

absence of platelet aggregation in response to ADP, adrenaline, thrombin, and collagen stimulation.[1,11] In the 1970s, multiple teams[12–15] studying the platelets of Glanzmann thrombasthenia patients identified a shared underlying deficiency of the membrane glycoprotein IIb/IIIa complex, now known as the $\alpha_{IIb}\beta_3$ integrin.

Role of $\alpha_{IIb}\beta_3$ Integrin

Wild-type platelets express approximately 50,000 copies of $\alpha_{IIb}\beta_3$ integrin at their surface membranes.[16,17] The α_{IIb} subunit, predominantly expressed within cells of the megakaryocytic lineage,[18] is produced as a pro-peptide comprised of a heavy and light chain linked together by disulfide bridges.[19–21] In addition to providing intramolecular stability, the integrity of the 674–687 disulfide bridge in particular has also been shown to be necessary for ultimate surface expression of the $\alpha_{IIb}\beta_3$ integrin.[22] The α_{IIb} subunit binds its β_3 partner via calcium-depending bonds prior to undergoing post-translational modifications, including O- and N-glycosylation, within the endoplasmic reticulum and Golgi apparatus.[20,21] Once integrated into the platelet membrane, each mature subunit within the final receptor complex contains a large globular extracellular domain, a single transmembrane domain, and a small cytoplasmic region that interacts with its neighboring cytoplasmic domain via salt bridge (Figure 1).[20] The cytoplasmic domains also interact with other cytoplasmic and cytoskeletal proteins[20,23] and facilitate both inside-out and outside-in signaling (Figure 2).[20,24,25] In its resting state, the $\alpha_{IIb}\beta_3$ integrin receptor exists in a "bent" conformation whose extracellular domains are clasped and have a low affinity for binding ligands.[1,26,27] A rise in the intracellular concentration of calcium leads to a talin-induced conformational change within the extracellular domains of both subunits, exposing their ligand binding sites.[26–28] This inside-out activation allows ligand binding sites on each subunit to bind the same fibrinogen molecule, which in turns binds identical ligand binding sites on other platelets to establish a platelet plug.[1] Meanwhile, fibrinogen binding also facilitates unclasping of the extracellular domains of the α_{IIb} and β_3 subunits[26,27,29,30] and triggers protein kinase C-mediated cytoskeletal changes and platelet granule secretion, ultimately resulting in platelet spreading and fibrin clot stabilization.[1] Clot formation and stabilization is further enhanced by β_3's ability to bind von Willebrand factor, fibronectin, and vitronectin.[1,28,31] Furthermore, β_3 promotes cleavage of Factor Xa, assisting with conversion of prothrombin to thrombin.[8,32–34] β_3 has also been shown to have a role in fibrin clot retraction that is independent from its ability to bind fibrinogen.[8,10,35–37] Given the myriad of interactions that must occur for the α_{IIb} and β_3 subunits to reach the platelet membrane and promote platelet aggregation, one can imagine the many opportunities for this process to
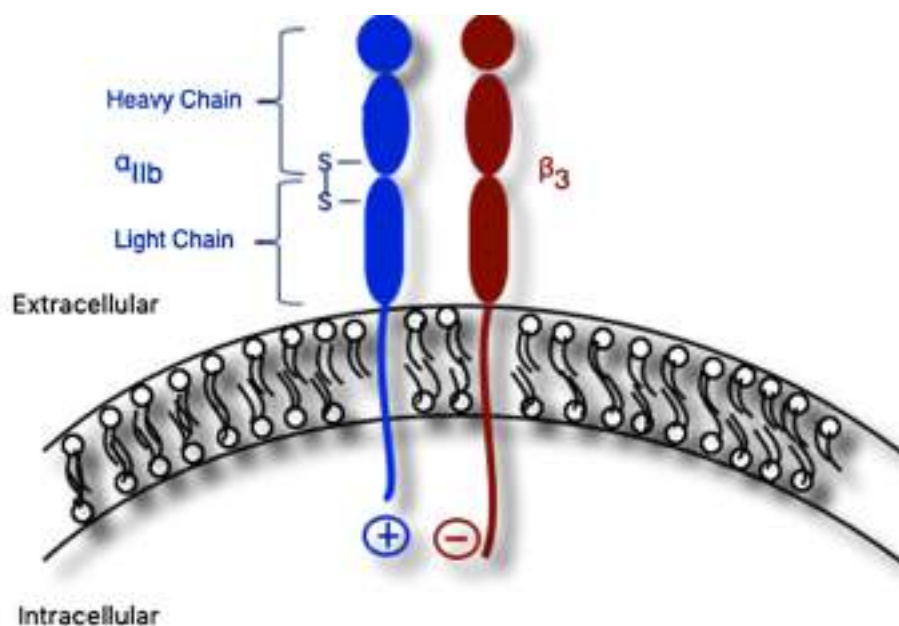
Figure 1 Schematic of $\alpha_{IIb}\beta_3$ integrin composed of α_{IIb} and β_3 subunits. The mature α_{IIb} subunit contains extracellular heavy and light chains linked together via disulfide bridge. Both subunits contain extracellular, transmembrane, and cytoplasmic domains; the latter domains are linked via salt bridge.
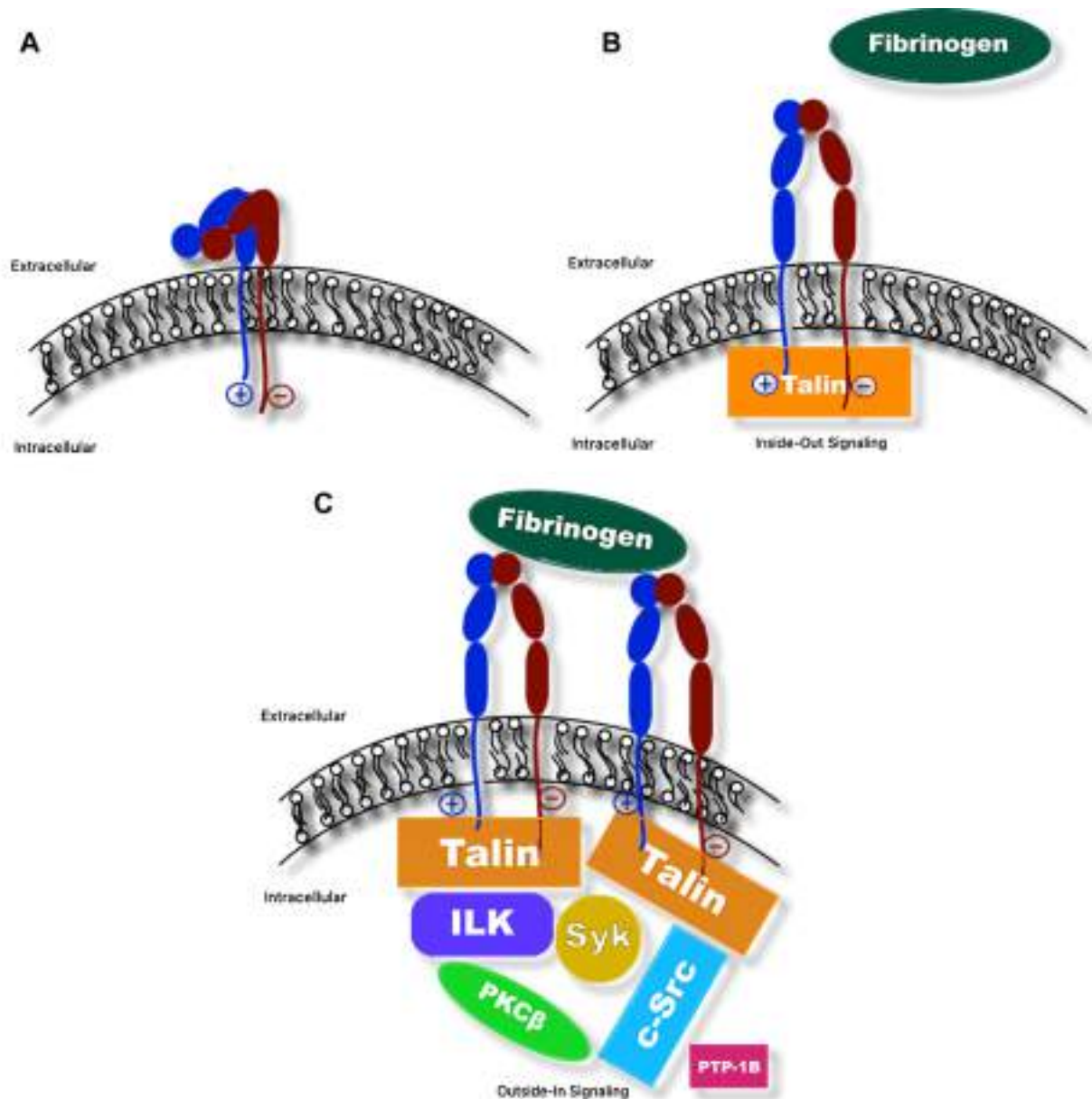
Figure 2 Schematic of $\alpha_{IIb}\beta_3$ integrin undergoing inside-out and outside-in signaling. (**A**) Bent confirmation of $\alpha_{IIb}\beta_3$ integrin with intact salt bridge linking cytosolic domains of the subunits (low affinity for binding fibrinogen). (**B**) Binding of intracellular protein talin disrupts salt bridge and triggers separation of the cytosolic region of β_3 from that of α_{IIb}, resulting in a conformational change of the $\alpha_{IIb}\beta_3$ integrin into the upright position. In this position, fibrinogen is able to bind extracellular domains (high affinity for binding fibrinogen; inside-out signaling). (**C**) Fibrinogen, in turn, binds additional $\alpha_{IIb}\beta_3$ integrins to facilitate platelet aggregation, resulting in activation and recruitment of additional intracellular and cytosolic proteins, such as c-Src tyrosine kinase (c-Src), integrin-linked kinase (ILK), spleen tyrosine kinase (Syk), protein kinase C (PKC), and protein tyrosine phosphatase (PTP1B) and others, to facilitate processes including cytoskeletal reorganization for platelet spreading, clot stabilization, and clot retraction (outside-in signaling).

become disrupted. Each of these opportunities for error, in turn, implies the potential for countless theoretical GT-causing genetic variants.

Genetics

The α_{IIb} and β_3 subunits are respectively encoded by *ITGA2B* (65 kbp) and *ITGB3* (17 kbp), which are both found on chromosome 17 (17q21.31 and 17q21.32, respectively; OMIM # 607759 and OMIM #173470). *ITGA2B* and *ITGB3* expression are not known to be coordinated,[3,31] however, and mutations in either of these genes can lead to forms of GT that are phenotypically indistinguishable.[1] GT inheritance is typically autosomal recessive and patients may exhibit homozygosity, particularly if

consanguinity is present, or compound heterozygosity.[8,20,31,38] *ITGB3* mutations are more common, presumably due to its relatively larger coding region of 30 exons in comparison to the 15 exons comprising *ITGA2B*.[1] As of February 2021, 475 *ITGA2B* and *ITBG3* GT-causing mutations have been catalogued in the Glanzmann Thrombasthenia Database (https://glanzmann.mcw.edu/) and most commonly include nonsense, missense, and splice site variants.[1,20] Large deletion and duplication mutations are rare.[1,20] Mutations leading to a GT phenotype can be manifested by dysfunctional gene expression, protein folding, post-translational processing, trafficking to the platelet membrane, and ligand binding.[1,8,39–43] In fact, missense mutations impeding such processes have helped researchers to identify functional coding sequences within *ITGA2B* and *ITBG3*.[20,44–46] For example, *ITGA2B* c.818G>A disrupts the calcium-binding site with the β_3 subunit and has been shown to result in lack of $\alpha_{IIb}\beta_3$ integrin expression at the platelet membrane.[8,39–41] Alternatively, defects within $\alpha_{IIb}\beta_3$ integrin extracellular ligand binding sites result in a qualitative variant of GT in which quantities of $\alpha_{IIb}\beta_3$ integrin at the platelet membrane are otherwise intact. Such differences in $\alpha_{IIb}\beta_3$ integrin expression at the level of the platelet membrane are the basis for distinguishing clinical types of hereditary GT (Table 1). In Type I GT, platelets' membrane expression of $\alpha_{IIb}\beta_3$ integrin is less than 5% of the wild-type quantity.[47,48] Type I GT is most common, representing 62–78% of GT cases.[1,3,4,20] Type II GT, in which 5–25% of normal of $\alpha_{IIb}\beta_3$ integrin expression is maintained,[4,8,20,47,48] represents about 12–16% of the GT population.[1,3,4,20] Type III represents a "variant" GT phenotype in which the $\alpha_{IIb}\beta_3$ integrin is present in sufficient quantities at the platelet membrane (ranging from 25% to 100% of reference levels),[8,20,47,48] but is qualitatively dysfunctional, and represents 8–22% of affected patients.[1,3,4,20] Mutations conferring a defective $\alpha_{IIb}\beta_3$ integrin result in varying clinical severities, but tend to involve ligand binding sites, such as *ITGB3* c.719G>A, and inside-out signaling, such as *ITGB3* c.2332T>C.[8,45,49,50] Interestingly, gain-of-function GT-like cases have also been described involving compound heterozygous *ITGA2B* and *ITBG3* mutations affecting membrane-adjacent residues resulting in auto-activation of $\alpha_{IIb}\beta_3$, reduced $\alpha_{IIb}\beta_3$ expression, and thrombocytopenia.[1,51–53] In a minority of patients, no mutation may be found, suggesting unidentified causes of GT that could perhaps be attributed to non-sequenced promoter or intron regions, mutated proteins that may help facilitate $\alpha_{IIb}\beta_3$ integrin development and transport, or modulators affecting $\alpha_{IIb}\beta_3$ integrin expression, such as miRNA or epigenetic changes.[20]

Acquired Glanzmann Thrombasthenia

Acquired GT is a disorder characterized by anti-$\alpha_{IIb}\beta_3$ complex-antibody-mediated platelet destruction.[57,58] More common than its hereditary counterpart, acquired GT can manifest as primary immune thrombocytopenia (ITP) or occur secondary to autoimmune disorders, malignancies, or organ transplants.[57–60] Certain medications, including the antimalarial quinine, antiarrhythmic quinidine, and various anticoagulants, including abciximab, have been identified as triggers for acquired GT.[1,58]

Clinical Presentation

Patients with hereditary GT tend to develop easy bruising and mucocutaneous bleeding symptoms (Table 2) early in life, with a mean age of diagnosis of 1 year of age and 15% of GT patients presenting beyond age 14 years.[2,61] Males with GT may be diagnosed as a result of post-circumcision hemorrhage.[8,62] Loss of primary teeth is

Table 1 Types of Glanzmann Thrombasthenia and Their Frequencies, with Examples of Genetic Mutations

Type of GT	Relative Expression of $\alpha_{IIb}\beta3$	Relative Frequency	Example of Functional $\alpha_{IIb}\beta3$ Defect
Type I	<5% versus Wild-type	62–78%	*ITGA2B* c.273G>D: $\alpha_{IIb}\beta3$ unable to transport to the platelet membrane[54]
Type II	5–25% versus Wild-type	12–16%	*ITGA2B* c.1772_1773insG: Premature stop codon leading to nonsense-mediated decay of mRNA[55]
Type III (Variant)	25–100% versus Wild-type (Qualitative Defect)	8–22%	*ITGB3* c.2259T>C: Defect in outside-in signaling via $\alpha_{IIb}\beta3$[56]

Abbreviation: GT, Glanzmann Thrombasthenia.

Table 2 Clinical Presentations of Glanzmann Thrombasthenia

Clinical Presentation	Note(s)
Easy Bruising	Following minor trauma
Mucocutaneous bleeding	Includes epistaxis; heavy, prolonged, and/or more frequent menstrual bleeding; gingival bleeding; gastrointestinal bleeding (less common)
Excessive bleeding post trauma or surgery	In young boys, may see persistent bleeding following circumcision
Intracranial hemorrhage	Very rare
Hematuria	Very rare
Hemarthrosis	Very rare
Organ bleeding	Very rare

another common source of bleeding during childhood.[8] In rare cases, abnormal bleeding may not occur until adulthood, when a patient's coagulation system is challenged by childbirth or another severe trauma.[4,20] Bruising provoked by mild trauma is the most common symptom experienced, followed by mucocutaneous bleeding.[4] Typically, bleeding symptoms are less severe than those seen in hemophilia patients,[2,63] although more than two-thirds of patients require one or more platelet and/or red blood cell transfusions over their lifetime.[20,64] Epistaxis is the most prevalent source of severe bleeding, affecting 60–80% of patients.[4] This symptom is most prominent during childhood,[4,65] when the nasal septum is most friable and also most likely to be subjected to the trauma of nose-picking.[1]

The majority of females with GT experience heavy, prolonged, and/or more frequent menstrual bleeding.[4] Gingival bleeding is also a source of concern, affecting up to 60% of patients, and may even result in iron deficiency anemia.[4] This symptom may be remedied with improved oral hygiene. Gastrointestinal bleeding is more rare, affecting only 10–28% of patients,[4,20] but may be particularly concerning in the presence of localized angiodysplasia.[8] Intracranial hemorrhages, hemarthroses, hematuria, and organ bleeds have all been described in GT patients, but are exceedingly rare.[1,4,8,20]

Diagnosis

As with any patient presenting with easy bruising and/or mucocutaneous bleeding, it is important to take a detailed history of bleeding symptoms. Diagnostic bleeding scores, which quantify a given patient's ongoing bleeding risk based on their historical symptoms and need for interventions, are generally useful for establishing a true bleeding

tendency although no cut-off values have been established for GT.[1,66] The ISTH/SSC bleeding assessment tool[67] has, however, demonstrated an ability to identify patients with inherited platelet disorders, once von Willebrand disease has been ruled out. History should also include the presence of any past or present autoimmune or malignant diagnoses, as well as current symptoms that may point to an undetected underlying systemic condition that could trigger acquired GT. Past infections should also be queried, as mutations involving integrin regulatory proteins, including kindlin-3 and calcium and diacylglycerol-regulated guanine nucleotide exchange factor, can affect both platelet and leukocyte integrin function, resulting in a simultaneous immunodeficiency;[58,68] these patients do not have GT. A medication history should include the use of antiplatelet medications, such as NSAIDs, and other anticoagulants that may suggest an alternative cause. Taking a family history and drawing a pedigree is encouraged, as it could provide critical information to help establish the presence of a familial bleeding disorder spanning multiple generations with a specific pattern of inheritance. When examining the patient, it is important to inspect the skin for signs of bruising as well as the mucocutaneous regions, including the nares, for evidence of bleeding. Additional areas of importance may be guided by history.

Preliminary investigations that are widely available and relatively inexpensive are necessary to help narrow the list of differential diagnoses (Figure 3), given the rarity of GT amongst haemostasis and coagulation disorders; for every one patient diagnosed with inherited GT, there are 1000 people diagnosed with von Willebrand disease,[69] 95 people diagnosed with immune thrombocytopenia,[70] and 85 people diagnosed with haemophilia A.[71] In terms of specific inherited platelet defects diagnosed per year, GT
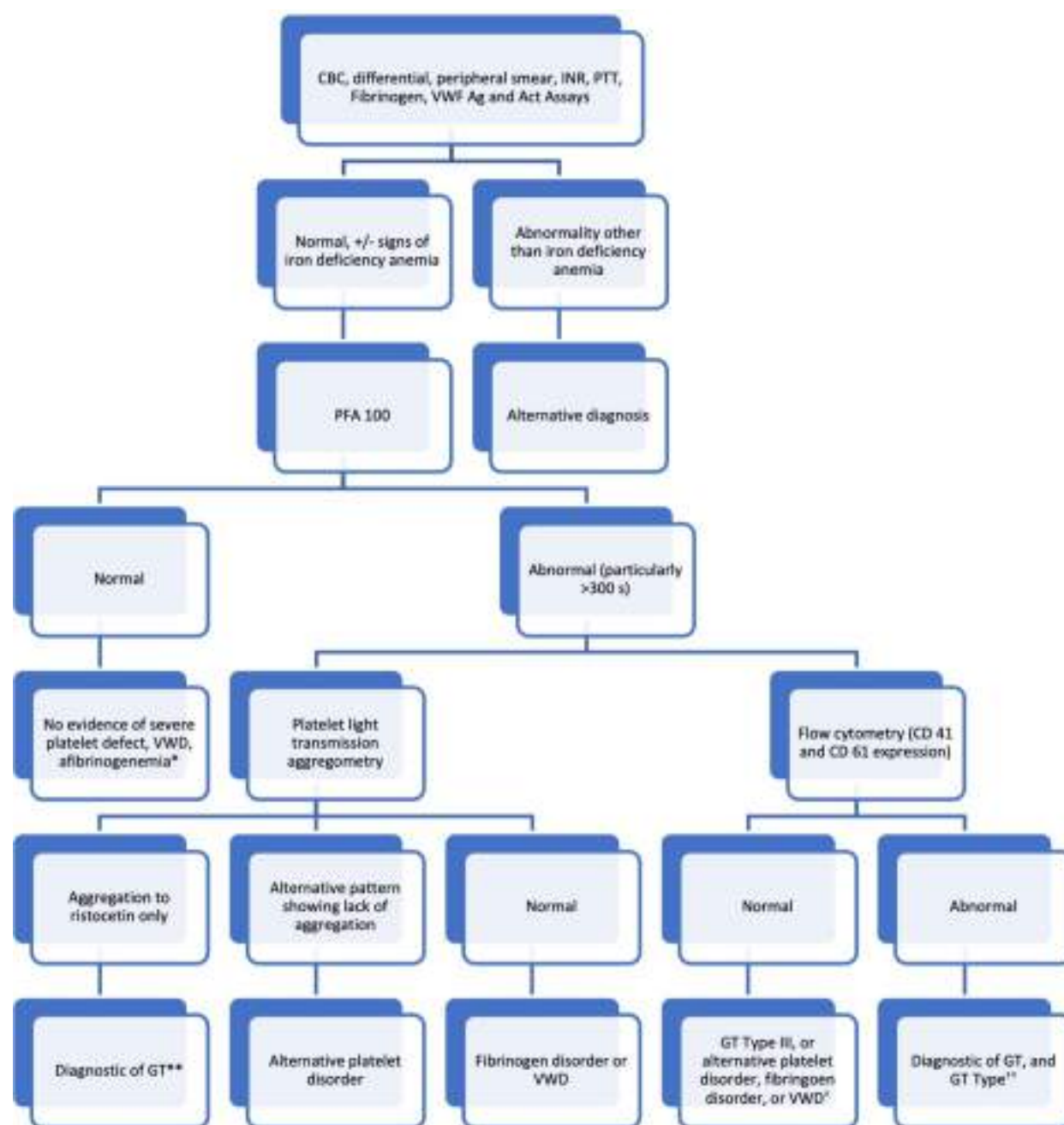
Figure 3 Diagnostic algorithm for GT. *Consider proceeding to platelet light transmission aggregometry if suspicion for platelet defect remains high. **Consider genetic testing to identify specific mutation of *ITGA2B* and *ITGB3* and/or flow cytometry to differentiate GT type. †Consider clot retraction assay (if available) and platelet light transmission aggregometry or genetic testing of *ITGA2B* and *ITGB3* to make the diagnosis of GT Type III. ††Consider genetic testing to identify specific mutation of *ITGA2B* or *ITGB3*.
Abbreviations: CBC, complete blood count; VWF Ag, von Willebrand factor antigen; VWF Act, von Willebrand factor activity; VWD, von Willebrand disease.

represents just 9.8% of this category.[72] As Dr. Glanzmann discovered,[7] complete blood count (CBC) and peripheral smear will typically reveal a normal quantity of platelets that are of a normal size and maintain a typical granular pattern, which helps differentiate GT from other platelet disorders, such as Bernard-Soulier and Grey Platelet

Syndromes. In some cases, the CBC might also suggest iron deficiency anemia (IDA), which may be a consequence and/or a cause of abnormal bleeding, as arachidonic acid-induced platelet aggregation has been shown to improve following treatment of IDA.[73,74] Additional CBC abnormalities would point towards

a diagnosis other than GT. Coagulation studies, including INR, PTT, and fibrinogen, are normal in GT and help rule out coagulation factor deficiencies and fibrinogen disorders that could be alternative, more common causes of bleeding and abnormal platelet aggregation. Additionally, normal von Willebrand factor antigen and von Willebrand factor activity assays are expected and eliminate the possibility of von Willebrand disease.

Additional screening tests may be helpful prior to confirming the diagnosis of GT with platelet light transmission aggregometry, which requires a large volume of blood, 3–4 hours, and a specialized laboratory.[75] A clot retraction assay can be performed using 1–2 mL of whole blood incubated overnight at 37 degrees Celsius, removing the newly formed clot, and quantifying the relative volume of serum within the remaining plasma sample that was extracted from the clot during the retraction process. The assay can also be performed using platelet rich plasma. Assuming a normal platelet quantity and adequate fibrinogen count and quality, an abnormal clot retraction is consistent with diagnosis of GT, as this assay specifically tests the outside-in signaling of the $\alpha_{IIb}\beta_3$ integrin.[76,77] Furthermore, a result of no clot retract versus low-to-normal clot retraction can help differentiate between Types I and II GT, respectively.[36] It should be noted, however, that this test has fallen out of favour, as it is only available is specialized laboratories and is no longer considered to be a required test in the diagnosis of GT.

Platelet function analyzer, or PFA 100, testing requires as little as 2 mL of blood and is a measure of primary hemostasis.[75,78] By timing platelet plug formation over a membrane in the presence of stimulants (collagen and epinephrine or collagen and ADP) under high shear conditions, samples affected by platelet disorders and von Willebrand disease will be differentiated.[78] While an abnormal PFA-100 is 100% sensitive for GT,[75] it does not distinguish GT from severe von Willebrand disease or afibrinogenemia.[1]

Platelet light transmission aggregometry is a gold standard for establishing a diagnosis of GT.[79] This test exposes a blood sample to various agonists to stimulate platelet plug formation and measures the subsequent transmission of light through the platelet suspension.[80] Platelet aggregation in the presence of ristocetin, but not in the presence of ADP, collagen, thrombin, or adrenaline (<10% of reference values) is pathognomonic for the disorder.[36,47,77] It may be necessary to repeat testing once to confirm the results or compare with a control sample in parallel, given

the multiple pre-analytical and analytical variables that may affect the outcome of this investigation.[1,80–83]

Once Glanzmann thrombasthenia has been diagnosed, there are additional tests that are helpful for further characterization of the disorder. Flow cytometry with monoclonal antibody panels against CD41 and CD61 can quantify deficiencies in αIIb and β3 expression at the platelet membranes, respectively, and allow for identification of Type I (<5% expression) and Type II (5–25% expression) GT.[84] Flow cytometry typically reveals at least 50% αIIb and β3 expression in Type III GT.[36,84] Differentiating GT type is particularly helpful for risk stratifying patients who may develop alloimmunization following platelet transfusion (see Platelet Refractoriness and Alloimmunization). Furthermore, flow cytometry can also be used to identify specific antibodies against $\alpha_{IIb}\beta_3$ in cases of acquired GT.[84,85] Genetic sequencing of *ITGA2B* and *ITGB3* may also be performed to confirm the specific mutations involved. Multiple groups[86–89] have recently developed high-throughput molecular diagnostic assays for patients with GT and other inherited bleeding disorders. It should be noted though that at this time, there does not seem to be a correlation between specific mutation and phenotype severity[8] and even family members sharing similar mutations have been shown to have significant variability amongst their clinical outcomes.[20]

Management
Routine Follow-Up and Anticipatory Guidance

Any patient diagnosed with GT should be referred to a tertiary care centre with a haematologist experienced in treating patients with inherited bleeding disorders. This centre must be able to manage patients outside of regular clinic hours should severe bleeding occur. During regular clinic hours, the patient should have access to a multidisciplinary team, including a nursing coordinator, physiotherapist, social worker, and psychologist as necessary.[2,90] Good oral hygiene and routine dental follow-up are also of the utmost importance.[91] Patients with GT can and should receive routine vaccinations with the additional step of providing 15 minutes of applied pressure to the site to encourage proper hemostasis.[90] Additional immunization against hepatitis A and B is encouraged, given heightened risk for exposure to blood products.[90] Patients should receive adequate teaching about GT, including education on preventing bleeds, such as avoiding

certain medications, including aspirin and NSAIDs, and high-impact physical activities. Patients should be counseled regarding recognition of bleeds and necessity of urgent medical intervention for prolonged or worrisome bleeding, as delay in treatment has been implicated in both hospital length of stay and overall treatment response.[92,93] Any patient with a diagnosis of GT, or any clinically significant bleeding disorder, should wear a MedicAlert bracelet or similar piece of identification to flag their condition to emergency medical personnel in the event of incapacitation.

Pregnant women who have been diagnosed with GT, or whose partners have been diagnosed with GT, should be considered for prenatal testing if the parents of the fetus are consanguineous. Prenatal diagnosis is routinely confirmed by genetic testing,[1] although flow cytometry has also been used.[94] Pregnant women who are known to have GT should also be screened for anti- $\alpha_{IIb}\beta_3$ antibodies via monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay regularly throughout gestation,[95] as these antibodies can cross the placenta and cause dangerously low platelet levels in the fetus.[31,96,97] Mothers must be counseled about this risk and for this reason, it is important to test the newborn's platelet count within the first few hours life[8] if maternal antibodies have been detected. While mothers with GT are not expected to experience increased bleeding during the pregnancy itself, specific planning surrounding labour and delivery is indicated in order to prevent excessive bleeding in the intra- and post-partum periods (see Site-Specific considerations below).

General Approach to Bleeding

Minor bleeding episodes may be initially managed at home using manual compression and/or antifibrinolytic therapy. Depending on the bleeding site, manual compression may be achieved by applying pressure with gauze or, in the cases of epistaxis, pinching the soft cartilages of the nares closed using the index finger and thumb. Cold compresses are not recommended, as they have been shown to impair haemostasis and coagulation in patients with and without bleeding disorders.[98,99] The use of oral antifibrinolytic therapy,[31,47] including tranexamic acid (TXA) and aminocaproic acid, can be easily administered in the home setting and may be critical for stopping a bleed in its early stages. Antifibrinolytics should not be administered to patients experiencing gross hematuria, however.[100,101]

GT patients with bleeding that is refractory to these methods should seek emergency medical attention.

Patients with GT experiencing severe bleeding require some combination of three main treatment options to achieve haemostasis: antifibrinolytics, recombinant activated factor VII (rF7a), and platelet transfusion. DDAVP has also been used,[47,102] although study results have been inconsistent[1] and further investigation is needed in this area. In addition to providing haemostasis, the need for red blood cell transfusion must always be considered. These transfusions should be sourced from washed or frozen red blood cells in order to remove any residual platelets that could trigger alloimmunization.[31] Unfortunately, the urgency of the situation may preclude the ability to wait for these products to become available and the risks and benefits of transfusing fresh non-washed pRBCs must be carefully weighed.

Antifibrinolytics, in addition to being available enterally, can be administered intravenously or topically using antifibrinolytic-soaked gauze or gel foam. Fibrin glue and topical thrombin are additional topical alternatives/complements to antifibrinolytics.

Platelet transfusion has been a mainstay of GT therapy for years. However, the benefit of providing functional platelets must be weighed against the risk of causing alloimmunization and rendering a patient refractory to subsequent platelet transfusions. GT Type I patients are at particular risk of alloimmunization against αIIb and β3 due to their inherent lack of self-expression of these antigens.[20,31] Given the possibly fatal implications of platelet refractoriness, platelet transfusions should be reserved for life-threatening hemorrhages in the GT population.[31] The use of platelet transfusions in girls and pre-menopausal women should be particularly avoided whenever possible given the added risk of transplacental antibody transfer causing future neonatal alloimmune thrombocytopenia.[1,96] If and when choosing to transfuse platelets, they should be HLA-matched, leukocyte-reduced whenever possible to avoid HLA-alloimmunization.[31] ABO compatibility offers an additional layer of protection[31] and patients who have received any blood products, including only red blood cells, should be regularly screened for antibodies. Furthermore, there have even been some reports of antibody presence in non-transfused patients,[20,31,103] which may have been infection-induced.

rF7a is an expensive[47,104,105] but efficacious treatment for patients with GT experiencing moderate to severe bleeding. rF7a has a long shelf life at room

temperature[106,107] and is approved in several counties for use in patients with hemophilia, congenital factor VII deficiency, and GT. In particular, it is approved in the United States for use in GT patients who are refractory to platelets as well as in Europe for patients with GT who cannot receive platelets. Various studies have reported partial or better responses to rF7a for 67–93%[104–106,108] of nonsurgical hemorrhages in GT patients, with or without the help of antifibrinolytics. Its efficacy has been found to be irrelevant to antibody presence.[105] rF7a's mechanism of action for GT patients is not entirely understood.[104] However, it is thought that through activation of factor X, rF7a facilitates generation of thrombin, which can then bind and activate GT platelets via intact GP1b receptors.[31,106,109,110]

It is important to note that dosing of rF7a varies based on indication, and patients with GT typically require fewer total doses than hemophilia patients with inhibitors do.[111] Patients with GT typically require 80–140 μg/kg intravenous every 2.5 hours or less until hemostasis is achieved.[61,104] Continuous infusions of rF7a have only been rarely used and are not well studied.[104]

Site-Specific Considerations

As epistaxis is a major source of bleeding in children with GT, efforts should be made to prevent over-drying of the nasal mucosa in affected patients. These methods include use of humidifiers, saline nasal sprays and Vaseline gel. Some patients may find it helpful to sleep in a seated position when experiencing mild nasal or oral bleeding. Major epistaxis requires the expertise of an otolaryngologist. A 2010 retrospective study[65] of 5 children with GT presenting on 63 occasions with epistaxis reported a hospitalization rate of 72%. Forty-two percent of these admissions required intensive care. While anterior nasal packing with or without topic hemostatic treatments were successful about one-third of the time, the administration of a bovine collagen matrix was deemed successful in just one-half of cases.

Oral cavity bleeding can commonly present in the setting of gingivitis. Daily flossing is therefore highly encouraged. Those with gingival bleeding may benefit from using antifibrinolytic therapy as a mouthwash.[2,5,6] Children are also at risk of oral bleeding with the routine loss of primary teeth. Loose teeth can be treated with fibrin glue to help mitigate blood loss.[3,112,113] Plastic splints can also be used to physically support hemostasis.[114]

Heavy uterine bleeding affects most women with GT. Efforts should be made to quantify this symptom using standardized scales, such as the Pictorial Blood Loss Assessment Chart.[115,116] GT patients with heavy menses should be treated first line with antifibrinolytics. Patients who have refractory bleeding should be reviewed by a gynecologist and considered for oral contraceptive therapy or hormonal intrauterine devices, with or without adjunctive antifibrinolytics. Intravenous high-dose estrogen therapy over 1–2 days is an effective measure for these patients and should be administered in consultation with a gynecologist.[35,117] It should be considered prior to administration of rF7a or platelet transfusion if bleeding is not life-threatening.

Bleeding associated with childbirth is a major concern in women with GT. Surprisingly, up to 50% of women with GT may not be diagnosed until facing the haemostatic challenges of labour and delivery.[1,96] Epidural anesthesia is contraindicated in this population due to additional bleeding risks and an alternative pain management plan should be arranged beforehand.[1,77] Women in labour for vaginal delivery or preoperative for caesarean section should be started on rF7a and antifibrinolytics in the presence of evidence of abnormal bleeding and may benefit from platelets as well. Platelets may continue to be required up to 7 days after delivery.[8,118,119] Women experiencing postpartum hemorrhage should be managed with packed red blood cells and uterotonics.[120]

Perioperative Bleeding

The rate of perioperative bleeding in patients with inherited functional defects has been reported at 24.8%, with cardiovascular and urological surgeries bearing a particularly significant risk.[121] The principles for preventing and managing surgical bleeds are similar to those for nonsurgical hemorrhages: patients tend to be managed with a combination of antifibrinolytics and rF7a, with or without platelets. One international retrospective review sponsored by Novo Nordisk Health Care AG involving 96 GT patients[122] who underwent 101 surgeries in which rF7a and platelets were used, with or without antifibrinolytics, reported 100% efficacy in achieving hemostasis, regardless of alloimmunization status. This success rate was maintained for minor procedures when only rF7a and antifibrinolytics were given to patients with a history of antibodies and refractoriness. The same study found that this success rate decreased to 88.9% when rF7a was used without antifibrinolytics. The group that received platelets with or without antifibrinolytics had a success

rate of just 67%. Another international survey in which ties to Novo Nordisk Health Care AG were disclosed reported 67% success in preventing surgical bleeding in 9 GT patients undergoing major operations and 92% of 25 GT patients undergoing minor procedures, with or without antifibrinolytics.[105] rF7a dosing in surgical patients is 80–90 ug/kg intravenously immediately prior to surgery, with at least 2 repeated doses every 2–6 hours;[123] some sources[61,106,122] recommend dosing as high as 140 u/kg. Patients undergoing major operations are expected to require additional doses, with one review[104] on rF7a use in surgical patients reporting median durations of treatment of 7 hours and 2 days for minor and major operations, respectively. In the post-operative period, haemostasis can be monitored clinically along with trending of hemoglobin.[47]

Platelet Refractoriness and Alloimmunization

Any GT patient presenting with refractoriness to platelet transfusion likely requires rF7a and should be considered for packed red blood cell transfusion. Anti-platelet antibodies, should always be suspected in the case of platelet refractoriness.[8] The prevalence of alloimmunization, due either to anti- $\alpha_{IIb}\beta_3$ or anti-HLA antibodies, is as high at 30% in the general GT population.[61] Patients who have developed HLA antibodies should be treated with HLA-compatible platelets. Alloimmunization against $\alpha_{IIb}\beta_3$ resulting in lack of response to all platelet transfusions, however, is a life-threatening complication for GT patients. Patients whose mutations prevent any $\alpha_{IIb}\beta_3$ expression at the platelet membrane are theoretically at the highest risk for developing anti-$\alpha_{IIb}\beta_3$ antibodies.[31] Women seem to be more affected than men, although this risk may be due to the prevalence of transfusions for uterine bleeding.[20,31] A relationship between number of past platelet transfusions and presence of platelet refractoriness in the GT population has not been established, however, and even residual platelets within red blood cell transfusions can trigger alloimmunization.[31,124,125] When anti-$\alpha_{IIb}\beta_3$ antibodies are present, platelet transfusions in conjunction with antifibrinolytics have still been successful 71% of the time for non-surgical hemorrhages.[106] However, rF7a has been shown to be effective in 91% of non-surgical bleeds, regardless of antibody status.[106] rF7a has similarly been shown to be successful in treating 88% of surgical bleeds in antibody patients.[122] Patients with antibodies have been shown to require more doses or rF7a than those without.[106,122]

Alloimmunization has been reported in up to 70% of pregnant women with GT.[1,61] Pregnant mothers with GT who are immunized by fetal platelet antigens will produce antibodies that can later cross the placenta and induce life-threatening thrombocytopenia and hemorrhage in the fetus.[31,96,97] Methods to lower antibody titre prenatally include plasma exchange, steroids, and intravenous immunoglobulin (IVIG),[8,95,126,127] with IVIG dosing of 0.5–1g/kg per week associated with 97.3% success.[95,128]

Thrombosis Secondary to Recombinant Activated Factor VII

The rate of thromboses secondary to rF7a use in GT patients fortunately remains low. One literature review of rF7a use in GT patients found a thromboembolic event rate of 1.4% amongst 221 bleeding episodes.[104] A review including data from the Glanzmann Thrombasthenia Registry found 5 cases of thromboembolism amongst 490 instances of rF7a use for nonsurgical bleeding and perioperative prophylaxis.[129] Two of these cases occurred in patients over the age of 65 years, with one featuring additional risk factors of immobilization, surgery, and continuous rF7a infusion. Thromboembolic diagnoses reported in GT patients receiving rF7a within the literature include deep vein thrombosis, pulmonary embolism, ureteric obstruction, and intracardiac thrombi.[105,130–132]

Curative Therapy

Bone marrow transplantation has proven to be a curative option in several GT patients, including those with anti-platelet antibodies.[31,133–139] In many cases, conditioning therapy has helped alleviate antibodies, which can otherwise threaten engraftment.[50,117] Transplant has typically been performed in the pediatric population, with chronic graft versus host disease being a major morbidity.

Though still in the experimental stages, gene therapy has been explored as an option for treating GT. In 2011, Fang et al[140] demonstrated increased $\alpha_{IIb}\beta_3$ expression in GT dog models who had been transfected with peripheral blood stem cells engineered to express human *ITGA2B*. Three years later, Sullivan et al[141] generated induced pluripotent stem (iPS) cells from peripheral monocytes of 2 GT patients and transfected the iPS cells with αIIb cDNA at the AAVS1 locus, accompanied by a megakaryocyte-specific promoter. Thereafter, these patients exhibited $\alpha_{IIb}\beta_3$ platelet expression surpassing 50% and 70%.

Future Steps

The diagnosis and management of patients with GT continues to have many associated challenges. Diagnosis requires testing in specialized laboratories and further genetic testing, at least at this point, does little to help predict severity. However, by continuing to grow the Glanzmann Thrombasthenia Registry, our understanding of GT's various mutations will become more developed. Currently, the mainstay of treatment remains supportive. However, as recognition of anti-platelet antibodies becomes more prevalent, the demand for curative options will certainly increase. To meet this demand, bone marrow transplant regimens will need to become more standardized for this population and recognized as a treatment option early in life. Moreover, the further development of gene therapy technology for GT patients will offer an alternative option to cure this otherwise lifelong disease.

Disclosure

The authors reported no conflicts of interest for this work.

References

1. Botero JP, Lee K, Branchford BR, et al. Glanzmann thrombasthenia: genetic basis and clinical correlates. *Haematologica*. 2020;105(4):888–894. doi:10.3324/haematol.2018.214239
2. Krause KA, Graham BC. Glanzmann Thrombasthenia. 2020. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021. PMID: 30855858.
3. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood*. 2011;118(23):5996–6005. doi:10.1182/blood-2011-07-365635
4. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood*. 1990;75(7):1383–1395. doi:10.1182/blood.V75.7.1383.1383
5. Lee A, Poon MC. Inherited platelet functional disorders: general principles and practical aspects of management. *Transfus Apher Sci*. 2018;57(4):494–501. doi:10.1016/j.transci.2018.07.010
6. Borhany M, Fatima H, Naz A, Patel H, Shamsi T. Pattern of bleeding and response to therapy in Glanzmann thrombasthenia. *Haemophilia*. 2012;18(6):e423–5. doi:10.1111/hae.12017
7. Glanzmann E. Hereditare hämorrhagische thrombasthenie. Ein Beitrag zur Pathologie der Blutplättchen. *Jb Kinderheilk*. 1918;88:133. German.
8. Bellucci S, Caen J. Molecular basis of Glanzmann's thrombasthenia and current strategies in treatment. *Blood Rev*. 2002;16 (3):193–202. doi:10.1016/s0268-960x(02)00030-9
9. Coller B. A brief history of ideas about platelets in health and disease. *Platelets*. 2007;170. doi:10.1016/B978-0-12-387837-3.00069-9
10. Stevens RF, Meyer S. Fanconi and Glanzmann: the men and their works. *Br J Haematol*. 2002;119(4):901–904. doi:10.1046/j.1365-2141.2002.03812.x
11. Caen J, Cousin C. ["In vivo" disorder of platelet adhesiveness in Willebrand's disease and Glanzmann's thrombasthenias. Trial interpretation]. *Nouv Rev Fr Hematol*. 1962;2:685–694. [Slovenian].
12. Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. *J Clin Invest*. 1977;60(3):535–545. doi:10.1172/JCI108805
13. Kunicki TJ, Aster RH. Deletion of the platelet-specific alloantigen PlA1 from platelets in Glanzmann's thrombasthenia. *J Clin Invest*. 1978;61(5):1225–1231. doi:10.1172/JCI109038
14. Hagen I, Solum NO. Further studies on the protein composition and surface structure of normal platelets and platelets from patients with Glanzmann's thrombasthenia and Bernard-Soulier syndrome. *Thromb Res*. 1978;13(5):845–855. doi:10.1016/0049-3848(78)90189-5
15. Nurden AT, Caen JP. The different glycoprotein abnormalities in thrombasthenic and Bernard-Soulier platelets. *Semin Hematol*. 1979;16(3):234–250.
16. Nurden AT. Inherited abnormalities of platelets. *Thromb Haemost*. 1999;82(2):468–480. doi:10.1055/s-0037-1615867
17. Coller BS, Cheresh DA, Asch E, Seligsohn U. Platelet vitronectin receptor expression differentiates Iraqi-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. *Blood*. 1991;77 (1):75–83. doi:10.1182/blood.V77.1.75.75
18. Nurden P, Poujol C, Nurden AT. The evolution of megakaryocytes to platelets. *Baillieres Clin Haematol*. 1997;10(1):1–27. doi:10.1016/s0950-3536(97)80048-0
19. Mitchell WB, Li J, French DL, Coller BS. alphaIIbbeta3 biogenesis is controlled by engagement of alphaIIb in the calnexin cycle via the N15-linked glycan. *Blood*. 2006;107(7):2713–2719. doi:10.1182/blood-2005-07-2990
20. Nurden AT, Pillois X, Fiore M, et al. Expanding the mutation spectrum affecting αIIbβ3 integrin in Glanzmann thrombasthenia: screening of the ITGA2B and ITGB3 genes in a large international cohort. *Hum Mutat*. 2015;36(5):548–561. doi:10.1002/humu.22776
21. Carrell NA, Fitzgerald LA, Steiner B, Erickson HP, Phillips DR. Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. *J Biol Chem*. 1985;260 (3):1743–1749. doi:10.1016/S0021-9258(18)89656-9
22. González-Manchón C, Fernández-Pinel M, Arias-Salgado EG, et al. Molecular genetic analysis of a compound heterozygote for the glycoprotein (GP) IIb gene associated with Glanzmann's thrombasthenia: disruption of the 674–687 disulfide bridge in GPIIb prevents surface exposure of GPIIb-IIIa complexes. *Blood*. 1999;93(3):866–875. doi:10.1182/blood.V93.3.866
23. Yang J, Ma YQ, Page RC, Misra S, Plow EF, Qin J. Structure of an integrin alphaIIb beta3 transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proc Natl Acad Sci U S A*. 2009;106(42):17729–17734. doi:10.1073/pnas.0909589106
24. Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. 2004;104(6):1606–1615. doi:10.1182/blood-2004-04-1257
25. Coller BS, Shattil SJ. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood*. 2008;112(8):3011–3025. doi:10.1182/blood-2008-06-077891
26. Armstrong PC, Peter K. GPIIb/IIIa inhibitors: from bench to bedside and back to bench again. *Thromb Haemost*. 2012;107 (5):808–814. doi:10.1160/TH11-10-0727
27. Zhu J, Negri A, Provasi D, Filizola M, Coller BS, Springer TA. Closed headpiece of integrin αIIbβ3 and its complex with an αIIbβ3-specific antagonist that does not induce opening. *Blood*. 2010;116(23):5050–5059. doi:10.1182/blood-2010-04-281154
28. Kieffer N, Phillips DR. Platelet membrane glycoproteins: functions in cellular interactions. *Annu Rev Cell Biol*. 1990;6:329–357. doi:10.1146/annurev.cb.06.110190.001553

29. Farrell DH, Thiagarajan P. Binding of recombinant fibrinogen mutants to platelets. *J Biol Chem*. 1994;269(1):226–231. doi:10.1016/S0021-9258(17)42338-6

30. Du X, Ginsberg MH. Integrin αIIbβ3 and platelet function. *Thromb Haemost*. 1997;78(1):96–100. doi:10.1055/s-0038-1657508

31. Fiore M, d'Oiron R, Pillois X, Alessi MC. Anti-α. *Br J Haematol*. 2018;181(2):173–182. doi:10.1111/bjh.15087

32. Gemmell CH, Sefton MV, Yeo EL. Platelet-derived microparticle formation involves glycoprotein IIb-IIIa. Inhibition by RGDS and a Glanzmann's thrombasthenia defect. *J Biol Chem*. 1993;268 (20):14586–14589. doi:10.1016/S0021-9258(18)82371-7

33. Reverter JC, Béguin S, Kessels H, Kumar R, Hemker HC, Coller BS. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and "clinical restenosis". *J Clin Invest*. 1996;98 (3):863–874. doi:10.1172/JCI118859

34. Byzova TV, Plow EF. Networking in the hemostatic system. Integrin alphaiibbeta3 binds prothrombin and influences its activation. *J Biol Chem*. 1997;272(43):27183–27188. doi:10.1074/jbc.272.43.27183

35. Solh T, Botsford A, Solh M. Glanzmann's thrombasthenia: pathogenesis, diagnosis, and current and emerging treatment options. *J Blood Med*. 2015;6:219–227. doi:10.2147/JBM.S71319

36. Fiore M, Nurden AT, Nurden P, Seligsohn U. Clinical utility gene card for: Glanzmann thrombasthenia. *Eur J Hum Genet*. 2012;20 (10). doi:10.1038/ejhg.2012.151

37. Ward CM, Kestin AS, Newman PJ. A Leu262Pro mutation in the integrin beta(3) subunit results in an alpha(IIb)-beta(3) complex that binds fibrin but not fibrinogen. *Blood*. 2000;96(1):161–169. doi:10.1182/blood.V96.1.161

38. Ruan J, Peyruchaud O, Alberio L, et al. Double heterozygosity of the GPIIb gene in a Swiss patient with Glanzmann's thrombasthenia. *Br J Haematol*. 1998;102(4):918–925. doi:10.1046/j.1365-2141.1998.00852.x

39. French DL. The molecular genetics of Glanzmann's thrombasthenia. *Platelets*. 1998;9(1):5–20. doi:10.1080/09537109876951

40. Schlegel N, Gayet O, Morel-Kopp MC, et al. The molecular genetic basis of Glanzmann's thrombasthenia in a gypsy population in France: identification of a new mutation on the alpha IIb gene. *Blood*. 1995;86(3):977–982. doi:10.1182/blood. V86.3.977.977

41. Vinciguerra C, Bordet JC, Beaune G, Grenier C, Dechavanne M, Négrier C. Description of 10 new mutations in platelet glycoprotein IIb (alphaIIb) and glycoprotein IIIa (beta3) genes. *Platelets*. 2001;12(8):486–495. doi:10.1080/095371001317126383

42. Fullard J, Murphy R, O'Neill S, Moran N, Ottridge B, Fitzgerald DJ. A Val193Met mutation in GPIIIa results in a GPIIb/IIIa receptor with a constitutively high affinity for a small ligand. *Br J Haematol*. 2001;115(1):131–139. doi:10.1046/j.1365-2141.2001.03075.x

43. Tadokoro S, Tomiyama Y, Honda S, et al. Missense mutations in the β3 subunit have a different impact on the expression and function between αIIbβ3 and αvβ3. *Blood*. 2002;99(3):931–938. doi:10.1182/blood.v99.3.931

44. Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger AL, Ginsberg MH. A beta 3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science*. 1990;249(4971):915–918. doi:10.1126/science.2392682

45. Bajt ML, Ginsberg MH, Frelinger AL, Berndt MC, Loftus JC. A spontaneous mutation of integrin alpha IIb beta 3 (platelet glycoprotein IIb-IIIa) helps define a ligand binding site. *J Biol Chem*. 1992;267(6):3789–3794. doi:10.1016/S0021-9258(19) 50595-6

46. Chen YP, Djaffar I, Pidard D, et al. Ser-752–>Pro mutation in the cytoplasmic domain of integrin beta 3 subunit and defective activation of platelet integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc Natl Acad Sci U S A*. 1992;89(21):10169–10173. doi:10.1073/ pnas.89.21.10169

47. Ganapule A, Jain P, Abubacker FN, et al. Surgical procedures in patients with Glanzmann's thrombasthenia: case series and literature review. *Blood Coagul Fibrinolysis*. 2017;28(2):171–175. doi:10.1097/MBC.0000000000000524

48. Casati V, D'Angelo A, Barbato L, et al. Perioperative management of a heterozygous carrier of Glanzmann's thrombasthenia submitted to coronary artery bypass grafting with cardiopulmonary bypass. *Anesth Analg*. 2006;103(2):309–311. doi:10.1213/01. ane.0000226087.11062.cd

49. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405–424. doi:10.1038/gim.2015.30

50. Nurden AT. Acquired antibodies to αIIbβ3 in Glanzmann thrombasthenia: from transfusion and pregnancy to bone marrow transplants and beyond. *Transfus Med Rev*. 2018;32(3):155–164. doi:10.1016/j.tmrv.2018.05.002

51. Kashiwagi H, Kunishima S, Kiyomizu K, et al. Demonstration of novel gain-of-function mutations of αIIbβ3: association with macrothrombocytopenia and glanzmann thrombasthenia-like phenotype. *Mol Genet Genomic Med*. 2013;1(2):77–86. doi:10.1002/ mgg3.9

52. Jayo A, Conde I, Lastres P, et al. L718P mutation in the membrane-proximal cytoplasmic tail of beta 3 promotes abnormal alpha IIb beta 3 clustering and lipid microdomain coalescence, and associates with a thrombasthenia-like phenotype. *Haematologica*. 2010;95(7):1158–1166. doi:10.3324/ haematol.2009.018572

53. Gresele P, Falcinelli E, Giannini S, et al. Dominant inheritance of a novel integrin beta3 mutation associated with a hereditary macrothrombocytopenia and platelet dysfunction in two Italian families. *Haematologica*. 2009;94(5):663–669. doi:10.3324/ haematol.2008.002246

54. Poncz M, Rifat S, Coller BS, et al. Glanzmann thrombasthenia secondary to a Gly273–>Asp mutation adjacent to the first calcium-binding domain of platelet glycoprotein IIb. *J Clin Invest*. 1994;93(1):172–179. doi:10.1172/JCI116942

55. Losonczy G, Rosenberg N, Boda Z, et al. Three novel mutations in the glycoprotein IIb gene in a patient with type II Glanzmann thrombasthenia. *Haematologica*. 2007;92(5):698–701. doi:10.3324/haematol.10847

56. Nurden P, Poujol C, Winckler J, Combrié R, Caen JP, Nurden AT. A Ser752–>Pro substitution in the cytoplasmic domain of beta3 in a Glanzmann thrombasthenia variant fails to prevent interactions between the alphaIIbbeta3 integrin and the platelet granule pool of fibrinogen. *Br J Haematol*. 2002;118(4):1143–1151. doi:10.1046/j.1365-2141.2002.03758.x

57. Sharma P, Kar R, Bhargava R, Ranjan R, Mishra PC, Saxena R. Acquired platelet dysfunction in 109 patients from a tertiary care referral hospital. *Clin Appl Thromb Hemost*. 2011;17(1):88–93. doi:10.1177/1076029610379397

58. Nurden AT. Acquired Glanzmann thrombasthenia: from antibodies to anti-platelet drugs. *Blood Rev*. 2019;36:10–22. doi:10.1016/j.blre.2019.03.004

59. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. *Blood*. 2009;113 (26):6511–6521. doi:10.1182/blood-2009-01-129155

60. Linge P, Fortin PR, Lood C, Bengtsson AA, Boilard E. The non-haemostatic role of platelets in systemic lupus erythematosus. *Nat Rev Rheumatol*. 2018;14(4):195–213. doi:10.1038/nrrheum.2018.38

61. Poon MC, Di Minno G, d'Oiron R, Zotz R. New insights into the treatment of Glanzmann thrombasthenia. *Transfus Med Rev*. 2016;30(2):92–99. doi:10.1016/j.tmrv.2016.01.001

62. Awidi AS. Increased incidence of Glanzmann's thrombasthenia in Jordan as compared with Scandinavia. *Scand J Haematol*. 1983;30(3):218–222. doi:10.1111/j.1600-0609.1983.tb01477.x

63. Iqbal I, Farhan S, Ahmed N. Glanzmann thrombasthenia: a clinicopathological profile. *J Coll Physicians Surg Pak*. 2016;26(8):647–650.

64. Franchini M, Favaloro EJ, Lippi G. Glanzmann thrombasthenia: an update. *Clin Chim Acta*. 2010;411(1–2):1–6. doi:10.1016/j.cca.2009.10.016

65. Rosas RR, Kurth MH, Sidman J. Treatment and outcomes for epistaxis in children with Glanzmann's thrombasthenia. *Laryngoscope*. 2010;120(12):2374–2377. doi:10.1002/lary.21034

66. Lowe GC, Lordkipanidzé M, Watson SP; group UGs. Utility of the ISTH bleeding assessment tool in predicting platelet defects in participants with suspected inherited platelet function disorders. *J Thromb Haemost*. 2013;11(9):1663–1668. doi:10.1111/jth.12332

67. Gresele P, Orsini S, Noris P, et al. Validation of the ISTH/SSC bleeding assessment tool for inherited platelet disorders: a communication from the platelet physiology SSC. *J Thromb Haemost*. 2020;18(3):732–739. doi:10.1111/jth.14683

68. Rognoni E, Ruppert R, Fässler R. The kindlin family: functions, signaling properties and implications for human disease. *J Cell Sci*. 2016;129(1):17–27. doi:10.1242/jcs.161190

69. Lillicrap D, James P. *Von Willebrand Disease: An Introduction for the Primary Care Physician*. Montreal, QC, Canada: World Federation of Hemophilia; 2009.

70. Michel M. Immune thrombocytopenic purpura: epidemiology and implications for patients. *Eur J Haematol Suppl*. 2009;71:3–7. doi:10.1111/j.1600-0609.2008.01206.x

71. Iorio A, Stonebraker JS, Chambost H, et al. Establishing the prevalence and prevalence at birth of hemophilia in males: a meta-analytic approach using national registries. *Ann Intern Med*. 2019;171(8):540–546. doi:10.7326/m19-1208

72. Gresele P, Harrison P, Bury L, et al. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. *J Thromb Haemost*. 2014;12(9):1562–1569. doi:10.1111/jth.12650

73. Kürekçi AE, Atay AA, Saríçí SU, Zeybek C, Köseoğlu V, Ozcan O. Effect of iron therapy on the whole blood platelet aggregation in infants with iron deficiency anemia. *Thromb Res*. 2000;97(5):281–285. doi:10.1016/s0049-3848(99)00150-4

74. Akay OM, Akin E, Mutlu FS, Gulbas Z. Effect of iron therapy on platelet function among iron-deficient women with unexplained menorrhagia. *Pathophysiol Haemost Thromb*. 2008;36(2):80–83. doi:10.1159/000173726

75. Moenen F, Vries MJA, Nelemans PJ, et al. Screening for platelet function disorders with multiplate and platelet function analyzer. *Platelets*. 2019;30(1):81–87. doi:10.1080/09537104.2017.1371290

76. McCabe White M, Jennings LK. *Platelet Protocols: Research and Clinical Laboratory Procedures*. Academic Press; 1999.

77. Bolton-Maggs PH, Chalmers EA, Collins PW, et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. *Br J Haematol*. 2006;135 (5):603–633. doi:10.1111/j.1365-2141.2006.06343.x

78. Favaloro EJ. Clinical utility of the PFA-100. *Semin Thromb Hemost*. 2008;34(8):709–733. doi:10.1055/s-0029-1145254

79. Mezzano D, Quiroga T, Pereira J. The level of laboratory testing required for diagnosis or exclusion of a platelet function disorder using platelet aggregation and secretion assays. *Semin Thromb Hemost*. 2009;35(2):242–254. doi:10.1055/s-0029-1220785

80. Cattaneo M, Lecchi A, Zighetti ML, Lussana F. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. *Haematologica*. 2007;92(5):694–697. doi:10.3324/haematol.10999

81. Dacie SJV, Lewis SM. *Practical Haematology*. 6th ed. Churchill Livingstone; 1984.

82. Cattaneo M. Inherited platelet-based bleeding disorders. *J Thromb Haemost*. 2003;1(7):1628–1636. doi:10.1046/j.1538-7836.2003.00266.x

83. Philp R. *Methods of Testing Proposed Anti Thrombotic Drugs*. CRC Press; 1981.

84. Mutreja D, Sharma RK, Purohit A, Aggarwal M, Saxena R. Evaluation of platelet surface glycoproteins in patients with Glanzmann thrombasthenia: association with bleeding symptoms. *Indian J Med Res*. 2017;145(5):629–634. doi:10.4103/ijmr.IJMR_718_14

85. Giannini S, Mezzasoma AM, Guglielmini G, Rossi R, Falcinelli E, Gresele P. A new case of acquired Glanzmann's thrombasthenia: diagnostic value of flow cytometry. *Cytometry B Clin Cytom*. 2008;74(3):194–199. doi:10.1002/cyto.b.20396

86. Owaidah T, Saleh M, Baz B, et al. Molecular yield of targeted sequencing for Glanzmann thrombasthenia patients. *NPJ Genom Med*. 2019;4:4. doi:10.1038/s41525-019-0079-6

87. Bastida JM, Lozano ML, Benito R, et al. Introducing high-throughput sequencing into mainstream genetic diagnosis practice in inherited platelet disorders. *Haematologica*. 2018;103 (1):148–162. doi:10.3324/haematol.2017.171132

88. Downes K, Megy K, Duarte D, et al. Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders. *Blood*. 2019;134(23):2082–2091. doi:10.1182/blood.2018891192

89. Simeoni I, Stephens JC, Hu F, et al. A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders. *Blood*. 2016;127(23):2791–2803. doi:10.1182/blood-2015-12-688267

90. Amesse C, Baillargeon L, Bissonnette D, et al. *Glanzmann Thrombasthenia: An Inherited Bleeding Disorder*. 2nd. Canadian Hemophilia Society & Canadian Association of Nurses in Hemophilia Care;2014.

91. Mehta DN, Bhatia R. Dental considerations in the management of Glanzmann's thrombasthenia. *Int J Clin Pediatr Dent*. 2010;3 (1):51–56. doi:10.5005/jp-journals-10005-1054

92. Almeida AM, Khair K, Hann I, Liesner R. The use of recombinant factor VIIa in children with inherited platelet function disorders. *Br J Haematol*. 2003;121(3):477–481. doi:10.1046/j.1365-2141.2003.04286.x

93. Jayakrishnan TT, Limonnik V, Shah D, Mewawalla P. Glanzmann's thrombasthenia: how listening to the patient is sometimes the simple key to good medicine! *Case Rep Med*. 2020;2020:4862987. doi:10.1155/2020/4862987

94. Kulkarni B, Ghosh K, Shetty S. Second trimester prenatal diagnosis in Glanzmann's thrombasthenia. *Haemophilia*. 2016;22(2): e99–e100. doi:10.1111/hae.12865

95. Soni P, Mantri S, Prabhudesai A, Patil R, Shanmukhaiah C, Shetty S. Triple jeopardy: a case of Glanzmann's thrombasthenia with anti-GPIIb-IIIa antibodies and HPA incompatibility resulting in stillbirth. *Thromb Res*. 2019;181:141–144. doi:10.1016/j.thromres.2019.07.022

96. Siddiq S, Clark A, Mumford A. A systematic review of the management and outcomes of pregnancy in Glanzmann thrombasthenia. *Haemophilia*. 2011;17(5):e858–69. doi:10.1111/j.1365-2516.2011.02516.x

97. Léticée N, Kaplan C, Lémery D. Pregnancy in mother with Glanzmann's thrombasthenia and isoantibody against GPIIb-IIIa: is there a foetal risk? *Eur J Obstet Gynecol Reprod Biol*. 2005;121(2):139–142. doi:10.1016/j.ejogrb.2005.02.011

98. Forsyth AL, Zourikian N, Valentino LA, Rivard GE. The effect of cooling on coagulation and haemostasis: should "Ice" be part of treatment of acute haemarthrosis in haemophilia? *Haemophilia*. 2012;18(6):843–850. doi:10.1111/j.1365-2516.2012.02918.x

99. Forsyth AL, Zourikian N, Rivard GE, Valentino LA. An 'ice age' concept? The use of ice in the treatment of acute haemarthrosis in haemophilia. *Haemophilia*. 2013;19(6):e393–6. doi:10.1111/hae.12265

100. Levy JH, Koster A, Quinones QJ, Milling TJ, Key NS. Antifibrinolytic therapy and perioperative considerations. *Anesthesiology*. 2018;128(3):657–670. doi:10.1097/ALN.0000000000001997

101. Tengborn L, Blombäck M, Berntorp E. Tranexamic acid–an old drug still going strong and making a revival. *Thromb Res*. 2015;135(2):231–242. doi:10.1016/j.thromres.2014.11.012

102. Lombardo VT, Sottilotta G. Recombinant activated factor VII combined with desmopressin in preventing bleeding from dental extraction in a patient with Glanzmann's thrombasthenia. *Clin Appl Thromb Hemost*. 2006;12(1):115–116. doi:10.1177/107602960601200120

103. Ghosh K, Kulkarni B, Shetty S, Nair S. Antiplatelet antibodies in cases of Glanzmann's thrombasthenia with and without a history of multiple platelet transfusion. *Indian J Hum Genet*. 2009;15(1):23–27. doi:10.4103/0971-6866.50866

104. Rajpurkar M, Chitlur M, Recht M, Cooper DL. Use of recombinant activated factor VII in patients with Glanzmann's thrombasthenia: a review of the literature. *Haemophilia*. 2014;20(4):464–471. doi:10.1111/hae.12473

105. Poon MC, D'Oiron R, Von Depka M, et al. Prophylactic and therapeutic recombinant factor VIIa administration to patients with Glanzmann's thrombasthenia: results of an international survey. *J Thromb Haemost*. 2004;2(7):1096–1103. doi:10.1111/j.1538-7836.2004.00767.x

106. Di Minno G, Zotz RB, d'Oiron R, et al. The international, prospective Glanzmann thrombasthenia registry: treatment modalities and outcomes of non-surgical bleeding episodes in patients with Glanzmann thrombasthenia. *Haematologica*. 2015;100(8):1031–1037. doi:10.3324/haematol.2014.121475

107. Dubick MA, Dorfman R, Klemcke HG, et al. Stability of activated recombinant human factor VII (rFVIIa) after 28 days storage at 24° C and 43° C. *Blood*. 2008;112:4074. doi:10.1182/blood.V112.11.4074.4074

108. Lak M, Scharling B, Blemings A, et al. Evaluation of rFVIIa (NovoSeven) in Glanzmann patients with thromboelastogram. *Haemophilia*. 2008;14(1):103–110. doi:10.1111/j.1365-2516.2007.01592.x

109. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, De Groot PG. Recombinant factor VIIa enhances deposition of platelets with congenital or acquired alpha IIb beta 3 deficiency to endothelial cell matrix and collagen under conditions of flow via tissue factor-independent thrombin generation. *Blood*. 2003;101(5):1864–1870. doi:10.1182/blood-2002-09-2761

110. Poon MC. Clinical use of recombinant human activated factor VII (rFVIIa) in the prevention and treatment of bleeding episodes in patients with Glanzmann's thrombasthenia. *Vasc Health Risk Manag*. 2007;3(5):655–664.

111. Birschmann I, Klamroth R, Eichler H, Schenk J, Kirchmaier CM, Halimeh S. Results of the WIRK prospective, non-interventional observational study of recombinant activated factor VII (rFVIIa) in patients with congenital haemophilia with inhibitors and other bleeding disorders. *Haemophilia*. 2013;19(5):679–685. doi:10.1111/hae.12156

112. Rakocz M, Lavie G, Martinowitz U. Glanzmann's thrombasthenia: the use of autologous fibrin glue in tooth extractions. *ASDC J Dent Child*. 1995;62(2):129–131.

113. Franco R, Miranda M, Di Renzo L, De Lorenzo A, Barlattani A, Bollero P. Glanzmann's thrombasthenia: the role of tranexamic acid in oral surgery. *Case Rep Dent*. 2018;2018:9370212. doi:10.1155/2018/9370212

114. Nurden AT. Glanzmann thrombasthenia. *Orphanet J Rare Dis*. 2006;1:10. doi:10.1186/1750-1172-1-10

115. Herman MC, Mak N, Geomini PM, et al. Is the Pictorial Blood Loss Assessment Chart (PBAC) score associated with treatment outcome after endometrial ablation for heavy menstrual bleeding? A cohort study. *BJOG*. 2017;124(2):277–282. doi:10.1111/1471-0528.14434

116. Higham JM, O'Brien PM, Shaw RW. Assessment of menstrual blood loss using a pictorial chart. *Br J Obstet Gynaecol*. 1990;97(8):734–739. doi:10.1111/j.1471-0528.1990.tb16249.x

117. Nurden AT, Pillois X, Wilcox DA. Glanzmann thrombasthenia: state of the art and future directions. *Semin Thromb Hemost*. 2013;39(6):642–655. doi:10.1055/s-0033-1353393

118. Sherer DM, Lerner R. Glanzmann's thrombasthenia in pregnancy: a case and review of the literature. *Am J Perinatol*. 1999;16(6):297–301. doi:10.1055/s-2007-993875

119. Boval B, Bellucci S, Boyer-Neumann C. Glanzmann's thrombasthenia and pregnancy: clinical observations and management of four affected women Congress of the International Society of Thrombosis and Hemostasis. *Thromb Haemost*. 2001;P1154.

120. Punt MC, Schuitema PCE, Bloemenkamp KWM, Kremer Hovinga ICL, van Galen KPM. Menstrual and obstetrical bleeding in women with inherited platelet receptor defects-A systematic review. *Haemophilia*. 2020;26(2):216–227. doi:10.1111/hae.13927

121. Orsini S, Noris P, Bury L, et al. Bleeding risk of surgery and its prevention in patients with inherited platelet disorders. *Haematologica*. 2017;102(7):1192–1203. doi:10.3324/haematol.2016.160754

122. Poon MC, d'Oiron R, Zotz RB, et al. The international, prospective Glanzmann thrombasthenia registry: treatment and outcomes in surgical intervention. *Haematologica*. 2015;100(8):1038–1044. doi:10.3324/haematol.2014.121384

123. Nordisk N, Inc. NovoSeven® RT, coagulation factor VIIa (Recombinant) [package insert]. Plainsboro, NJ; 2019.

124. Santoro C, Rago A, Biondo F, et al. Prevalence of alloimmunization anti-HLA and anti-integrin αIIbβ3 in Glanzmann thromboasthenia patients. *Haemophilia*. 2010;16(5):805–812. doi:10.1111/j.1365-2516.2010.02230.x

125. Laurian Y, Tisseron-Maury B, Bibi Triki T, Kaplan C, Gaudelus J. Red blood cell transfusion in patients with type 1 Glanzmann's thrombasthenia. *J Thromb Haemost*. 2005;3(10):2346–2347. doi:10.1111/j.1538-7836.2005.01579.x

126. Vivier M, Treisser A, Naett M, et al. [Glanzmann's thrombasthenia and pregnancy. Contribution of plasma exchange before scheduled cesarean section]. *J Gynecol Obstet Biol Reprod*. 1989;18(4):507–513. [French].

127. Winkelhorst D, Murphy MF, Greinacher A, et al. Antenatal management in fetal and neonatal alloimmune thrombocytopenia: a systematic review. *Blood*. 2017;129(11):1538–1547. doi:10.1182/blood-2016-10-739656

128. Rayment R, Brunskill SJ, Soothill PW, Roberts DJ, Bussel JB, Murphy MF. Antenatal interventions for fetomaternal alloimmune thrombocytopenia. *Cochrane Database Syst Rev*. 2011;5:CD004226. doi:10.1002/14651858.CD004226.pub3

129. Poon MC. The Use of recombinant activated factor VII in patients with Glanzmann's thrombasthenia. *Thromb Haemost*. 2021;121(3):332–340. doi:10.1055/s-0040-1718373

130. Browning L, Dogra S, Bourbonnais J The use of recombinant factor VIIa use in a patient with Glanzmann's Thrombasthenia and diffuse alveolar hemorrhage. Poster Presentation at Congress of Society of Critical Care Medicine; 2010; USA.

131. Wertz D, Boveroux P, Péters P, Lenelle J, Franssen C. Surgical resection of a sphenoid wing meningioma in a patient with Glanzmann thrombasthenia. *Acta Anaesthesiol Belg.* 2011;62 (2):83–86.

132. Phillips R, Richards M. Venous thrombosis in Glanzmann's thrombasthenia. *Haemophilia.* 2007;13(6):758–759. doi:10.1111/j.1365-2516.2007.01555.x

133. Bellucci S, Damaj G, Boval B, et al. Bone marrow transplantation in severe Glanzmann's thrombasthenia with antiplatelet alloimmunization. *Bone Marrow Transplant.* 2000;25(3):327–330. doi:10.1038/sj.bmt.1702139

134. Bellucci S, Devergie A, Gluckman E, et al. Complete correction of Glanzmann's thrombasthenia by allogeneic bone-marrow transplantation. *Br J Haematol.* 1985;59(4):635–641. doi:10.1111/j.1365-2141.1985.tb07358.x

135. Flood VH, Johnson FL, Boshkov LK, et al. Sustained engraftment post bone marrow transplant despite anti-platelet antibodies in Glanzmann thrombasthenia. *Pediatr Blood Cancer.* 2005;45 (7):971–975. doi:10.1002/pbc.20365

136. Ishaqi MK, El-Hayek M, Gassas A, et al. Allogeneic stem cell transplantation for Glanzmann thrombasthenia. *Pediatr Blood Cancer.* 2009;52(5):682–683. doi:10.1002/pbc.21888

137. Kitko CL, Levine JE, Matthews DC, Carpenter PA. Successful unrelated donor cord blood transplantation for Glanzmann's thrombasthenia. *Pediatr Transplant.* 2011;15(3):e42–6. doi:10.1111/j.1399-3046.2009.01251.x

138. Wiegering V, Sauer K, Winkler B, Eyrich M, Schlegel PG. Indication for allogeneic stem cell transplantation in Glanzmann's thrombasthenia. *Hamostaseologie.* 2013;33 (4):305–312. doi:10.5482/HAMO-12-08-0014

139. Ramzi M, Dehghani M, Haghighat S, Nejad HH. Stem cell transplant in severe Glanzmann thrombasthenia in an adult patient. *Exp Clin Transplant.* 2016;14(6):688–690. doi:10.6002/ect.2014.0165

140. Fang J, Jensen ES, Boudreaux MK, et al. Platelet gene therapy improves hemostatic function for integrin alphaIIbbeta3-deficient dogs. *Proc Natl Acad Sci U S A.* 2011;108(23):9583–9588. doi:10.1073/pnas.1016394108

141. Sullivan SK, Mills JA, Koukouritaki SB, et al. High-level transgene expression in induced pluripotent stem cell-derived megakaryocytes: correction of Glanzmann thrombasthenia. *Blood.* 2014;123(5):753–757. doi:10.1182/blood-2013-10-530725

Ketorolac-fluconazole: A New Combination Reverting Resistance in *Candida albicans* from Acute Myeloid Leukemia Patients on Induction Chemotherapy: In vitro Study

Shereen A Sayed, Ehsan A B Hassan, Muhamad R Abdel Hameed, Michael N Agban, Mostafa F Mohammed Saleh, Hayam H Mohammed, Abu-Baker M Abdel-Aal & Sherein G Elgendy

ORIGINAL RESEARCH

Ketorolac-fluconazole: A New Combination Reverting Resistance in *Candida albicans* from Acute Myeloid Leukemia Patients on Induction Chemotherapy: In vitro Study

Shereen A Sayed[1]
Ehsan AB Hassan[2]
Muhamad R Abdel Hameed [ID][3]
Michael N Agban[2]
Mostafa F Mohammed Saleh [ID][3]
Hayam H Mohammed[4]
Abu-Baker M Abdel-Aal[5]
Sherein G Elgendy [ID][2]

[1]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt; [2]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt; [3]Department of Internal Medicine & Hematology Unit, Assiut University Hospitals and Bone Marrow Transplantation Unit, South Egypt Cancer Institute, Assiut University, Assiut, Egypt; [4]Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut, Egypt; [5]Department of Organic Chemistry, Faculty of Pharmacy, Assiut University, Assiut, Egypt

Correspondence: Muhamad R Abdel Hameed
Department of Internal Medicine & Hematology Unit, Assiut University Hospitals and Bone Marrow Transplantation Unit, South Egypt Cancer Institute, Assiut University, Assiut, 71515, Egypt
Tel +20 1097510010
Fax +20 88-2080278
Email dr.muhamadramadan@yahoo.com

Sherein G Elgendy
Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, 71515, Egypt
Tel +20 1021887728
Fax +20 88-2080278
Email Shereinelgendy@yahoo.com

Background and Objectives: *Candida albicans* is a significant source of morbidity and mortality for patients with acute myeloid leukemia (AML). Prolonged use of fluconazole as empirical antifungal prophylaxis in AML patients leads to overexpression of efflux pump genes that resulted in the emergence of azole-resistant species. Consequently, the introduction of a new strategy to improve the management of *C. albicans* infections is an urgent need. Nonsteroidal anti-inflammatory drug (NSAID) ketorolac is associated with a reduction in cancer relapses. The present study was performed to investigate the use of ketorolac-fluconazole combination to reverse fluconazole resistance in *C. albicans* isolated from AML patients on induction chemotherapy.

Patients and Methods: One hundred and seventy AML patients were evaluated. Fifty *C. albicans* were isolated and subjected to disc diffusion assay and broth microdilution for fluconazole alone and combined with different concentrations of ketorolac. Efflux pump gene (*CDR1, CDR2*, and *MDR1*) expressions were quantified by real-time PCR.

Results: The tested ketorolac acted synergistically with fluconazole against resistant *C. albicans* with the minimum inhibitory concentration (MIC) of fluconazole decreased from >160 μg/mL to 0.3–1.25 μg/mL in (93.8%) of resistant isolates with fractional inhibitory concentration index (FICI) value of 0.25. The majority of the resistant isolates overexpressed *CDR1* (71.1%) and *MDR1* (60%).

Conclusion: Ketorolac-fluconazole in vitro combination would be a promising strategy for further clinical in vivo trials to overcome fluconazole resistance in AML patients on induction chemotherapy.

Keywords: ketorolac, fluconazole resistance, acute myeloid leukemia, *CDR1*, *MDR1*

Introduction

Acute myeloid leukemia (AML) is a hematological disease caused by the clonal expansion of myeloblasts in the peripheral blood, bone marrow, or other tissues. It is characterized by various chromosomal abnormalities and gene mutations.[1] The typical clinical manifestations of AML are fever, fatigue, and bleeding caused by the expansion of blasts and decreased normal hematopoiesis in the bone marrow. Treatment of AML by combination chemotherapy results in persistent neutropenia, which further increases the risk of opportunistic infections.[2]

Candida spp. is an important opportunistic human pathogen that causes oro-pharyngeal candidiasis (OPC), vulvovaginitis, and invasive infections in AML

patients.[3] The of American Infectious Diseases Society has approved fluconazole as a primary drug of choice for prophylaxis and treatment of candidiasis.[4]

Fluconazole has several advantages over other antifungal drugs including the safety, oral bioavailability, cost, and ability to cross the blood–brain barrier.[5] Fluconazole inhibits the cytochrome P450 enzyme lanosterol demethylase, a critical enzyme in the synthesis of ergosterol which is encoded by the *ERG11* gene.[6] However, the extensive use of fluconazole as empirical therapy in cancer patients, especially in AML patients, has increased the incidence of resistance to the drug among different fungal strains, especially *Candida albicans*.[7] Fluconazole resistance has the potential to cross over to other azoles including voriconazole and itraconazole.[8]

There are multiple mechanisms for azole resistance, the major one is overexpression of plasma membrane efflux pumps.[9] The ATP-binding cassette (ABC) pumps and the major facilitator superfamily (MFS) transporters are the two main families of efflux proteins. They differ in the source of energy used for their activity and encoded by *Candida* drug resistance (*CDR1* and *CDR2*) and multidrug resistance (*MDR1*) genes. They are located in the plasma membrane and are responsible for pumping the drug out of the fungal cell decreasing its intracellular concentration which leads to treatment failure.[10]

Several studies[11,12] have stated the ability of some drugs to reverse azole resistance in *C. albicans*. Generally, these drug concentrations required to reverse the azole resistance are above the therapeutic concentrations. Moreover, some of these drugs can result in serious side effects, such as those caused by cyclosporine and tacrolimus.[13] Remarkably, a few studies have reported the ability of some nonsteroidal anti-inflammatory drugs (NSAIDs) to act synergistically with different antifungals. For example, ibuprofen was found to exhibit synergistic activity with azoles against *Candida spp*.[14] NSAIDs have antipyretic, analgesic, and can be used alone or in combination with other drugs for the treatment of cancer. They also have direct and indirect antimicrobial effects.[15] The antifungal activities of NSAIDs against *Candida* spp. include the reduction of extracellular polysaccharide, hyphal, and biofilm formations.[16]

To repurpose drugs and explore new leads in the field of antifungal drug discovery; we explored another nonsteroidal anti-inflammatory drug "ketorolac" to reverse azole resistance in *C. albicans*. Ketorolac is a potent analgesic, antipyretic, and moderate anti-inflammatory drug used in

the treatment of severe cancer pain. The simple use of this safe and effective anti-inflammatory agent might eliminate most early cancer relapses.[17]

In the current study, we investigate a new strategy to improve the management of *C. albicans* infections through the use of in vitro ketorolac-fluconazole in combination to reverse fluconazole resistance in *C. albicans* isolated from AML patients and overexpressing efflux pumps genes (*CDR1, CDR2*, and *MDR1*) assessed by quantitative real-time qRT-PCR.

Materials and Methods
Ethical Statement

This study was approved by the Local Ethics Committee (no. 17100543), Faculty of Medicine, Assiut University in accordance with the provisions of the Declaration of Helsinki. Informed written consent was obtained from all patients before enrolment in the study.

Patients

This study included 170 AML patients admitted from October 29, 2016, to March 11, 2020, to the Department of Internal Medicine (Hematology Unit), and South Egypt Cancer Institute (SECI) in Assiut University, Assiut, Egypt. All newly diagnosed non-M3 AML patients (aged 18–69 years) were enrolled in this study. The patients were diagnosed according to the WHO criteria for AML.[18] Standard induction chemotherapy for 170 non-M3 AML patients in this study was idarubicin 12 mg/m^2 per day for two to three days, and cytarabine 100 mg/m^2/day for five to seven days. Patients received prophylactic treatment during the period of neutropenia following chemotherapy in the form of sulfamethoxazole (400 mg/trimethoprim 80 mg once or twice daily). Patients received empirical azole prophylaxis, fluconazole (400 mg PO/IV per day).[19] During induction chemotherapy, granulocyte colony-stimulating factor (G-CSF) was used in a few cases showing poor performance status and not applied routinely for all patients. The definition of treatment response generally followed the European Leukemia Network (ELN) 2010 recommendation.[20] Complete remission (CR) was defined as a blast count less than 5% in the bone marrow. Partial remission (PR) was defined as a decrease in bone marrow blasts by 50% but still remaining in a range of 5–25%. Resistant disease (RD) was referred to those who failed to achieve CR or PR in bone marrow examination after chemotherapy. The undefined response was referred to

466

Journal of Blood Medicine 2021:12

those who had no available results of bone marrow examination after chemotherapy. Exclusion criteria including AML (M3), AML with antecedent hematologic malignancy, Patients with possible risk factors of candidiasis, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) positive, diabetes, and autoimmune diseases. Patients with contraindication to fluconazole therapy as abnormal heart rhythm, prolonged QT interval on EKG, abnormal liver function tests, pregnancy, and chronic kidney disease: moderate to severe stage. Data were collected and included the following parameters: age, gender, and, cytogenetic results at diagnosis, induction regimens, and treatment response, Table 1.

Standard Control Strain

C. albicans ATCC 10231 was obtained from MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University, Egypt.

Table 1 Clinical Data of 170 Patients with Newly Diagnosed Acute Myeloid Leukemia

Character	N (%)
Gender	72 F/98 M
Median Age in years (range)	49 (18–69)
AML Diagnosis	
AML (M0) Undifferentiated acute myeloblastic leukemia	11 (6.47)
AML (M1) Acute myeloblastic leukemia with minimal maturation	33 (19.41)
AML (M2) Acute myeloblastic leukemia with maturation	49 (28.82)
AML (M4) Myelomonocytic	51 (30)
AML (M5) Monocytic	19 (11.18)
AML (M6) Acute erythroid leukemia	4 (2.36)
AML (M7) Acute megakaryocytic leukemia	3 (1.76)
Cytogenetic/Molecular Risk	
Favorable risk	29 (17.05)
Intermediate risk	64 (37.67)
Unfavorable risk	31 (18.23)
Not available	46 (27.05)
Neutropenic Fever	116 (68.23)
Response of Induction Chemotherapy	
Complete remission (CR)	81 (47.64)
Partial remission (PR)	20 (11.76)
Resistant disease (RD)	46 (27.05)
Undefined response	23 (13.52)

Clinical Sample Collection and Processing

Samples collected from the patients according to their clinical presentation and different localizing symptoms. Vaginal swabs from patients with vaginal infection. Oropharyngeal swabs from patients with oral thrush were rubbed on the candidiasis lesion. Swabs were streaked on Sabouraud dextrose agar medium supplemented with chloramphenicol (0.5 g/L) (SDA, HiMedia, India) and incubated at 30°C for 48 h.[21] *Candida* spp. shows typical creamy white pasty colonies with characteristic yeasty odor. Each isolated *Candida* spp. was individually stored at −20°C with 20% glycerol.[22]

Phenotypic Identification of *Candida albicans* Strains

(a) Germ tube test: by inoculating yeasts in small tubes containing 0.5 mL of human serum (Sigma-Aldrich, Germany) containing 0.5% glucose and incubated at 37°C for two to three hours. It is positive for *C.albicans* or *C. dubliniensis*.[22]

(b) Corn meal agar (CMA): by inoculating yeasts on CMA containing Tween 80 (Difco, USA), for four to seven days to ensure production of chlamydospores. It is positive for *C. albicans* or *C. dubliniensis*.[22]

(c) CHROMagar® *Candida* medium: (CHROMagar, Paris, France) which allows selective isolation and identification of *C. albicans, C. dubliniensis, C. tropicalis*, and *C. krusei* by morphology and color reaction. The strains were identified as *C. albicans* or *C. dubliniensis* for green colonies, *C. tropicalis* for steel blue colonies, *C. krusei* colonies showing rose color.[21,22]

(d) Growth at 45°C: Growth at 45°C has been considered a useful test for the differentiation of *C. dubliniensis* (no growth) from *C. albicans* (growth).[21,22]

(e) Sugar assimilation test: using API 20C AUX (bioMérieux SA, France) according to the manufacturer's instructions.[23]

Genotypic Identification of *Candida albicans* Strains by Polymerase Chain Reaction (PCR)

The species specific primer pair sequence for the amplification of the 25S rRNA gene was described by Mannarelli and Kurtzman;[24] (5′TGTTGCTCTCTCGGGGGCGGCC G3′ and 5′ AGATCATTATGCCAACATCCTAGGTTAA

A 3′). It is specific for *C. albicans* and amplifies a 175-bp DNA fragment. DNA extraction was done by a commercial QIAamp DNA Mini Kit (Qiagen, Germany). DNA amplification performed using the Thermocycler T100 gradient system (BioRadT100, USA). PCR reaction mixture and PCR amplification conditions were performed according to the method described by Marinho et al.[22] Amplification product visualized by electrophoresis on 2% agarose gel using a 100-bp ladder molecular weight ladder (Gen Ruler 100bp DNA ladder plus).

Antifungal Susceptibility Test
Disk Diffusion Method
Adopted by the Clinical and Laboratory Standards Institute (CLSI), the M44-A2 protocol[25] was used to evaluate the degree of fungal sensitivity for four common azoles. Antifungal discs were obtained from (HiMedia, India). The response to the antifungal agents was determined via the interpretive breakpoints[26] described in Table 2.

Determination of MICs by Broth Microdilution
The MICs of fluconazole (Sigma, USA) and ketorolac (Sigma, USA) separately were identified by the protocol recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[27] The test was conducted in 96-well microtiter plates with yeast (2.5×10^5 CFU/mL) in RPMI-1640 medium (PH 7.0) buffered with MOPS (Sigma, USA) and supplemented with glucose to a final concentration of 2%. RPMI-1640 containing wells were considered negative controls, and a drug-free well was set as growth control. After 24 h of incubation at 35°C, MIC values were determined with a spectrophotometer (wavelength of 450 nm; ETI System Fast Reader ELX, BioTek, US) as the lowest concentration of drug that resulted in >50% inhibition of growth relative

to that of the growth control. MIC interpretive criteria of fluconazole for *C. albicans* were those described in the document E.DEF 7.3.1 [available on the EUCAST website: http://www.eucast.org]. MIC ≤2.0 mg/L was considered to be sensitive, MIC between 2.0 and 4.0 mg/L was considered to be intermediate and that >4.0 mg/L was considered resistant.

Fractional Inhibitory Concentration Index (FICI)
The intensity of interaction between ketorolac and fluconazole antifungals against azole-resistant *C. albicans* clinical isolates was determined briefly as follow, drugs were serially diluted 2-fold in RPMI-1640 medium, and 0.31–160 µg/mL fluconazole and 0.16–10 µg/mL ketorolac were added to the wells. Subsequently, yeast at a final concentration of 2.5×10^5 CFU/mL was added to each well. Wells containing only RPMI-1640 medium served as negative controls, and a drug-free well was set as a growth control. After 24 h of incubation at 35°C, MICs were determined as described above. To evaluate the intensity of the drug interactions, the fractional inhibitory concentration index (FICI) model was used to analyze the obtained data. The FICI model is based on the Loewe additivity theory[28] and is expressed as $FICI=FIC_A+FIC_B=(A/MIC_A)+(B/MIC_B)$ where A and B are the MIC of each drug in combination (in a single well), and MIC_A and MIC_B are the MIC of each drug individually. The drug interaction is interpreted as synergistic when FICI ≤0.5, indifferent when FICI >0.5–4.0, and antagonistic when FICI >4.0.[29]

Efflux Pump Genes Expression Analysis by qRT-PCR
Reverse Transcriptase (RT)-PCR
The reverse transcription was performed using HiSenScript™ RH(-) cDNA Synthesis Kit (iNtRON, Cat. no. 25014) according to the manufacturer's instructions.

Table 2 Interpretative Breakpoints of Disk Diffusion Method for Fluconazole, Itraconazole, Miconazole, and Voriconazole[26]

Antifungal	Abbr.	Conc./Disc	Zone Diameter in mm		
			S	I	R
Fluconazole	FU	25 µg	≥19	15–18	≤14
Itraconazole	IT	10 µg	≥23	14–22	≤13
Miconazole	MIC	10 µg	≥20	12–19	≤11
Voriconazole	VRC	1 µg	≥17	14–16	≤13

Abbreviations: S, susceptible; I, intermediate; R, resistant.

Reverse transcription was performed at 45°C for 60 min, followed by 80°C for 10 min. The cDNA products were stored at −20°C for later use as templates for quantitative real-time PCR (qPCR).

Real-Time PCR

The procedures of qRT-PCR analysis were described in a previous study.[30] Expression levels of the target genes (*CDR1, CDR2*, and *MDR1*) and the housekeeping gene (*ACT1*, used as a normalizing gene) were assessed by quantitative real-time RT-PCR (qRT-PCR). The primers (analysis) used tested through the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and listed in Table 3.[31] The expression of target genes was carried out using SensiFAST SYBR® No-ROX (Bioline, USA) using a 7500 Fast Real-Time PCR System (Applied Biosystems). The fold change in expression of these genes was calculated according to the $2^{\wedge}(-\Delta\Delta CT)$ method using *ACT1* as a control gene to normalize cDNA levels.[32] Fluconazole-susceptible *C. albicans* clinical isolate (MIC, 0.5 mg/L) was used as a reference isolate for gene expression analysis. Real-time PCR reaction mix for (*CDR1, CDR2, MDR1* and ACT1) contained 0.5 μL of cDNA,1.5 μL of 2 μm primer and 7.5 μL of 2×SYBR Green Master Mix in a final volume of 15 μL and qRT-PCR was performed using the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 seconds, 50°C for 15 seconds for *CDR1, CDR2*, and *ACT1*, or 55°C for 15 seconds for *MDR1*, and 60°C for 30 seconds.

Statistical Analysis

Data entry and data analysis get done using SPSS (Statistical Package for Social Science) version 19. Data were presented as a number, percentage, mean, median, standard deviation, and standard error. Chi-squared test and Fisher's exact test were used for comparing qualitative variables. The Mann–Whitney *U*-test was used to compare nonparametric tests. *P*-value considered statistically significant when $P<0.05$.

Results
Patient Population

The demographic and clinical characteristics of 170 non-M3-AML patients who received induction chemotherapy admitted to Clinical Hematology Unit, Internal Medicine Department, Assiut University Hospital, and South Egypt Cancer Institute (SECI) presented in Table 1. The median age was 49 years (range: 18–69 years). Female patients' were 72 (42.35%) while male patients were 98 (57.65%). The diagnosis was performed according to the WHO criteria for AML. There were mainly AML M4, AML M2, AML M1 and AML M5 (51 (30%), 49 (28.82%), 33 (19.41%) and 19 (11.17%) respectively. A total of 39 patients (22.9%) had favorable cytogenetic risk, 74 (43.52%) intermediate cytogenetic risk, and 41 (24.11%) unfavorable risk.

The collected samples were 76 oropharyngeal swabs, 42 vaginal swabs. Seventy-five samples were diagnosed as yeast infection from which 50 isolates were diagnosed as *Candida albicans* by phenotypic tests and PCR.

Antifungal Susceptibility Test
Disk Diffusion Method

The pattern of antifungal resistance to tested azoles for *Candida albicans* isolates was relatively high 94% for both fluconazole and voriconazole, 74% for miconazole and itraconazole as shown in Table 4.

Ketorolac Acted Synergistically with Fluconazole Against Resistant *C. albicans* in vitro

The minimal inhibitory concentrations (MICs) of ketorolac and fluconazole against resistant *C. albicans* are listed in Table 5. The MIC of fluconazole was all >160 μg/mL for 94% of tested *C. albicans* isolates, indicating strong resistance of these *C. albicans* isolates. The MIC of ketorolac was >10 μg/mL. However, when used in combination with fluconazole, ketorolac could significantly decrease the MICs of fluconazole from >160 μg/mL to 0.3–1.25 μg/mL, indicating a significantly increased sensitivity of resistant *C. albicans* to fluconazole caused by ketorolac. When the MIC of FLC was decreased to <2 μg/mL, the

Table 3 Gene-specific Primers Used for RT- qPCR[31]

Gene	Primer Sequence
CDR1	F: 5′– TGCCAAACAATCCAACAA–3′ R: 5′–CGACGGATCACCTTTCATACGA–3′
CDR2	F: 5′– AAGGTTT TGATGCTACTGC–3′ R: 5′–GTCGGACATGTGGCTCAAA–3′
MDR1	F: 5′–GTGTTGGCCCATTGGTTTTCAGTC–3′ R: 5′–CCAAAGCA GTGGGGATTTGTAG–3′
ACT1	F: 5′–AAGAATTGATTTGGCTGGTAGAGA–3′ R: 5′-TGGCAGAAGATTGAGAAGAAGTTT–3′

Table 4 Resistance Patterns of 50 *C. albicans* Using a Kirby–Bauer Disk Diffusion Method for Azoles

Total (n= 50)	Fluconazole (25 μg/Disc) N (%)	Miconazole (10 μg/Disc) N (%)	Voriconazole (1 μg/Disc) N (%)	Itraconazole (10 μg/Disc) N (%)
Sensitive (S)	3 (6)	3 (6)	3 (6)	3 (6)
Intermediate (I)	0 (0)	10 (20)	0 (0)	10 (20)
Resistant (R)	47 (94)	37 (74)	47 (94)	37 (74)

Note: Data are presented as number (%) of isolates resistant to antimicrobial indicated.

Table 5 In vitro Interaction of Ketorolac with Fluconazole Against Resistant *C. albicans*

Mean MIC (μg/mL)		Mean MIC (μg/mL)		Mean FICI	IN
Alone		Combination			
Ketorolac	Fluconazole	Ketorolac	Fluconazole		
>10	>160	2.5	0.62	0.25	SYN

Abbreviations: IN, interpretation; SYN, synergism.

concentration of ketorolac required was 2.5 μg/mL. Moreover, the FICI values obtained from the FICI model were <0.5, showing a strong synergism induced by ketorolac plus fluconazole.

Efflux Pump Genes Expression Analysis

CDR1, CDR2, and *MDR1* gene expression levels were quantified for all 50 isolates and normalized relative to the housekeeping gene, *ACT1*. This study found that the *MDR1* gene showed the most gene overexpression (88%) followed by *CDR1* gene (84%), and finally *CDR2* gene (12%). When comparing the relationship between fluconazole MICs and the expression of efflux-related genes (Figure 1), it was clear that isolates with higher fluconazole MIC (>4.0 mg/L) showed higher expression levels of *CDR1* and *MDR1*, Table 6. These results confirm those expression levels of efflux-related genes *CDR1* and *MDR1* agree with fluconazole MICs in the *C. albicans* isolates.

Discussion

In Egypt, leukemia comprises (10%) all malignancies, with AML representing (16.9%).[33] This study included 170, non-M3 (acute promyelocytic) AML patients. The ages ranged from 18 to 69 years, with a mean of 49 years. A slight male predominance was noted (female to male ratio was 1:1.36). The commonest FAB subgroup was M4 followed by M2. Another study at National Cancer Institute (NCI)-Egypt on 82 adult AML patients showed epidemiological characteristics of AML Egyptian patients, slightly similar to our data. Their ages ranged between 18 and 68 years with a median of 34 years. The male to female ratio was 1.05:1, and the commonest FAB subgroups reported were M1 and M2.[34]

Another Egyptian study included ninety AML patients. The ages ranged from 18 to 76 years, with a mean of 37.8 years. A slight female predominance was noted (male to female ratio was 1:1.3). The commonest FAB subgroup was M2 followed by M5.[35]

Response of induction chemotherapy in the current study, were complete remission (CR), partial remission, resistant disease and undefined response: 81 (47.64%), 20 (11.76%), 46 (27.05%) and 23 (13.52%), respectively. The time elapsed between presentation and start of treatment ranged from 4 to 85 days, with a mean of 13 days. The treatment delay in most patients of our study was mainly due to uncontrolled infection. An Egyptian study showed CR achievement in (57.5%) of AML patients, which is a slightly higher rate than that of our study.[35] It can be explained that we adopted treatment strategy interruption with neutropenic fever onset, which reduced treatment-related mortality at the expense of response rate. Also, slightly longer duration from onset of presentation to treatment beginning when compared with such study.

A retrospective study on 1317 AML patients had time elapsed between presentation and start of treatment with a median of four days, and a range of 1–78 days. The longer time was associated with worse CR in patients younger than 60 years, and this effect was more pronounced with duration of five days or more.[36] It is crucial that public health systems in developing countries (DC), including
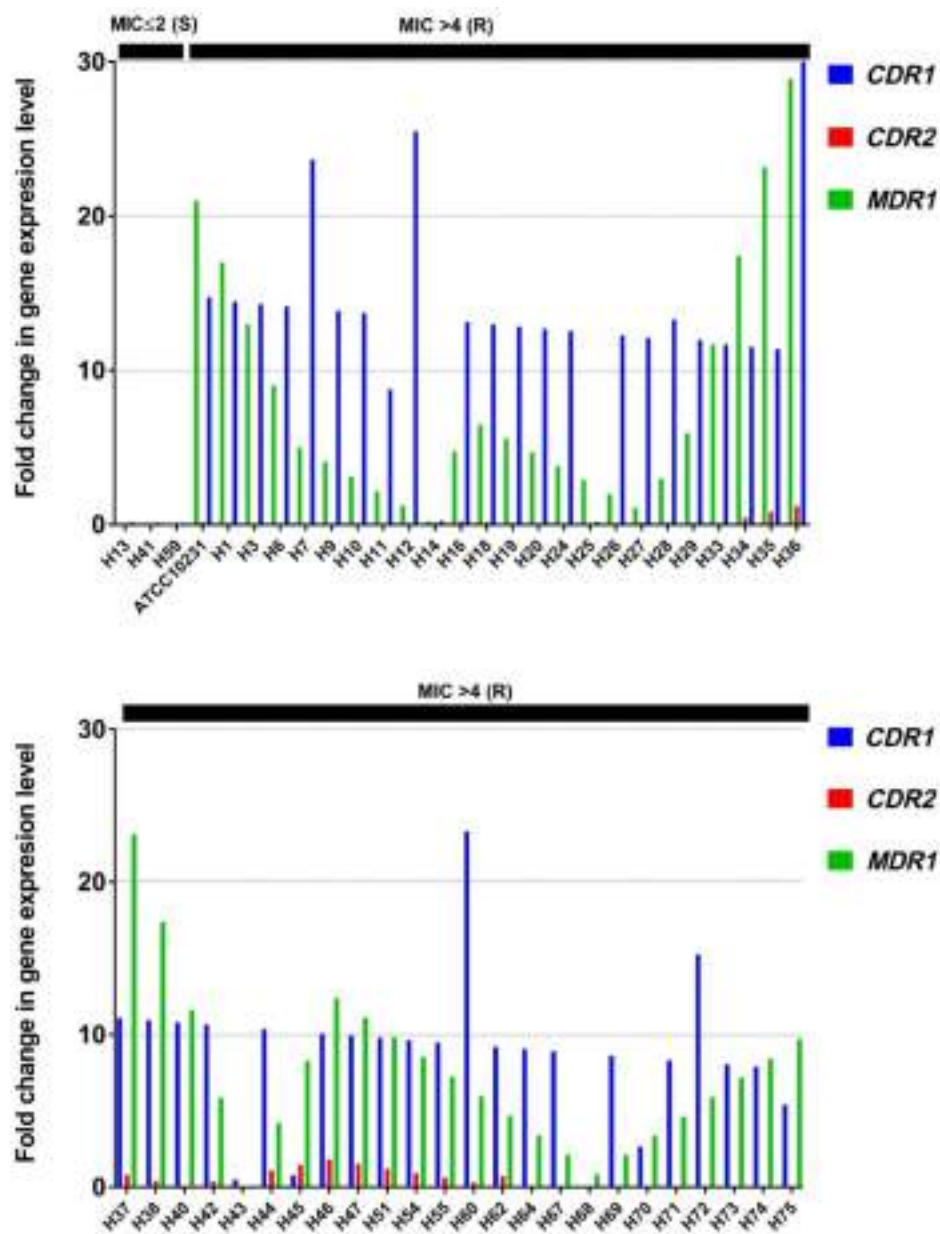
Figure 1 Efflux pump gene expression of *C. albicans* isolates in relation to the MIC of fluconazole.

Egypt, turn to larger epidemiological studies to better understand how the disease characteristics interact with socioeconomic factors. An appropriate health system must shorten the time from diagnosis to treatment to ensure a better outcome of induction therapy and more successful result in AML.

C. albicans is an important cause of morbidity and mortality for AML patients. The rise of multidrug resistant organisms causes a challenge in the treatment of infective diseases[37] Resistance among *C. albicans* represents a serious therapeutic problem that is mainly attributed to the overexpression of efflux pump genes encoded by *CDR1, CDR2* (related to azole cross-resistance), and *MDR1* genes (confined to selective resistance to fluconazole).[38]

In our study, in vitro susceptibility testing for *C. albicans* isolated from AML patients to four azoles using disc diffusion method showed a high level of resistance pattern that was 94% for both fluconazole and voriconazole, 74% for miconazole and itraconazole. Also, the MIC of fluconazole was all >160 μg/mL for 94% of tested *C. albicans* isolates, indicating a very high resistance of these *C. albicans* isolates, which is consistent with 86.2% and

Table 6 The Level of the Efflux Pumps Gene Expression in Resistant and Sensitive Strains in the Human Study Group

Gene	Mean ±SE	Median	P-value
CDR1			
Resistant strains	11.16±0.9	11.02	0.006**
Sensitive strains	0.13±0.12	0.13	
CDR2			
Resistant strains	0.33±0.07	0.1	0.078*
Sensitive strains	0.02±0.003	0.02	
MDR1			
Resistant strains	7.81±0.96	5.88	0.004***
Sensitive strains	0.37±0.003	0.04	

Notes: *Nonsignificant ($P>0.05$), **significant ($P<0.05$), ***highly significant ($P<0.005$).

75.9 fluconazole resistance among *C. albicans* isolated in two recent local studies in Egypt, respectively.[39,40]

All the MICs obtained to support the antibiogram of isolates to fluconazole, which indicates that disk diffusion has a good correlation with MIC. Pfaller et al showed similar results.[41]

Many researchers indicated that prolonged therapy and increased use of antifungals for prophylaxis or treatment of recurrent candidiasis are the most common risk factors to azole resistance.[42] To overcome fungal resistance, research on antifungal sensitizers has attracted considerable attention.[43] The need for novel antifungal regimens to overcome resistance prompted us to study the activity of ketorolac, which has a superior effect on pain control in cancer patients that suffer from frequent and recurrent *Candida* infections which showed a high level of resistance to the most common group of antifungals, the azoles, especially fluconazole.

In vitro, we found that ketorolac acted synergistically with fluconazole against tested *C. albicans* isolates, as interpreted by the FICI as it decreased the MIC of fluconazole by >4 folds against 93.6% of resistant isolates (new finding). This is superior to the recently reported 60.9% reversal of fluconazole resistance using ibuprofen by Sharma et al.[44]

The MIC of ketorolac was >10 μg/mL. However, when used in combination with fluconazole, ketorolac could significantly decrease the MICs of fluconazole from >160 μg/mL to 0.3–1.25 μg/mL, indicating a significantly increased sensitivity of resistant *C. albicans* to fluconazole caused by ketorolac. When the MIC of FLC was decreased to <2 μg/mL, the concentration of ketorolac required was 2.5 μg/mL. Similar study on the combination of ibuprofen with fluconazole showed synergic activity in 8/12 of studied *Candida spp.* including four of the five fluconazole-resistant strains. The MICs of fluconazole in fluconazole-resistant *Candida spp.*

decreased 2 to 128-fold when the drug was associated with ibuprofen. Also the MICs for ibuprofen decreased 64-fold for the 12 studied *Candida spp*. They reported the practicability of using ibuprofen in combination with fluconazole in the treatment of *Candida* infections.[14]

The continuous emergence of resistance to conventional drugs through efflux pumps leads to increasing efforts directed toward discovering efflux inhibitory molecules.[45] This study found that the MDR1 gene was the gene that showed the most overexpression (88%) followed by *CDR1* gene (84%), and finally *CDR2* gene (12%). This finding is suggesting that *CDR1* protein contributes more than *CDR2* protein in resistance by ABC family of efflux pump which agreed with the finding of Tsao et al,[46] and Holmes et al[47] who conclude that in *C. albicans* Cdr1p efflux activity makes a greater contribution than Cdr2p to resistance to fluconazole and Cdr1p was present in greater amounts (2 to 20-fold) than Cdr2p. This result was contradictory with Chau et al[30] who found that *CDR2* was overexpressed in the majority of the patient isolates. This can be explained by that reported by Niimi et al[48] who found that the strains hyperexpression *CDR2* showed decreased susceptibility to caspofungin in agar plate drug resistance assays because ABC transporters confer resistance to a wide range of structurally unrelated xenobiotics so *CDR2* may be related to other antifungal resistance.

In three *C. albicans* isolates, although their resistance profile to fluconazole, they exhibited downregulation of genes of efflux pump which suggested different azole resistance mechanisms as those belonging to *ERG11* which was described.[49]

Conclusion

To our knowledge, the current study is the first in vitro report on the use of ketorolac in reverting fluconazole resistance in

C. albicans isolated from AML patients. Resistance of *C. albicans* to azole antifungals is associated with overexpression of efflux pump genes especially *CDR1* and *MDR1*. Ketorolac concentration as low as (2.5 μg/mL) was able to revert resistance in 93.8% of tested strains, so the current study recommends for the next step to run clinical studies based on the in vivo ketorolac-fluconazole combination therapy for AML patients.

Data Sharing Statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

Dr Shereen A Sayed and Dr Hayam H Mohammed report grants from Faculty of Medicine Assiut University, during the conduct of the study. Dr Ehsan AB Hassan reports that a grant was obtained from Faculty of Medicine, Assiut University, for all the authors in this study. The authors declare no other potential conflicts of interest in this work.

References

1. Grove CS, Vassiliou GS. Acute myeloid leukemia: a paradigm for the clonal evolution of cancer? *Dis Model Mech*. 2014;7(8):941–951.
2. Ohtake S, Miyawaki S, Fujita H, et al. Randomized study of induction therapy comparing standard dose idarubicin with high dose daunorubicin in adult patients with previously untreated acute myeloid leukemia: the JALSG AML 201 study. *Blood*. 2011;117:2358–2365. doi:10.1182/blood-2010-03-273243
3. Wang ES. Common fungal infection in patients with leukemia. *Clin Adv Hematol Oncol*. 2017;5(15):352.
4. Pappas PG, Kauffman CA, Andes DR, et al. Clinical practice guidline for the management of candidiasis: 2016 update by the Infectious Disease Society of America. *Clin Infect Dis*. 2016;62(4):e1–e50.
5. Shao PL, Huang LM, Hsueh PR. Recent advances and challenges in the treatment of invasive fungal infection. *Int J Antimicrob Agents*. 2007;30(6):487–489. doi:10.1016/j.ijantimicag.2007.07.019
6. Paul S, Kannan I, Mohanram K. Eextensive ERG11 mutation associated with fluconazole- resistant Candida albicans isolated from HIV infected patients. *Curr Med Mycol*. 2019;5(3):1.
7. Goncalves SS, Souza ACR, Chowdhary A, Meis JF, Colombo AL. Epidemiology and molecular mechanism of antifungal resistant in Candida and Aspergillus. *Mycoses*. 2016;59(4):198–219.
8. Kanafani ZA, Perfect JR. Resistance to antifungal agents: mechanism and clinical impact. *Clin Infect Dis*. 2008;46(1):120–128. doi:10.1086/524071
9. Rogers PD, Barker KS. Genome wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in Candida albicans clinical isolates. *Antimicrob Agents Chemother*. 2003;47(4):1220–1227. doi:10.1128/AAC.47.4.1220-1227.2003
10. Akins RA. An update on antifungal target and mechanism of resistance in Candida albicans. *Med Mycol*. 2005;43(4):285–318. doi:10.1080/13693780500138971
11. Liu S, Yue L, Gu W, Li X, Zhang L, Sun S. Synergesic effect pffluconazole and calcium channel blocker against resistant Candida albicans. *PLoS One*. 2016;11(3):e0150859. doi:10.1371/journal.pone.0150859
12. Chen X, Ren B, Chen M, et al. ASDCD:Antifugal synergistic drug combination database. *PLoS One*. 2014;9(1):e86499. doi:10.1371/journal.pone.0086499
13. Rodrgues- Diez R, Gonzales-Guerrero C, Ocana-Salceda C, et al. Calcineurin inhibitors cyclosporine A and tacrolimus induce vascular inflammation and endothelial activation through TLR4 signaling. *Sci Rep*. 2016;6(1):1–16. doi:10.1038/s41598-016-0001-8
14. Pina-vaz C, Sansonetty F, Roderigues AG, Martinez-de-oliveria J, Fonseca AF, Mardh P-A. Antifungal activity of ibuprofen alone, and in combination with fluconazole against candida species. *J Med Microbiol*. 2000;49(9):831–840. doi:10.1099/0022-1317-49-9-831
15. Hersh E, Hammond B, Fleury A. Antimicrobial activity of flurbirofen, and ibuprofen in vitro against six common periodontal pathogens. *J Clin Dent*. 1991;3(1):1–5.
16. Ahangarkani F, Khodavaisy S, Mahmoudi S, et al. Indifferent effect of non-steroidal anti-inflammatory drugs (NSAIDs) combined with fluconazole against multidrug resistant Candida auris. *Curr Med Mycol*. 2019;5(3):26.
17. Retsky M, Rogers R, Demicheli R, et al. NSAID analgesic ketorolac used perioperatively may suppress early breast cancer replace: particular relevance to triple negative subgroup. *Breast Cancer Res Treat*. 2012;134(2):881–888. doi:10.1007/s10549-012-2094-5
18. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues*. 4th ed. Lyon: IARC; 2008:130–139.
19. Rotstein S, Bow EJ, Laverdiere M, et al. Randomized placebo-controlled trial of fluconazole prophylaxis for neutropenic cancer patients: benefit based on purpose and intensity of cytotoxic therapy. The Canadian fluconazole prophylaxis study group. *Clin Infect Dis*. 1999;28:331–340. doi:10.1086/515128
20. Dohner H, Estey EH, Amadori S, et al. Dianosis and management of acute myeloid leukemia in adults: recommendation from an international expert panel, on behalf of the European Leukemia Net. *Blood*. 2010;115:453–474.
21. Power D, Johnsen J. *Difco TM, BBL TM, Manual: Manual of Microbiological Culture Media*. Sparks: Becton Dickinson and company; 2009.

22. Marinho SA, Teixeira AB, Santos OS, et al. Identification of Candida spp. by phenotypic tests and PCR. *Braz j Microbiol.* 2010;41 (2):286=294. doi:10.1590/S1517-83822010000200004

23. Pincus D, Orenga S, Chatellier S. Yeast identification past present and future methods. *Med Mycol.* 2007;45(2):97–121. doi:10.1080/13693780601059936

24. Mannarelli BM, Kurtzman CP. Rapid identification of Candida albicans and other human pathogenic yeast by using short oligonucleotides in a PCR. *J Clin Microbiol.* 1998;1(36):1634–1641. doi:10.1128/JCM.36.6.1634-1641.1998

25. CLSI. *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeast; Approved Guideline.* M44=A2 ed. Wayne, PA: Clinical and laboratory standards Institute; 2009.

26. Ellis D. Antifungal susceptibility profile Australian antifungal susceptibility data for Candida isolates from recurrent vulvovaginal candidiasis (2007–2009) using the CLIS M44-A2 disc susceptibility standard for yeast. Mycology online. Adelaide, Australia: The University of Adelaide; 2011.

27. Meletiadis J, Mouton J, Lagrou JW, Hamal K, Petr Guinea J; EUCAST. Subcommittee on Antifungal susceptibility testing of the ESCMID European committee for Antimicrobial susceptibility testing. *Def.* 2017;7(1).

28. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother.* 2003;52(1):1. doi:10.1093/jac/dkg301

29. Ghen Y-L, Lehman VN, Averette AF, Perfect JR, Heitman J. Posaconazole exhibits In vitro and in vivo synergistic antifungal activity with caspofungin or FK506 against Candida albicans. *PLoS One.* 2013;3:e57672.

30. Chau AS, Mendrick CA, Sabatelli FJ, Loebenberg D, Mcnicholas PM. Application of real time quantitative PCR to molecular analysis of Candida albicans strain exhibiting reduced susceptibility to azoles. *Antimicrob Agents Chemother.* 2004;48:2124–2131. doi:10.1128/AAC.48.6.2124-2131.2004

31. Rocha MFG, Banderia SP, De Alencar LP, et al. Azole resistance in Candida albicans from animals: highlights on efflux pump activity and gene over-expression. *Mycoses.* 2017;60:462–468. doi:10.1111/myc.12611

32. Pfaffi MW. A new mathematical model for relative quantification in real time PCR. *Nucleic Acids Res.* 2001;29(9):e45–e. doi:10.1093/nar/29.9.e45

33. Ibrahim AS, Khaled HM, Mikhail NM, Baraka H, Kamel H. Cancer Incidence in Egypt: resultsof the National Population –Based Cancer Registry Program. *J Cancer Epidemiol.* 2014;2014:1–8. doi:10.1155/2014/437971

34. Acharya UH, Kanaan MN, Cui H, Roe DJ. Does the region of diagnosis correlate with overall survival in patients with Acute Myeloid Leukemia (AML)-a SEER review of AML diagnosis from 2004–2007. *Blood.* 2014;124:1291. doi:10.1182/blood.V124.21.1291.1291

35. Zawam H, Salama R, Alsirafy SA, Bishr MK. Treatment outcome of Acute Myeloid Leukemia in Egypt: adeveloping country perspective. *Int J Cancer Treat.* 2018;1(1):53–59.

36. Sekeres MA, Elson P, Kalaycio ME. Time from diagnosis to treatment initiation predicts survival in younger, but not older, acute myeloid leukemia patients. *Blood.* 2009;113:28–36. doi:10.1182/blood-2008-05-157065

37. Donadu MG, Usai D, Marchetti M, et al. Antifungal activity of oil macerates of North Sardinia plants against Candida species isolated from clinical patients with candidiasis. *Nat Prod Res.* 2020;34 (22):3280–3284. doi:10.1080/14786419.2018.1557175

38. Sanglard D, Odds FC. Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis.* 2002;2(2):73–85. doi:10.1016/S1473-3099(02)00181-0

39. Farhan MA, Moharram AM, Salah T, Shaaban OM. Relation between antifungal resistance and enzymatic activity of yeast causing oral and vaginal mycosis. *Assiut Univ J Bot Microbiol.* 2019;48(1):1–16.

40. Farhan MA, Moharram AM, Salah T, Shaaban OM. Types of yeasts that cause vulvovaginal candidiasis in chronic users of corticosteroids. *Med Mycol.* 2019;57(6):681–687. doi:10.1093/mmy/myy117

41. Pfaller M, Diekema DJ, Ostrosky-Zeichner L, et al. Correlation of MIC with outcome of Candida species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interactive MIC breakpoint. *J Clin Microbiol.* 2008;46(8):2620–2629. doi:10.1128/JCM.00566-08

42. Ehrstrom S, Yu A, Rylander E. Glucose in vaginal secretion before and after oral glucose tolerance testing in women with and without recurrent vulvovaginal candidiasis. *Obstet Gynecol.* 2006;108 (6):1432–1437. doi:10.1097/01.AOG.0000246800.38892.fc

43. Lu M, Yan H, Yu C, Yuan L, Sun S. Proton pump inhibitors act synergistically with fluconazole against resistant Candida albicans. *Sci Rep.* 2020;10(1):498. doi:10.1038/s41598-019-57174-4

44. Sharma M, Biswas D, Kotwal A, et al. Ibuprafen- mediated reversal of fluconazole resistance in clinical isolates of Candida. *J Clin Diagn Res.* 2015;9(1):DC20.

45. Usai D, Donadu M, Bua A, et al. Enhancement of antimicrobial activity of pump inhibitor associating drugs. *J Infect Dev Ctries.* 2019;13(02):162–164. doi:10.3855/jidc.11102

46. Tsao S, Rahkhoodaee F, Raymond M. Relative contribution of the candida albicans ABC tr ansporters Cdr1p and Cdr2p to clinical azole rsistence. *Antimicrob Agents Chemother.* 2009;53(4):1344–1352. doi:10.1128/AAC.00926-08

47. Holmes AR, Lin Y-H, Niimi K, et al. ABC transporters Cdr1p contributes more than Cdr2p does to fluconazole efflux in fluconazole–resistant Candida albicans clinical isolates. *Antimicrob Agents Chemother.* 2008;52(11):3851–3862. doi:10.1128/AAC.00463-08

48. Niimi K, Maki K, Ikeda F, et al. Overexpression of Candida albicans CDR1, CDR2, orMDR1 does not produce significant changes in echinocandin susceptibility. *Antimicrob Agents Chemother.* 2006;50 (4):1148–1155. doi:10.1128/AAC.50.4.1148-1155.2006

49. Xiang M-J, Liu J-Y, Ni P-H, et al. Erg11 mutations associated with azoles resistence in clinical isolates of candida albicans. *FEMS Yeast Res.* 2013;13(4):386–393. doi:10.1111/1567-1364.12042

Blood Donation and Associated Factors Among Employees Working at Negele Arsi General Hospital and Medical College, Southeast Ethiopia: A Cross-sectional Study

Ashebir Nigussie Yirgu, Kassim Hussien Mohammed, Sisay Degno Diriba, Abdella Kumbi Babso & Abdella Amano Abdo

ORIGINAL RESEARCH

Blood Donation and Associated Factors Among Employees Working at Negele Arsi General Hospital and Medical College, Southeast Ethiopia: A Cross-sectional Study

Ashebir Nigussie Yirgu[1]
Kassim Hussien Mohammed[2]
Sisay Degno Diriba [3]
Abdella Kumbi Babso[4]
Abdella Amano Abdo [5]

[1]Negele Arsi General Hospital and Medical College, Department of Anesthesia, Negele Arsi, Ethiopia; [2]Department of Sociology, Negele Arsi General Hospital and Medical College, Negele Arsi, Ethiopia; [3]MaddaWalabu University, Shashemene Campus, School of Health Science, Department of Public Health, Shashemene, Ethiopia; [4]Negele Arsi General Hospital and Medical College, Department of Gynecology and Obstetrics, Negele Arsi, Ethiopia; [5]Hawassa University, College of Medicine and Health Science, School of Public Health, Hawassa, Ethiopia

Background: Blood is a specialized body fluid in humans. Securing voluntary, nonpaid blood donation is an important national goal to prevent blood shortages. The donated blood plays a big role during surgery, accidents, delivery, bleeding cases, and the like. Currently, in many developing and developed countries, the blood supply is critically insufficient. Hence, the aim of this study was to assess the practice of blood donation and associated factors among employees (clinical and nonclinical) at Negele Arsi General Hospital and Medical College.

Methods: A facility-based cross-sectional study was conducted from August 1 to 20, 2020. Self-administered and interview-administered questionnaires were used to collect the data. A stratified sampling method was employed to select 122 participants. Data were entered into EpiData 3.1 software and the analysis was done using SPSS version 25. Bivariable and multivariable binary logistic regression analysis with 95%CI was carried out.

Results: Among 122 employees who participated in the study, 39% have ever donated blood. Clinical staff were eight times more likely to donate blood compared to nonclinical staff (AOR=7.81, 95%CI: 2.15–28.39). Those who had one to five years work experience were 85% (AOR=0.15, 95% CI:0.03-0.74) less likely to donate blood compared to > 11 years of work experience.Those with inadequate knowledge were 71.0% (AOR=0.29, 95%CI: 0.09–0.89) less likely to donate blood compared to those with adequate knowledge. Those with an unfavorable attitude were 68.0% (AOR=0.32, 95%CI: 0.11–0.92) less likely to donate blood compared to those with a favorable attitude.

Conclusion: Generally, blood donation practice was low in the study area. The professional category, work experience, knowledge, and attitude were significantly associated with the practice of blood donation. Therefore, a blood donation campaign should be prepared to strengthen the practice.

Keywords: blood donation, practice, associated factors, employees, hospital, Negele Arsi

Introduction

Blood is one of the most valuable donations. Blood transfusion is essential and life-saving support within the health-care system, which saves millions of lives each year. The need for equitable and timely access to safe blood is universal but is not available to many patients requiring transfusion as part of their clinical management.[1]

Correspondence: Ashebir Nigussie Yirgu
Email ashebirnigussie3@gmail.com

Donated blood is very important for pregnant women during bleeding and childbirth, in the young age group suffering from severe anemia secondary to malaria and malnutrition, and in case of trauma, emergencies, accidents, and advanced surgical procedures like cardiovascular surgery and transplantation.[2]

World Health Organization (WHO) recommends that; by 2020 countries achieve 100% anonymous nonpaid voluntary blood donation, focusing on young people. This is because young people are healthy, active, self-motivated, and open, and constitute a greater proportion of the population.[3]

According to the WHO report of 2014, globally there are around a total of 10,000 blood centers. Globally, in 2013 around 108 million blood donations were collected. However, nearly half of these were collected in high-income countries.[4]

In developing countries, donors usually give blood when family or friends want a transfusion. However, in the developed countries, most blood donors are voluntary nonpaid donors who donate blood for their population.[5]

To ensure safe, adequate, and sustainable blood supplies all over the country, health workers have a significant role in different ways. Even if health workers are too low in numbers, they are vital to mobilize the community towards blood donation.

All clinical and nonclinical hospital employees are expected to be more aware than the general population and take the lead to create awareness on blood donation. Therefore, the aim of this study was to assess the practice and associated factors of blood donation among employees at Negele Arsi General Hospital and Medical College (NAGHMC), Ethiopia.

Materials and Methods
Study Setting
Negele Arsi General Hospital and Medical College is located in Oromia regional state in west Arsi zone, Negele Arsi town, 250km from Addis Ababa, the capital city of Ethiopia. This is the only private hospital in the town. The facility provides medical education training in addition to medical services. This hospital provides service for different clients who comes from Bale, East Arsi, Guji, West Arsi, and other zones of Oromia regional state. The hospital provides service as a general hospital and is open 24 hours for emergency services.

It has around 162 staff working in different fields: 64 clinical staff (9 physicians, 35 nurses, 8 laboratories, 6 pharmacists, 3 anesthetists, and 3 radiology technicians) and 98 nonclinical staff.

Study Design and Period
This was an institution-based cross-sectional study design and was conducted at NAGHMC from August 1 to 20, 2020.

Population
The study population were all employees providing services at NAGHMC. Those employees on annual leave were excluded from the study.

Sample Size Determination and Sampling Procedure
The sample size was determined using a single proportion formula based on the prevalence from the previous study conducted in Debre Markos town[6] using 56.6% for knowledge level, using 95% confidence interval level (Zα/2=1.96) and margin of error to be 5% (d=0.05). So, n=(1.96)^2×0.565×(1–0.565)/(0.05)^2=378.

Since the source population (162) was less than 10,000 (N<10,000), a sample size correction formula was applied and add a 10% nonresponse rate, then the final sample size was determined to be 125. A stratified sampling technique was used to select the study subject. Study participants sample was stratified as clinical and nonclinical employees and then simple random sampling was used based on proportional allocation to select study participants from each group.

Data Collection Technique and Procedure
The data was collected using self-administered and interviewer-administered questionnaires. The questionnaire included sociodemographic characteristics and also questions on knowledge, attitude, and practice toward blood donation. Knowledge of blood donors was assessed by summing up the response of each individual among 12 questions. Those who scored mean and less than mean value were categorized as having inadequate knowledge and those who scored above mean were categorized as having adequate knowledge. Also, attitude toward blood donors was assessed from 10 questions. Those who responded below mean were considered as having unfavorable attitude and those scored mean and above as

having a favorable attitude. Also, the practice of study participants toward blood donation was assessed through the experience of blood donation, reasons for donating, and not donating blood.

Data Quality Assurance

The quality of data was assured by proper designing and pretesting on 5% of the questionnaires. The training was given to data collectors as well as a supervisor by the principal investigator. During the data collection period, all completed questionnaires were reviewed and cross-checked for completeness every day.

Data Processing and Analyzing

The data were cleaned and checked for completeness and entered into EpiData 3.1. SPSS version 25 was used to analyze data. Data was presented using text, tables, figures, and summary measures such as mean, standard deviation, and proportion. Bivariable and multivariable binary logistic regression model were used to identify factors associated with practice of blood donation. Odds ratio (OR) and 95% confidence interval (CI) were used to measure the strength of association between dependent and independent variables. In multivariable logistic regression P-value ≤ 0.05 was considered for statistical significance.

Ethical Consideration

The study was conducted in accordance to the Declaration of Helsinki. Ethical clearance was obtained from the Ethical Review Committee of NAGHMC. Also, verbal consent was obtained from all study participants, after they had been informed about the objective of the study. Confidentiality of the participants' information was kept throughout the research processes.

Results
Sociodemographic Characteristic of Respondents

A total of 122 employees responded to the questionnaires, with a response rate of 97.6%. Among the participants, 40.2% were clinical staff and 59.8% were nonclinical staff. More than two in five (42.6%) were within the age range of 18–25 years, and 62 (50.8%) of the participants were females. More than half (54.1%) of the study participants had a monthly income of between 1000 and 3000

Ethiopian Birr (ETB). Nearly three-quarters (72.1%) of the participants had work experience of one to five years (Table 1).

Knowledge Level of Study Participants About Blood Donation

Out of the total study participants, 83 (68%) had adequate knowledge whereas 39 (32%) had inadequate knowledge about blood donation. The majority, 96 (78.7%) of the study participants knew that blood donation is good for health and 41% said the minimum weight for donation is ≥ 45 kg. Most of the respondents, 97 (79.5%) had adequate knowledge about the risk of infection transmission during a blood transfusion (Table 2).

The Attitude of Study Participants Toward Blood Donation

Two-thirds (67.2%) of the respondents had a favorable attitude toward blood donation. Among respondents, 81 (66.5%) feel that blood donation was good and 102 (83.6%) of the study participants think that voluntary blood donation was the best source of blood (Table 3).

Blood Donation Practice of Study Participants

Among the participants, 48 (39.3%), 95%CI: 30.97–48.22 had ever donated blood. Of the total respondents, 26 (21.3%) of donors had donated blood two to four times. Among donors, nearly one-quarter (23.0%) donated last year. After donating blood, 35 (28.7%) reported that they feel happy, five (4.1%) feel fatigued, and eight (6.6%) feel mixed. More than one out of five (22.0%) of participants mentioned lack of adequate information about blood donation as a reason for not donating the blood (Figure 1).

Factors Associated with Blood Donation Practice Among Employees of NAGHMC

Those variables that had a P-value ≤ 0.25 in binary logistic regression were transferred to multivariable logistic regression. Among the variables, category of the profession, one to five-years' work experience, knowledge level, and attitude of the study participants were significantly associated with the practice of blood donation in multivariable logistic regression.

Table 1 Distribution of Sociodemographic Characteristics of Employees in Negele Arsi General Hospital and Medical College, August 2020, Negele Arsi, Southeast Ethiopia (N=122)

Variables	Category	Frequency	Percentage
Age	18–25	52	42.6
	26–30	32	26.2
	31–35	14	11.5
	>36	24	19.7
Sex	Male	60	49.2
	Female	62	50.8
Marital status	Married	65	53.3
	Single	51	41.8
	Others[a]	6	4.9
Religion	Orthodox	28	23.0
	Muslim	60	49.2
	Protestant	23	18.9
	Others[b]	11	9.0
Monthly income	1000 to <3000 ETB	66	54.1
	3000 to <5000 ETB	28	23
	5000 to <10,000 ETB	16	13.1
	>10,000 ETB	12	9.8
Level of education	Below secondary school	33	27.1
	Diploma	42	34.4
	First degree and above	47	38.5
Experience in years	1–5 years	88	72.1
	6–10 years	21	17.2
	>11 years	13	10.6
Staff category	Clinical staff	49	40.2
	Nonclinical staff	73	59.8

Notes: [a]Shows those divorced and widowed, [b]Shows Adventist and Catholic religions.

Accordingly, clinical staff were eight times more likely to donate blood than nonclinical staff (AOR=7.81, 95% CI: 2.15–28.39). Those who had one to five years of experience were 85.0% less likely to donate blood compared to >11 years' work experience (AOR=0.15, 95% CI: 0.03–0.74). Those with inadequate knowledge were 71.0% less likely to donate blood compared to those with adequate knowledge (AOR=0.29, 95%CI: 0.09–0.89). Participants who have an unfavorable attitude toward blood donation were 68.0% less likely to donate blood compared to those with a favorable attitude. (AOR=0.32, 95%CI: 0.11–0.92) (Table 4).

Discussion

In our study, among the total study participants, the practice of employees toward blood donation was 39.3%. The variables significantly associated with the practice of blood donation were the category of the professions, between one and five years' experience, knowledge, and attitude of the respondents.

In this study, concerning practice towards blood donation, 39.3% had ever donated blood and this finding is supported by the study conducted on the physician of University of Benin Teaching Hospital in Nigeria (41.4%)[7] and study among health professionals in Addis Ababa Tikur Anbessa specialized hospital in which blood donation practice was 38.3%.[8] The finding of this study also supported by a study conducted in the University of Gondar Hospital (33.2%),[9] and Tigray regional hospital (47.8%)[10] of the health professionals practiced blood donation.

In this study, years of experience had a significant association with blood donation practice. Those

Table 2 Level of Knowledge on Blood Donation Among Employees in Negele Arsi General Hospital and Medical College, August 2020, Negele Arsi, Southeast Ethiopia (N=122)

Knowledge Questions	Response	FrequencyN (%)
Blood donation is good for health	Yes No I do not know	96 (78.7) 14 (11.5) 12 (9.8)
How many types of blood groups are there?	Four Eight I do not know	95 (77.9) 6 (4.9) 21 (17.2)
Do you know your blood group?	Yes No	93 (76.2) 29 (23.8)
Which blood group is a universal donor?	O AB A B I do not know	68 (55.7) 7 (5.7) 1 (0.8) 3 (2.5) 43 (35.2)
What is the minimum weight for blood donation?	≥ 45 kg ≥ 50 kg I do not know	50 (41) 50 (41) 22 (18)
For donating blood, should the donor be fasting?	Yes No I do not know	19 (15.6) 84 (68.9) 19 (15.6)
Is there a risk of transmission of infection by receiving blood transfusion?	Yes No	97 (79.5) 25 (20.5)
Among the following which is acquired by receiving infected blood?	HIV/Hepatitis/Malaria Cancer/I do not know	103 (84.4) 19 (15.6)
How often blood can be safely donated by a person?	Every 3 months Every 6 months Annually I do not know	75 (61.4) 20 (16.4) 6 (4.9) 21 (17.2)
Who can donate blood?	Age 18–65 years Men and women Anybody healthy	29 (23.8) 11 (9) 82 (67.2)
What volume of blood is collected during each donation?	<500 mL 500–1000 mL I do not know	59 (48.4) 21 (17.2) 42 (34.4)
What is the duration takes during process of donating blood?	<20 20–60 min I do not know	35 (28.7) 51 (41.8) 36 (29.5)

employees who had work experience between one and five years were less likely to donate blood compared to more than 10 years' experience. A similar finding was reported from a study conducted at the University of Gondar.[9] This might be because those who had less work experience may not have adequate exposure to the necessity of blood in their work environment.

Category of the profession is also another factor associated with blood donation. Clinical workers were more likely donate blood compared to nonclinical workers. This finding was in line with a study conducted in

Table 3 Attitude Toward Blood Donation in Employees at Negele Arsi General Hospital and Medical College, August 2020, Negele Arsi, Southeast Ethiopia (N=122)

Attitude Questions	Category	Frequency N (%)
What do you feel about blood donation?	Feeling good Feeling bad Feeling Neutral Have no idea	81 (66.5) 6 (4.9) 24 (19.5) 11 (9.1)
What do you think is the best source of blood donors?	Voluntary Remunerated Replacement I do not know	102 (83.6) 1 (0.8) 5 (4.1) 14 (11.5)
Could harm occur to a blood donor during or after donation?	Yes No I do not know	53 (43.4) 57 (46.7) 12 (9.8)
Do you think that blood donation leads to anemia?	Yes No	38 (31.1) 84 (68.8)
Should patient relatives be asked to donate?	Yes No I do not know	73 (59.8) 39 (32) 10 (8.2)
Willingness to encourage others to donate blood?	Yes No	101 (82.8) 21 (17.2)
Would you like to donate blood in the future?	Yes No	115 (94.3) 7 (5.7)
Would you like to donate blood to only family members or friends?	Yes No	25 (20.5) 97 (79.5)
If you asked, do you willing to donate blood to an unknown person?	Yes No	108 (88.5) 14 (11.5)
Do you think as good to give incentives to those who donate blood?	Yes No	41 (33.6) 81 (66.4)

ALERT Hospital, Addis Ababa[11] in which health professionals were more likely donate blood compared to non-health professionals. This could be due to the fact that health professionals had good awareness about the importance of blood dation and closely work with individuals in need of blood.

Those study participants with inadequate knowledge of blood donation were less likely to donate blood compared to those with adequate knowledge. This finding was in line with a study conducted in a different part of Ethiopia[9,10,12] where the participants' knowledge showed significant association with blood donation. This can be explained by the fact that those who had inadequate knowledge about blood donation could not understand the benefit of blood donation for those in need.

In the current study, the attitude of the respondents was significantly associated with blood donation practice. Those respondents with unfavorable attitudes were less likely to donate blood compared to those with a favorable attitude. This finding is supported by the study conducted in the University of Gondar hospital,[9] Tigray regional state hospitals,[10] and ALERT Hospital[11] where the attitude of health professionals had a significant association with the practice of blood donation. This could be explained by having a good attitude can motivate individuals to donate blood to save others' lives.

Conclusions

Overall, in Negele Arsi General Hospital and Medical College practice of employees towards blood donation

480

Journal of Blood Medicine 2021:12
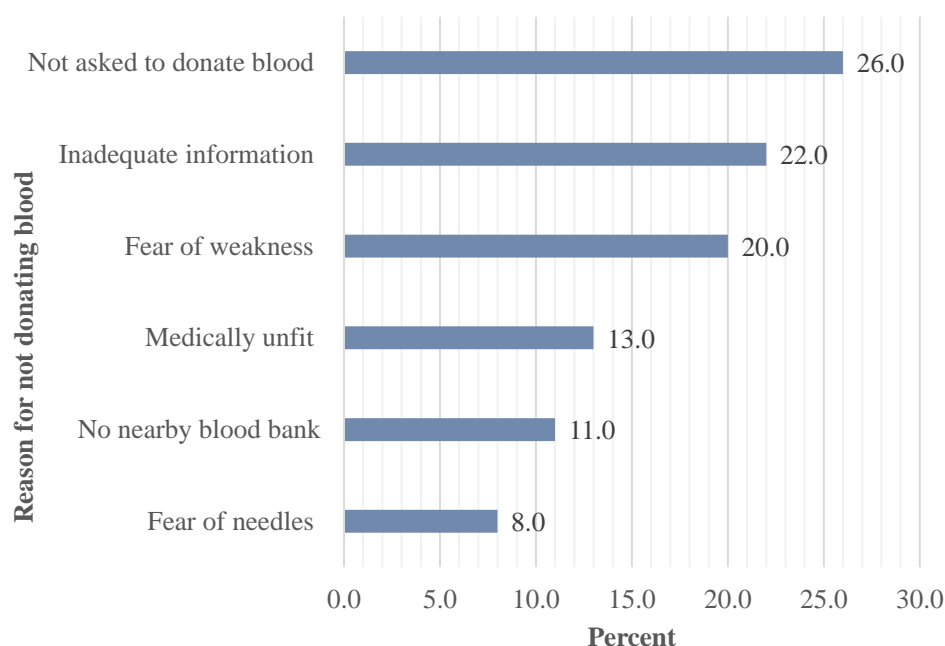
Figure 1 Reasons for not donating blood among employees at Negele Arsi General Hospital and Medical College, August 2020, Negele Arsi, Southeast Ethiopia.

was low. Year of experience, category of professions, knowledge, and attitude of employees were significantly associated with the practice of blood donation. Therefore,

staff updating training to empower their knowledge are important. The hospital has to prepare a blood donation campaign and a need for an active education program.

Table 4 Factors Associated with Practice on Blood Donation Among Employees in Negele Arsi General Hospital and Medical College, Ethiopia, 2020

Variables	Category	Practice Level		COR (95%CI)	AOR (95%CI)
		Yes	No		
Income	1000–3000	20	46	0.217 (0.059–0.806)	0.27 (0.03–2.78)
	3000–5000	13	16	0.375 (0.091–1.543)	0.77 (0.12–5.26)
	5000–10000	7	8	0.500 (0.106–2.355)	0.79 (0.13–4.76)
	>10000	8	4	1	1
Category of the profession	Clinical	30	19	4.825 (2.204–10.559)	7.81 (2.15–28.39)[a]
	Nonclinical	18	55	1	1
Experience in years	1–5	29	59	0.218 (0.062–0.769)	0.15 (0.03–0.74)[a]
	5–10	10	11	0.404 (0.094–1.733)	0.23 (0.04–1.42)
	≥11	9	4	1	1
Level of education	Below secondary	6	27	0.253 (0.088–0.724)	0.81 (0.14–4.80)
	Diploma	20	22	1.033 (0.449–2.378)	1.58 (0.39–6.40)
	First Degree and above	22	25	1	1
Knowledge level	Inadequate	7	32	0.224 (0.089–0.565)	0.29 (0.09–0.89)[a]
	Adequate	41	42	1	1
Attitude	Unfavorable	9	31	0.32 (0.136–0.756)	0.32 (0.11–0.92)[a]
	Favorable	39	43	1	1

Note: [a]Shows that this variable was significantly associated with practice of blood donation.

Abbreviation

AOR, adjusted odds ratio; CI, confidence interval; COR, crude odds ratio; KAP, knowledge, attitude, and practice; NAGHMC, Negele Arsi General Hospital and Medical College.

Data Sharing Statement

The data sets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Author Contributions

All authors made a momentous involvement to the work reported, that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; participate in drafting and revising of the article; all reviewed and agreed on all version during the process of publication; all agreed on the journal type to which the article has been submitted; and agreed to take responsibility for the contents of the article.

Disclosure

The authors report no conflicts of interest in this work.

References

1. World Health Organization. Universal access to safe blood transfusion; 2008.
2. Loua A, Nikiema JB, Sougou A, Kasilo OJM. Transfusion in the WHO African Region. *Transfus Clin Biol*. 2019;26(3):155–159. doi:10.1016/j.tracli.2019.06.191
3. WHO Guidelines Approved by the Guidelines Review Committee. In towards 100% voluntary blood donation: a global framework for action. Geneva: World Health Organization; 2010.
4. Roberts N, James S, Delaney M, Fitzmaurice C. The global need and availability of blood products: a modelling study. *Lancet Haematol*. 2019;6(12):e606–e615. doi:10.1016/s2352-3026(19)30200-5
5. Salaudeen AG, Odeh E. Knowledge and behavior towards voluntary blood donation among students of a tertiary institution in Nigeria. *Niger J Clin Pract*. 2011;14(3):303–307. doi:10.4103/1119-3077.86773
6. Jemberu YA, Esmael A, Ahmed KY. Knowledge, attitude and practice towards blood donation and associated factors among adults in Debre Markos town, Northwest Ethiopia. *BMC Hematol*. 2016;16 (1):23. doi:10.1186/s12878-016-0062-8
7. Nwogoh B, Aigberadion U, Nwannadi AI. Knowledge, attitude, and practice of voluntary blood donation among healthcare workers at the University of Benin Teaching Hospital, Benin City, Nigeria. *J Blood Transfus*. 2013;797830. doi:10.1155/2013/797830
8. Teklu SJ. College of Health Sciences, Addis Ababa University. Knowledge, attitude, practice and associated factors of blood donation among health care workers in Tikur Anbessa specialized hospital. Addis Ababa, Ethiopia; 2015.
9. Arage G, Ibrahim S, Adimasu E. Blood donation practice and its associated factors among health professionals of University of Gondar Hospital, Northwest Ethiopia: a cross sectional study. *BMC Res Notes*. 2017;10(1):294. doi:10.1186/s13104-017-2618-5
10. Tadesse T, Berhane T, Abraha TH, et al. Blood donation practice and associated factors among health professionals in Tigray regional state public hospitals, northern Ethiopia. *BMC Res Notes*. 2018;11(1):677. doi:10.1186/s13104-018-3786-7
11. Teferi MY, Woldesenbet SG, Feleke SA, Abebe EH, Adane HT, Alemayehu CM. Assessment of the level and factors associated with knowledge, attitude and practice of blood donation among medical and paramedical personnel in ALERT Hospital, Ethiopia. *J Public Health Res*. 2021;10(1):1860. doi:10.4081/jphr.2021.1860
12. Bantayehu D, Affairs H. Knowledge, attitude, and practice of voluntary blood donation and associated factors among health care providers in Addis Ababa health facilities. Ethiopia; 2015.

Efficacy, Safety and Pharmacokinetic Results of a Phase III, Open-Label, Multicenter Study with a Plasma-Derived Von Willebrand Factor (VWF)/ Factor VIII (FVIII) Concentrate in Pediatric Patients <12 Years of Age with Hemophilia A (SWIFTLY-HA Study)

Claudia Djambas Khayat, Genadi Iosava, Irina Romashevskaya, Oleksandra Stasyshyn, Marta Julia Lopez, Maria Teresa Pompa, Tobias Rogosch & Wilfried Seifert

Open Access Full Text Article

ORIGINAL RESEARCH

Efficacy, Safety and Pharmacokinetic Results of a Phase III, Open-Label, Multicenter Study with a Plasma-Derived Von Willebrand Factor (VWF)/ Factor VIII (FVIII) Concentrate in Pediatric Patients <12 Years of Age with Hemophilia A (SWIFTLY-HA Study)

Claudia Djambas Khayat[1]
Genadi Iosava[2]
Irina Romashevskaya [3]
Oleksandra Stasyshyn [4]
Marta Julia Lopez[5]
Maria Teresa Pompa[6]
Tobias Rogosch [7]
Wilfried Seifert[7]

[1]Hospital Hôtel Dieu de France, Saint Joseph University, Beirut, Lebanon; [2]Joint Stock Hematology and Transfusiology Research Institute, Tbilisi, Georgia; [3]Republican Research Centre of Radiation Medicine and Human Ecology, Gomel, Belarus; [4]Institute of Blood Pathology and Transfusion Medicine, Lviv, Ukraine; [5]Guatemala City Hospital Roosevelt, Guatemala, Guatemala; [6]Monterrey Nuevo Leon OCA Hospital (MIRC), Monterrey Nuevo Leon, Mexico; [7]CSL Behring, Clinical Research and Development, Marburg, Germany

Correspondence: Claudia Djambas Khayat
Hospital Hôtel Dieu de France, Saint Joseph University, Beirut, Lebanon
Tel +9611613027
Email claudiakhayat@yahoo.fr

Background: Plasma-derived von Willebrand factor/factor VIII (pdVWF/FVIII; VONCENTO®, CSL Behring) is a high-concentration, low-volume, high-purity concentrate, with a high level of VWF high-molecular-weight multimers and a VWF/FVIII ratio of ~2.4:1.

Methods: This study (NCT01229007) investigated the pharmacokinetics (PK), efficacy and safety of pdVWF/FVIII in 35 previously treated (minimum 20 exposure days [EDs]) pediatric patients (<12 years) with severe hemophilia A. PK was evaluated with a single 50 IU FVIII/kg dose of pdVWF/FVIII. Efficacy and safety analyses were performed during on-demand treatment (n=17) or prophylaxis (n=18) for up to 100 EDs with a maximum study duration of 12 months.

Results: PK profiles were similar for patients aged <6 years and those aged 6–12 years, and, as expected, the youngest patients had an increased clearance. On-demand patients reported 320 non-surgical bleeding (NSB) events and received a median number of 29.0 infusions (median dose 34.2 IU FVIII/kg). Hemostatic efficacy was assessed by the investigator as excellent/good in all cases (24%/76%). The 18 patients in the prophylaxis arm experienced 173 NSB events (97 NSBs [56%] in three patients). Five patients (28%) had no NSB events. Overall, patients received a median number of 92 infusions (median dose 30.6 IU FVIII/kg). The majority of bleeds (92%) were successfully controlled with only one infusion. Hemostatic efficacy was assessed by the investigator as excellent (86%) or good (14%). Inhibitors occurred in three patients of which two were transient (low titer) and one persisted (high titer). These three patients had known risk factors for inhibitor development.

Conclusion: This study demonstrated comparable PK profiles for pediatric patients aged <6 years and aged 6–12 years, and an excellent efficacy and safety profile in this population. The adverse events reported were mostly mild to moderate with inhibitor rates within the expected incidence range.

Keywords: hemophilia A, von Willebrand factor, factor VIII, on-demand therapy, prophylaxis, hemostatic efficacy

Plain Language Summary

Hemophilia A is an inherited bleeding disorder where the main symptom is spontaneous bleeding into the joints or muscles. Repeated bleeding episodes can be acutely painful and lead to long-term damage; therefore, the aim of hemophilia treatment is to prevent and manage these bleeding episodes to improve quality of life. One treatment option to control excessive bleeding for patients with hemophilia A is the use of von Willebrand factor/factor VIII (VWF/FVIII) concentrates derived from human plasma. Our study evaluated the safety and efficacy of a plasma-derived (pd)VWF/FVIII in children (aged <12 years old) with severe hemophilia A who had previously received hemophilia treatment with a FVIII product for a minimum of 20 exposure days. Treatment was either on-demand, to treat bleeding episodes as they arose, or prophylactic at regular intervals, with the aim of preventing bleeding. We also compared how children in different age groups (<6 versus 6–12 years old) responded to a one-off dose of pdVWF/FVIII. Hemostatic efficacy was assessed as excellent or good for both on-demand (24% and 76%, respectively) and prophylactic (86% and 14%, respectively) treatment with pdVWF/FVIII. Three patients developed inhibitors to FVIII, of which two were transient and one persisted; no other safety findings of concern were noted. These findings support those seen in adult/adolescent patients with severe hemophilia A. Comparable pharmacokinetic profiles were observed between the two age groups studied. Overall, these results demonstrate a favorable benefit-risk profile for pdVWF/FVIII, and support its use to treat bleeding events in children with hemophilia A.

Introduction

Hemophilia A is an X-chromosome-linked, congenital bleeding disorder, which is caused by decreased activity of factor VIII (FVIII) in plasma (FVIII:C) due to mutations in the FVIII gene. The incidence is estimated to be 1 in 5000 live male births.[1,2] Severe hemophilia A is characterized by a FVIII:C plasma level less than 1% of normal levels.[3] Intracranial, muscle and joint bleeding can occur in these patients, even with the minimal activities of daily life. Treatment for hemophilia is aimed at preventing and managing bleeding episodes and their subsequent complications. Prophylaxis with factor replacement therapy has been shown in many studies to prevent or at least reduce the progression of damage to target sites, such as joints.[4,5] A systematic review and meta-analysis of six randomized controlled trials demonstrated that prophylaxis started early in childhood preserves joint function as compared with on-demand treatment, due to a reduction in total bleeds and bleeding into joints, resulting in improved quality of life.[6]

A number of virus-depleted/inactivated plasma-derived FVIII and recombinant FVIII containing replacement products are currently available.[2,7–9] FVIII inhibitor development is the most significant treatment complication of these products seen in these patients.[9] The development of inhibitors in patients with hemophilia A is correlated with a variety of endogenous and exogenous risk factors.[10,11] The advantages and disadvantages of plasma-derived versus recombinant FVIII products are still an area of controversy, especially since the von Willebrand factor (VWF) present in different concentrations in some of the plasma-derived products might reduce inhibitor development.[11–19]

Plasma-derived VWF/FVIII concentrate (pdVWF/FVIII; Voncento®, CSL Behring, Germany) is a high-concentration, low-volume, high-purity VWF/FVIII concentrate which contains a large proportion of high-molecular-weight VWF multimers and a VWF:FVIII ratio of ~2.4:1.[20] The efficacy and safety of pdVWF/FVIII has been previously demonstrated in adults with hemophilia A.[20] In this study, the efficacy, safety and pharmacokinetics (PK) of pdVWF/FVIII were investigated in pediatric patients aged 0 to <12 years with severe hemophilia A (FVIII:C <1%) who had received limited previous FVIII treatment for a minimum of 20 exposure days (EDs).

Materials and Methods
Study Design

The SWIFTLY-HA study (NCT01229007) was conducted in eight centers in Ukraine (n=2), Belarus (n=2), Guatemala, Mexico, Georgia and Lebanon between August 2010 to July 2014. This study was carried out in accordance with the International Conference on Harmonization Good Clinical Practice guidelines, the Declaration of Helsinki (2008), and standard operating procedures for clinical research and development at CSL Behring. Ethics approval, individual written informed consent from patient's legal guardian (as all patients were <12 years of age), and approval by the Independent Ethics Committee/Institutional Review Board of the participating centers (Ethics Committee of the State Institution "Republican Scientific and Practical Center of Pediatric Oncology and Hematology" and Ethics Committee of State Institution "Republican Scientific and Practical Center of Radiation Medicine and Human Ecology", Belarus; Joint Stock Company the Institute of Haematology and Transfusiology, Georgia; Hospital Roosevelt Independent Ethics Committee, Guatemala; University of Saint-Joseph

Table 1 Guidelines for Dosage During the Efficacy Component[77]

Degree of Hemorrhage/ Type of Surgical Procedure[a]	FVIII Level Required (%)	Dose (IU/kg b.w.)	Frequency of Dosing (Per Day)	Duration of Treatment (Days)
Hemorrhage				
Early hemarthrosis, muscle bleeding, or oral bleeding	20–40	10–20	Repeat every 12–24 h	At least 1 day, until the bleeding episode, as indicated by pain, was resolved or healing was achieved
More extensive hemarthrosis, muscle bleeding, or hematoma	30–60	15–30	Repeat infusion every 12–24 h	For 3–4 days or more until pain and acute disability were resolved
Life-threatening hemorrhages	60–100	30–50	Repeat infusion every 8–24 h	Until threat was resolved
Surgery				
Minor, including tooth extraction	30–60	15–30	Every 24 h	At least 1 day, until healing was achieved
Major	80–100 (pre- and post- operative)	40–50	Repeat infusion every 8–24 h	Until adequate wound healing, then therapy for at least another 7 days to maintain a FVIII activity of 30–60% (IU/dL)
Prophylaxis[b]	–	20–40		At intervals of 2–3 days

Notes: [a]Guidelines were based on the Draft Guideline on Core SPC for Human Plasma Derived and Recombinant Coagulation Factor VIII Products (CPMP/BPWG/1619/ 1999). [b]In some cases, especially in younger patients, shorter dosage intervals or higher doses may have been necessary.
Abbreviations: b.w., body weight; FVIII, factor VIII; IU, international unit.

Beirut Ethics Committee, Lebanon; OCA Hospital Monterrey International Research Center Ethics & Research Committee, Mexico; Ministry of Health of Ukraine Central Ethics Commission, Ukraine) were obtained prior to enrollment.

Male patients were eligible for enrollment if they were <12 years of age with severe hemophilia A (FVIII:C <1%) and had received previous FVIII treatment for a minimum of 20 EDs. Patients with evidence of vaccination against hepatitis A and B or presence of antibodies against hepatitis A and B were included and provided signed informed consent (children <1 year of age could have been included without evidence of vaccination against hepatitis A); patients with no evidence of previous vaccination and/or no protective antibody titer against hepatitis A and B were vaccinated at screening. Patients were excluded from study participation if they received infusion of any FVIII product, cryoprecipitate, whole blood, plasma, or desmopressin acetate in the 4 days prior to Day 1, or presented with a known history of FVIII inhibitors, or with a FVIII inhibitor level >0.6 Bethesda Units (BU)/mL at screening; the intake of aspirin or other non-steroidal anti-inflammatory drugs

within 7 days of study drug administration also resulted in exclusion from study participation.

The efficacy and safety of pdVWF/FVIII was assessed in an open-label study comprising an on-demand and a prophylactic treatment arm. The on-demand regimen consisted of either immediate (irregular) treatment with pdVWF/FVIII of a non-surgical spontaneous or traumatic bleeding event, or a preventive (irregular) treatment with pdVWF/FVIII to prevent an anticipated bleeding event (eg, before expected physical activity that was associated with an increased bleeding risk). In the prophylaxis regimen, patients were given regular treatment with pdVWF/FVIII every 2–3 days. In addition, pdVWF/ FVIII was given to prevent and treat any surgical bleeding events. The general dosing recommendation for the treatment or prophylaxis of spontaneous or trauma-induced hemorrhages and for surgeries is shown in Table 1. Assignment to the prophylaxis or on-demand arm of the study, as well as each patient's treatment regimen and individual dose was determined by the investigator, based on the reason for use.

This study consisted of three periods (Figure 1): i) a screening period of up to 35 days; ii) a PK component of up to 3 days consisting of a single dose of pdVWF/FVIII on Day 1 with PK
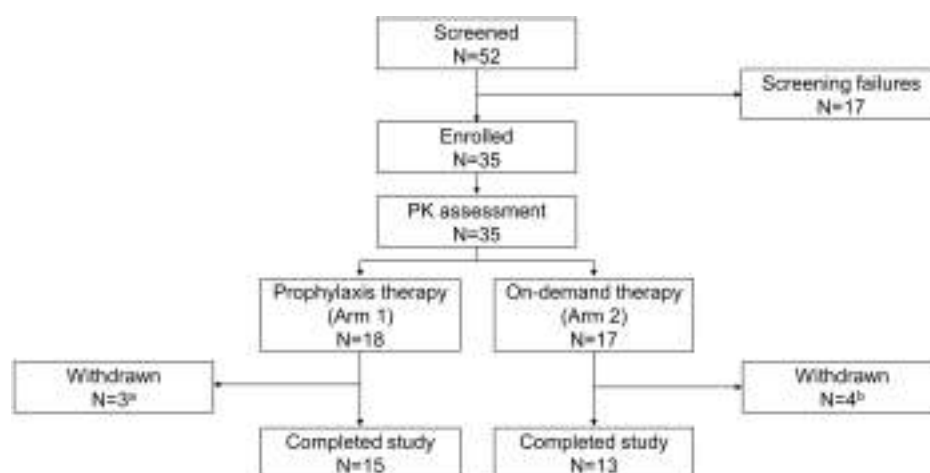
Figure 1 Study design.
Notes: [a]Due to site closure (n=2) and non-compliance with prophylaxis regimen (n=1). [b]Due to FVIII inhibitor development (n=2) and withdrew their consent (n=2).
Abbreviations: N, total number of patients; PK, pharmacokinetics.

samples collected on Days 1, 2, and 3; iii) an efficacy and safety component of 6 to 12 months (on-demand/preventive therapy or as regular prophylaxis) corresponding to approximately 50 and 100 EDs to pdVWF/FVIII.

The primary objectives were to assess the hemostatic efficacy of pdVWF/FVIII in its usage over ≥50 EDs, and to investigate the pharmacokinetic profile of pdVWF/FVIII. The secondary objective was to assess the safety of pdVWF/FVIII in this patient population.

Pharmacokinetics

The actual PK dose was calculated using the volume of each administered batch multiplied by the assigned potency of the specific batch, as determined at the batch release testing. PK parameters for FVIII:C were calculated by a non-compartmental infusion model from plasma concentration values collected after an initial single bolus intravenous infusion (administered at a maximum infusion speed of 6 mL/min) of 50 IU FVIII/kg body weight on Day 1. PK samples were drawn prior to the first dose of pdVWF/FVIII and then at 0.5, 4, 8, 24, and 48 h after the end of the first dose according to the European Medicines Agency guideline for FVIII products.[21] Chromogenic FVIII:C assay was performed in a central laboratory (Medilys Laborgesellschaft – Asklepios Institut, Hamburg, Germany).

Efficacy Assessments

The clinical efficacy parameters assessed in the study were hemostasis assessment (for each non-surgical bleeding [NSB] event and surgical event by the investigator and patient/

caregiver who filled in a home therapy diary), study product usage, blood product transfusion requirements, and surgeon's assessment of blood loss during a surgical procedure ("less", "equivalent", or "more" compared with the expected blood loss from a patient without a bleeding disorder undergoing the same procedure). Clinical assessments of hemostatic efficacy were based on a four-point grading scale: "excellent" if hemostasis was achieved/cessation of bleeding occurred; "good" if slight oozing or partial but adequate control of bleeding occurred and no additional product was required for unplanned treatment; "moderate" if moderate bleeding or moderate control of bleeding occurred and additional product was required for unplanned treatment; "none" in cases of severe uncontrolled bleeding. The severity of NSB events was assessed as major or minor by the investigator, according to guidance provided. If the patient was unable to self-administer or required more than two doses of pdVWF/FVIII to control an NSB event, they were to be treated at the study center and the investigator conducted daily hemostatic efficacy assessments. The patient visited the study site every 3 months and the investigator made a retrospective assessment of the patient's response to pdVWF/FVIII for each NSB event as documented in the home diary; ratings were based on the four-point efficacy grading scale described above. For each surgical procedure, the investigator was required to provide a daily assessment of patient response to pdVWF/FVIII during the inpatient period, with an overall investigator assessment performed at patient discharge, using the four-point efficacy grading scale. For prophylactic treatment, the annualized spontaneous bleeding rate (AsBR) was calculated.

Safety

All patients who received at least one dose of pdVWF/FVIII were included in the safety analysis. Safety assessments included the reporting of adverse drug reactions (an adverse event at least possibly related to pdVWF/FVIII), serious adverse events (SAEs, an adverse event that is life-threatening, requires/prolongs hospitalization, results in persistent/significant disability/incapacity, is a congenital anomaly/birth defect, or is considered medically significant), the presence of FVIII inhibitors, laboratory parameters (such as biochemistry, hematology, and urinalysis), and a physical examination and vital signs assessment. The extent of exposure to the study drug included administrations of pdVWF/FVIII during the PK and efficacy analyses. All medications taken 30 days prior to screening and during the entire study duration were recorded.

FVIII:C, the presence of FVIII inhibitors, and the investigation of seroconversion for virus markers (indicative of hepatitis A, B, and C virus infection) were assessed/conducted in a central laboratory (Medilys Laborgesellschaft – Asklepios Institut, Hamburg, Germany). Presence of FVIII inhibitors was analyzed using the Bethesda method (Nijmegen modification). Virology reference samples were collected at Day 1 and at the final visit, but were not analyzed unless deemed necessary.

Statistical Analyses

Descriptive statistics were used to summarize continuous variables (mean and standard deviation, and/or median and range). Categorical variables are presented as numbers and percentages in frequency tables. Summaries are based on observed data (missing data were not replaced). Formal statistical tests were not performed. Descriptive statistics for PK parameters were additionally stratified by age group (<6 years, 6 to <12 years).

Results

Patients

A total of 35 patients were enrolled, 18 patients were treated in the prophylaxis arm and 17 patients were treated in the on-demand arm (Figure 1). Patients participated in the study for a median (range) of 300 (1–388) days. A total of 28 patients completed the study (15 in the prophylaxis arm and 13 in the on-demand arm). In the prophylaxis arm, two patients who had developed a low titer FVIII inhibitor were withdrawn due to site closure and one patient who was non-compliant to treatment was withdrawn. In the on-demand arm, one patient was withdrawn due to a pre-existing FVIII inhibitor, one patient because he developed a high titer FVIII inhibitor and two patients withdrew their consent.

Baseline patient characteristics are shown in Table 2. In the on-demand arm, 11 patients (65%) had less than 50

Table 2 Patient Characteristics

Variables	Prophylaxis N=18	On-Demand N=17	Total N=35
Age [years]			
Mean (SD)	7.3 (2.68)	4.9 (3.43)	6.1 (3.26)
Median (range)	7.5 (2–11)	4.0 (0–11)	6.0 (0–11)
0 to <6 years, n (%)	4 (22.2)	12 (70.6)	16 (45.7)
6 to <12 years, n (%)	14 (77.8)	5 (29.4)	19 (54.3)
Ethnic origin, n (%)			
Caucasian	15 (83.3)	17 (100)	32 (91.4)
Hispanic	3 (16.7)	0	3 (8.6)
Weight [kg]			
Mean (SD)	29.1 (13.7)	22.6 (11.4)	26.0 (12.9)
Median (range)	25.5 (14.0–66.5)	18.5 (8.5–50.0)	23.0 (8.5–66.5)
Prior treatment regimen, n (%)			
Prophylaxis	7 (39)	0	7 (20)
On-demand	7 (39)	17 (100)	24 (69)
Not reported	4 (22)	0	4 (11)

Abbreviations: n, number of patients or events with characteristic; N, total number of patients; SD, standard deviation.
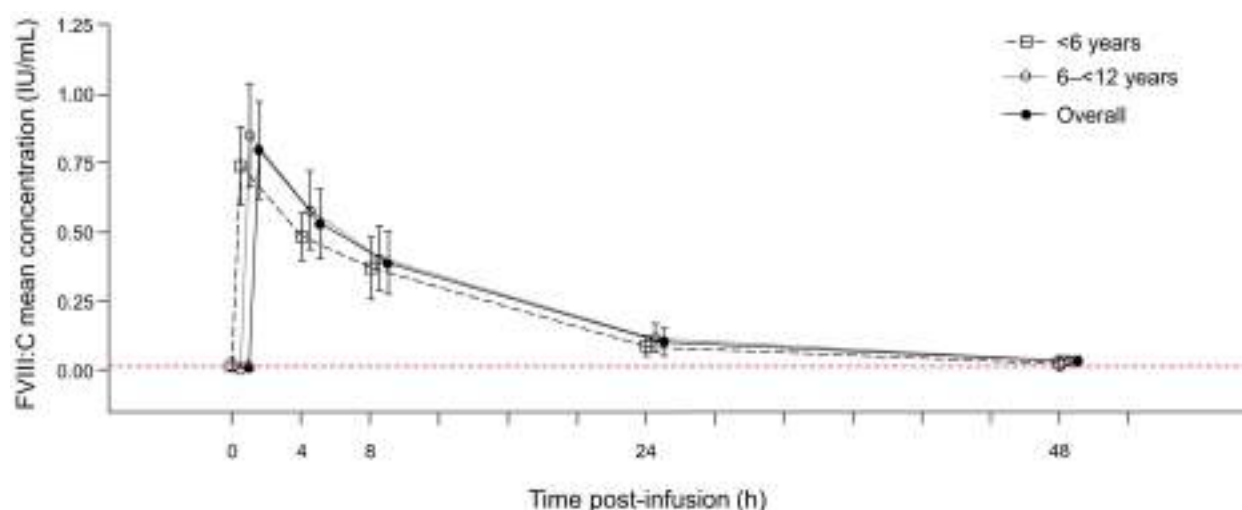
Figure 2 Mean (SD) concentration profiles (IU/mL) of FVIII:C by age group and overall.
Notes: Solid line with circles: overall population; black dashed line with squares: 0 to <6 years; black dashed line with circles: 6 to <12 years; red dashed line: lower limit of quantitation (0.008 IU/mL).
Abbreviations: FVIII:C, factor VIII: coagulant activity; IU, international unit; PK, pharmacokinetics; SD, standard deviation.

EDs to previous FVIII treatment. In the prophylaxis arm, 2 patients (11%) had less than 50 EDs. The proportion of patients aged <6 years was lower in the prophylaxis arm (22.2%) than in the on-demand arm (70.6%), resulting in a higher mean age in the prophylaxis arm (7.3 years) than in the on-demand arm (4.9 years).

Pharmacokinetics

The PK population comprised 31 patients, excluding four patients who had received a lower dose than planned (n=2), no PK concentrations were available (n=1) or the patient was excluded due to a pre-existing FVIII inhibitor (n=1). Concentration-time curves showed similar profiles for the overall PK population and the two age groups (Figure 2). In the overall PK population, FVIII:C PK parameters were raised immediately following pdVWF/FVIII infusion, reaching a median C_{max} of 0.76 IU/mL at the first sampling point 30 min post-dose, and then declined over time with a median $t_{1/2}$ of 9.78 h (Table 3). Median IR was 0.016 (IU/mL)/(IU/kg), median CL was 5.44 mL/h/kg, and median V_{ss} was 73.7 mL/kg. Median values for PK parameters were slightly higher in the 6 to <12 years age group compared to the 0 to <6 years age group (with the exception of CL and V_{ss}, Table 3).

Hemostatic Efficacy

On-demand arm: The 17 patients who were treated on-demand reported 320 NSB events and received a median number of 29.0 infusions (median dose of 34.2 IU FVIII/kg [range: 22.7–

49.7 IU/kg]). Two of the NSB events did not require treatment, and are not included in the efficacy evaluation. A total of 75 (23.4%) NSB events required >1 infusion of pdVWF/FVIII, up to a maximum of seven infusions; all remaining NSB events were treated with 1 infusion. Hemostatic efficacy was assessed by the investigator for all 318 events that were treated with pdVWF/FVIII (Table 4). The hemostatic efficacy was reported by the investigator as either excellent (77 events [24.2%]) or good (241 [75.8%]) in all cases. A similar distribution of hemostatic efficacy outcomes was also seen within bleeding event categories for type, severity, or location, with the exception of the 98 major bleeding events, where the hemostatic efficacy of a relatively higher proportion of events was assessed as good (95.9%). There were no relevant differences in the evaluation of hemostatic efficacy between patients aged <6 years and 6–<12 years.

Prophylaxis arm: The 18 patients on prophylaxis treatment received a median number of 92 infusions at a median average dose of 30.6 IU FVIII/kg per infusion (range: 21.1–58.7 IU/kg). The patients experienced 173 NSB events; fewer than 1 out of 3 events (31%) were spontaneous bleedings. More than half of these spontaneous NSB events (56%) were reported by three patients (see Table 5). The first of these patients was 9 years old, and experienced an AsBR of 24.91. Prior to study enrollment, this patient was receiving prophylaxis and had reported hemarthrosis of the right elbow, left and right ankle, left-hand finger, and both wrists. Additionally, during the study the patient was incompliant with his prophylaxis schedule. The second patient (10 years old) had an AsBR of 9.36,

Table 3 PK Parameters of FVIII:C in Patients <6 (N=15) and 6–12 Years of Age (N=16)

	<6 Years			6–12 Years			Overall		
Parameter	**N**	**Median**	**Range**	**N**	**Median**	**Range**	**N**	**Median**	**Range**
Dose administered (IU/kg)	15	50.1	47.5–54.5	16	50.0	47.5–54.5	31	50.0	47.5–54.5
Incremental recovery (IU/mL)/(IU/kg)	15	0.015	0.009–0.019	16	0.016	0.010–0.026	31	0.016	0.009–0.026
Half-life (h)	15	9.62	7.75–18.20	16	10.00	8.89–12.50	31	9.78	7.75–18.20
AUC_{0-48} (h*IU/mL)	15	8.23	3.96–11.04	16	9.90	6.17–17.62	31	8.80	3.96–17.62
C_{max} (IU/mL)	15	0.75	0.46–0.94	16	0.84	0.51–1.21	31	0.76	0.46–1.21
Total clearance (mL/(h*kg)	15	6.22	4.22–11.34	16	4.88	2.54–7.74	31	5.44	2.54–11.34
V_{ss} (mL/kg)	15	75.3	63.8–197.2	16	71.9	42.1–109.3	31	73.7	42.1–197.2

Abbreviations: AUC, area under the curve; C_{max}, maximum plasma concentration; C_{min}, minimum plasma concentration; FVIII:C, factor VIII: coagulant activity; IU, international unit; MRT, mean residence time; N, number of patients; t_{max}, time to maximum concentration; V_{ss}, volume of distribution at steady state.

Table 4 Investigator's Assessment of Hemostatic Efficacy per Bleeding Event in the On-Demand and Prophylaxis Arms

	On-Demand Arm					Prophylaxis Arm				
		Number (%) of NSB Events					**Number (%) of NSB Events**			
Bleeding Type	**Number of NSB Events**	**Excellent**	**Good**	**Moderate**	**None**	**Number of NSB Events**	**Excellent**	**Good**	**Moderate**	**None**
All NSB events	318	77 (24.2)	241 (75.8)	0	0	172	148 (86.0)	23 (13.4)	1 (0.6)	0
Spontaneous	123	26 (21.1)	97 (78.9)	0	0	54	42 (77.8)	11 (20.4)	1 (1.9)	0
Trauma	194	51 (26.3)	143 (73.7)	0	0	118	106 (89.8)	12 (10.2)	0	0
Post-surgery	1	0	1 (100.0)	0	0	0	0	0	0	0
Major	98	4 (4.1)	94 (95.9)	0	0	85	70 (82.4)	15 (17.6)	0	0
Minor	220	73 (33.2)	147 (66.8)	0	0	87	78 (89.7)	8 (9.2)	1 (1.1)	0
Joint	176	31 (17.6)	145 (82.4)	0	0	143	122 (85.3)	20 (14.0)	1 (0.7)	0
Mucosal	73	24 (32.9)	49 (67.1)	0	0	13	12 (92.3)	1 (7.7)	0	0
Muscle	67	21 (31.3)	46 (68.7)	0	0	14	12 (85.7)	2 (14.3)	0	0
Other	2	1 (50.0)	1 (50.0)	0	0	2	2 (100.0)	0	0	0

Notes: Percentages were based on the number of bleeding events of the considered type with available investigator's assessment. Bleeding events for which no treatment was needed (on-demand arm, n=2; prophylaxis arm, n=1) were not considered for this table.
Abbreviations: N, number of patients; NSB, non-surgical bleeding.

and prior to this study had received prophylaxis and reported hemarthrosis of both knees and both ankles. The third of these patients was 11 years old with an AsBR of 8.05; prior to his enrollment in this study, the patient had received on-demand treatment and reported hemarthrosis of the right knee, both elbows, both ankles, and the right hip. Seven of the 18 patients (39%) switched to prophylaxis treatment with pdVWF/FVIII from prophylaxis with a prior treatment. One NSB event

(0.6%), a minor trauma-induced oral bleed, did not require treatment and this event was not assessed for efficacy. The majority of all NSB events (159 events, 91.9%) required only one infusion for hemostatic control, the remainder required 2 infusions (13 events, 7.5%). Hemostatic efficacy was assessed by the investigator as excellent (86%) or as good (14%) (Table 4). The AsBR for patients who completed the study and the frequency of prophylaxis dosing per patient is shown

Table 5 Annualized Spontaneous Bleeding Rate and Prophylaxis Schedule (Number of Doses per Week per Individual Patient Who Completed the Study)

	Study Month	1	2	3	4	5	6	7	8	9	10	11	12	AsBR
Age (Years)														
2	Spontaneous NSB	0	0	0	0	0	0							0.00
	Prophylaxis/week	3.7	3.3	2.8	3.0	2.8	3.0							
3	Spontaneous NSB	0	0	0	0	I	I	2	0	0	0	0		4.35
	Prophylaxis/week	1.9	1.4	1.9	2.3	1.6	1.2	1.6	1.9	1.4	2.6	1.4		
4	Spontaneous NSB	I	0	0	0	0	0	0	0	0	I			2.32
	Prophylaxis/week	1.2	2.3	1.6	2.3	2.3	2.8	1.6	2.3	1.6	2.6			
5	Spontaneous NSB	0	I	0	I	0	I	0	0	I	0	0		4.24
	Prophylaxis/week	1.2	1.6	1.6	2.1	2.1	1.6	1.9	0.9	1.9	1.4	1.2		
6	Spontaneous NSB	0	0	0	0	0	0							0.00
	Prophylaxis/week	3.3	3.0	2.6	2.6	3.3	4.2							
7	Spontaneous NSB	0	0	0	0	0	0							0.00
	Prophylaxis/week	2.1	2.6	2.3	2.6	2.3	2.8							
7	Spontaneous NSB	0	0	0	0	0	0	0	0					0.00
	Prophylaxis/week	2.8	3.3	3.3	3.0	2.3	1.9	3.3	0.2					
8	Spontaneous NSB	0	0	0	0	0	0							0.00
	Prophylaxis/week	3.7	3.3	3.5	3.7	3.5	4.0							
8	Spontaneous NSB	0	0	0	0	0	0	0	I	I				2.64
	Prophylaxis/week	2.3	3.3	4.4	1.9	1.9	5.1	2.3	2.8	1.2				
9	Spontaneous NSB	2	I	0	0	I	I	4	0	3	5	2	7	24.91*
	Prophylaxis/week	0.9	0.7	2.3	3.0	0.2	0.9	1.4	1.2	0.0	0.2	0.2	0.2	
10	Spontaneous NSB	0	0	0	0	0	0							0.00
	Prophylaxis/week	2.1	2.6	2.3	2.6	1.9	2.3							
10	Spontaneous NSB	2	I	3	0	0	0	0						9.36*
	Prophylaxis/week	3.0	2.8	2.1	3.0	3.0	2.6	3.3						
10	Spontaneous NSB	0	0	0	0	0	0	0						0.00
	Prophylaxis/week	3.3	3.3	3.0	3.5	2.6	2.1	0.9						
10	Spontaneous NSB	0	0	0	0	0	0	0	0	I	0	0		1.15
	Prophylaxis/week	1.9	1.6	1.6	1.6	1.9	2.3	0.9	2.1	2.3	2.6	0.5		
I I	Spontaneous NSB	0	2	3	0	0	0	I	0	0				8.05*
	Prophylaxis/week	3.0	1.9	2.8	2.3	2.1	1.9	3.3	1.9	1.9				

Notes: Spontaneous NSB events are highlighted in grey; *More than half of the reported NSB events (56%) were reported by these three patients.
Abbreviations: AsBR, annualized spontaneous bleeding rate; NSB, non-surgical bleeding.

in Table 5. Seven patients (47%) had no spontaneous NSB events.

Surgical events: During the study, five patients underwent a total of five surgeries: two major (synovectomy of the right knee and elongation of the Achilles tendon) and three minor (one dental surgery and two tooth extractions). At discharge, the investigator assessed the hemostatic efficacy for two of the minor surgical events as excellent and for the remaining major and minor surgical events as good. Blood loss during surgery was assessed as less (two surgical events) or as comparable (three surgical events) to the expected blood loss from a patient without a bleeding disorder undergoing the same procedure in all surgical events. No patient required blood product transfusions.

Safety

The median (range) number of EDs for patients in the prophylaxis arm was 91.5 (15–117), with 2 patients having <50 EDs and 16 patients having ≥50 EDs. For the on-demand arm, the median (range) was 29.0 (1–89) days, with 12 patients having <50 EDs and 5 having ≥50 EDs.

Overall, pdVWF/FVIII was well tolerated. During this study, 12 patients (66.7%) on prophylaxis treatment reported 64 treatment-emergent adverse events (TEAEs), and 11 patients (64.7%) in the on-demand treatment arm reported 33 TEAEs. The most frequently reported TEAEs overall were cough (eight patients), pyrexia (six patients), and rhinitis, FVIII inhibition, and rash (four patients each). Four patients had an adverse drug reaction (three cases of FVIII inhibition and one case of rash). Five patients experienced one SAE each (four cases of FVIII inhibition [one of which was pre-existing and so not considered related to pdVWF/FVIII] and one tibia fracture). There were no clinically relevant differences in the TEAE reporting profile between patients aged <6 years and those aged 6–<12 years.

One patient (<1 year old) had a pre-existing FVIII inhibitor detected in the pre-treatment PK blood sample (30.4 BU/mL), which the investigator considered related to prior FVIII (treatment given for a total of 20 EDs), and was withdrawn from the study. Two patients from the prophylaxis arm developed a low titer, transient inhibitor. The first patient (6 years old) developed a low titer FVIII inhibitor of 1.9 BU/mL in the third month of treatment following 18 EDs with pdVWF/FVIII. At the time of inhibitor development, the patient had a total of 38 EDs to FVIII (20 EDs with a prior FVIII product and 18 EDs with pdVWF/FVIII), and experienced an additional 7 EDs with pdVWF/FVIII before the inhibitor was confirmed and the patient withdrew from prophylaxis treatment. The inhibitor

was confirmed at month 4 (1.7 BU/mL), but had decreased to normal values (<0.6 BU/mL) at months 6 and 10. DNA analysis identified a mutation in exon 25 of the FVIII gene. The low number of EDs represented a risk factor for inhibitor development; no other risk factors were identified. The second patient (10 years old) developed a low titer FVIII inhibitor of 2.1 BU/mL in month 1, following a total of 30 EDs to FVIII (22 EDs with a prior FVIII and 8 EDs with pdVWF/FVIII). The patient experienced a further 7 EDs with pdVWF/FVIII prior to confirmation of the inhibitor and withdrawal from prophylaxis treatment. The patient's low titer inhibitor persisted to month 8, with titers of 1.9–2.8 BU/mL. Risk factors for inhibitor development in this patient included an intron 22 inversion in the FVIII gene, a mutation associated with a higher risk of inhibitor development, and the low number of EDs to FVIII products; no other risk factors were identified. Three bleeding events occurred during the time period between inhibitor testing and withdrawal from prophylaxis treatment in these two patients. The hemostatic efficacy of pdVWF/FVIII for these bleeds was also excellent despite the presence of the inhibitor. Both inhibitors were transient and the titer decreased to normal levels again (<0.6 BU/mL). One patient (4 years old) from the on-demand arm developed a high titer FVIII inhibitor (461 BU/mL) during the treatment phase after 8 EDs. The high titer inhibitor was confirmed on two subsequent occasions (2048 BU/mL, 1556 BU/mL), and the patient was withdrawn from the study. The patient was found to have an intron 22 inversion in the FVIII gene, with no other identified risk factors for inhibitor development. Prior to the study, the patient had been treated with a prior FVIII product for a total of 48 EDs, but had not developed inhibitors. The Independent Data Monitoring Committee concluded that the development of inhibitors did not constitute a safety concern for the product, as the development of an inhibitor is an adverse event associated with FVIII replacement and this incidence was in concordance with information from the scientific literature. No other safety findings of concern were observed. None of the patients experienced a thromboembolic event or anaphylactic reaction, and there were no events of suspected transmission of infectious agents.

Discussion

This PK, efficacy, and safety study was designed to investigate the use of pdVWF/FVIII for the prevention and treatment of bleeding events in FVIII pre-treated pediatric patients (0 to 12 years of age) with severe hemophilia A. pdVWF/FVIII is a plasma-derived, high-concentration, low-volume, high-purity concentrate which contains

VWF:FVIII in a ratio ~2.4:1.[20] It was anticipated that in children pdVWF/FVIII would have a similar PK, efficacy and safety profile to that in the adult population.

The PK evaluation demonstrated comparable PK profiles for the children aged <6 years and aged 6 to 12 years. Differences were noted between the PK results from our pediatric study and those in the study of adult/adolescent hemophilia A patients:[20] IR of FVIII was lower in pediatric patients (0.016 [IU/mL]/[IU/kg]) than in adults/adolescents (0.021 [IU/mL]/[IU/kg]), $t_{1/2}$ was shorter (9.78 vs 13.4 h) and CL was higher (5.44 vs 3.92 mL/h/kg). It has been confirmed that weight-normalized CL of FVIII decreases during growth and continues to decline slightly during adulthood. Elimination $t_{1/2}$, which is inversely related to CL, thus follows an opposite trend.[22,23] The differences in $t_{1/2}$ between our pediatric study and the adult/adolescent study may have an effect on the dose of FVIII required to maintain a desired trough level during prophylaxis and indicates that the dose of FVIII per kilogram body weight required for adequate prophylaxis probably changes throughout a subject's life. The variance in $t_{1/2}$, however, suggests that if the FVIII dose for prophylaxis is to be based on $t_{1/2}$, then $t_{1/2}$ should be measured in each patient, rather than be based on the patient's age. Interestingly, a recent study in pediatric patients with hemophilia A reported that genetic determinants of VWF clearance and FVIII binding can modify FVIII PK, further supporting an individualized approach to dosing.[24]

This study, as well as the adult/adolescent study, showed that, despite slight age-related differences in the observed PK parameters, efficacy results were very similar across all age groups. The hemostatic efficacy of pdVWF/FVIII was assessed by the investigator as excellent or good for all NSB and surgical events without any relevant differences between patients aged <6 or 6 to 12 years. These hemostatic efficacy results are also in line with those seen in adult/adolescent previously treated patients (PTPs) with hemophilia A treated with the same pdVWF/FVIII in whom the investigator assessed the efficacy as either excellent or good in at least 96% of the bleeding events.[20] Comparable hemostatic efficacy (excellent or good in 96.7% of bleeding events) was also reported by investigators in a study of adult/adolescent PTPs with severe hemophilia A who were treated with a lower concentration plasma-derived VWF/FVIII (1:1) concentrate (Wilate®, Octapharma, Switzerland).[25]

Prophylaxis is considered the optimal treatment strategy for pediatric patients with severe hemophilia to prevent bleeding.[26,27] A prophylactic regimen must account for each patient's unique bleeding pattern, PK profile, adherence to treatment and level of physical activity. In this study, 18 patients experienced 173 NSB events when they were on a prophylaxis regimen; 97 NSB events (56%) were reported by three patients who had a high AsBR. Of these, one patient was non-compliant with his prophylaxis regimen and the other two patients already had hemarthrosis at enrollment and experienced their bleeds during the first 3 months of the study. Seven patients switched to prophylaxis with pdVWF/FVIII from a prior product. These data clearly demonstrate the benefit of secondary prophylaxis, although the demanding medical regimen can lead to imperfect compliance.

Safety results in this study were reflective of the pediatric population, with cough and pyrexia being the most frequently reported TEAEs. Two patients in the prophylaxis arm developed a transient low titer FVIII inhibitor and one patient in the on-demand arm developed a high titer inhibitor during the study; additionally, another patient in the on-demand arm was diagnosed with a pre-existing inhibitor. Both inhibitor patients in the on-demand arm withdrew from the study, no other TEAEs led to withdrawal from the study and no deaths occurred during the study. With the exception of the development of inhibitors in three patients, safety results were comparable with those seen in adult/adolescent PTPs with hemophilia A;[20] comparable safety results were also reported in adult/adolescent PTPs with severe hemophilia A who were treated with a lower concentration plasma-derived VWF:FVIII (1:1).[25]

Despite knowledge of several well-established risk factors for inhibitor development, why some patients develop an inhibitor and others do not remains unclear. In previously untreated patients with severe hemophilia A, the first 50 EDs are when patients are at highest risk of inhibitor development.[28] The three patients who developed inhibitors during our study only had limited exposure to FVIII products prior to the study (20, 22, and 48 EDs, respectively). Mutations such as null mutations, large deletions, nonsense mutations, and intron 22 inversions are also associated with a high prevalence of inhibitors.[29] This was evidenced in our study as the two patients who developed a low titer inhibitor during prophylaxis treatment with pdVWF/FVIII had a mutation in exon 25 and an intron 22 inversion of the FVIII gene, respectively, while the patient who developed a high titer inhibitor during on-demand treatment with pdVWF/FVIII had an intron 22 inversion of the FVIII gene. Although genetics play a role, the discordance in inhibitor development between monozygotic twins observed in an international study of brothers with hemophilia A demonstrates that other

treatment-related variables may also play a role in inhibitor development.[30] Age at first infusion (<6 months vs >12 months) and prophylaxis versus no prophylaxis have also been identified as potential risk factors, with a higher rate of inhibitor development in those who receive their first FVIII infusion before 6 months of age[31] and a reduced risk of inhibitor development in those who receive regular prophylaxis.[32] However, later analyses have offered conflicting results, with some studies showing no effects on inhibitor development linked to these factors, and so it is not clear whether or not first infusion at an early age or regular prophylaxis offer a protective effect.[33] In our study, the patient with a pre-existing FVIII inhibitor, who developed FVIII inhibitors after a FVIII product taken between screening and start of treatment with pdVWF/FVIII, was at high risk because he had started his first FVIII on-demand treatment at a very young age (<8 months) and all four patients had less than 50 EDs prior to study entry. Although the published studies designed to associate treatment conditions with inhibitor development are indicative, the association between FVIII administration and inhibitor formation is hard to predict until the genetic factors that underlie inhibitor development are better understood and can be used to properly stratify patients.

Limitations to our study include the low patient numbers, the disparity in age between the patients in the prophylaxis versus on-demand arms and the uneven distribution of patients with more or less than 50 EDs within these treatment arms. There are also few studies of VWF/FVIII concentrates in patients with hemophilia A, particularly in pediatrics. Further studies to confirm our observed efficacy, safety and PK parameters of VWF/FVIII in pediatric patients with severe hemophilia A would be beneficial.

Conclusions

In summary, pdVWF/FVIII was observed to be efficacious as both on-demand and prophylaxis therapy in pediatric patients with hemophilia A, with the hemostatic efficacy assessed as either excellent or good in all cases. Two patients developed a transient low titer FVIII inhibitor. One high-risk patient developed a high titer inhibitor associated with pdVWF/FVIII treatment which is within the expected incidence range of minimally pre-treated pediatric patients. No other safety findings of concern were observed. The efficacy and safety profile of pdVWF/FVIII was similar to that of the adult/adolescent population. These results provide evidence for use of pdVWF/

FVIII to treat and prevent bleeding events in pediatric patients with hemophilia A, supporting the favorable benefit-risk profile of this concentrate.

Abbreviations

AE, adverse event; AsBR, annualized spontaneous bleeding rate; AUC, area under the concentration curve; CL, total clearance of the drug from the body; C_{max}, maximum plasma concentration; FVIII, factor VIII; FVIII:C, factor VIII: coagulant activity; IR, incremental recovery; MRT, mean residence time; NSB, non-surgical bleeding; PK, pharmacokinetics; SAE, serious adverse event; SD, standard deviation; $t_{1/2}$, half-life; TEAE, treatment-emergent adverse event; t_{max}, time to maximum concentration; V_{ss}, volume of distribution at steady state; VWF, von Willebrand factor.

Data Sharing Statement

CSL will only consider requests to share Individual Patient Data (IPD) that are received from systematic review groups or bona-fide researchers. CSL will not process or act on IPD requests until 12 months after article publication on a public website. An IPD request will not be considered by CSL unless the proposed research question seeks to answer a significant and unknown medical science or patient care question. Applicable country-specific privacy and other laws and regulations will be considered and may prevent sharing of IPD.

Requests for use of the IPD will be reviewed by an internal CSL review committee. If the request is approved, and the researcher agrees to the applicable terms and conditions in a data sharing agreement, IPD that has been appropriately anonymized will be made available. Supporting documents including study protocol and Statistical Analysis Plan will also be provided.

For information on the process and requirements for submitting a voluntary data sharing request for IPD, please contact CSL at clinicaltrials@cslbehring.com.

Disclosure

Claudia Djambas Khayat has received investigator fees from CSL Behring. Tobias Rogosch and Wilfried Seifert are employees of CSL Behring. Genadi Iosava, Irina Romashevskaya, Oleksandra Stasyshyn, Maria Teresa Pompa and Marta Julia Lopez have no conflicts of interest.

References

1. Mannucci PM, Tuddenham EG. The hemophilias – from royal genes to gene therapy. *N Engl J Med*. 2001;344(23):1773–1779. doi:10.1056/NEJM200106073442307
2. Lieuw K. Many factor VIII products available in the treatment of hemophilia A: an embarrassment of riches? *J Blood Med*. 2017;8:67–73. doi:10.2147/JBM.S103796
3. White GC 2nd, Rosendaal F, Aledort LM, et al. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost*. 2001;85(3):560. doi:10.1055/s-0037-1615621
4. Berntorp E, Astermark J, Bjorkman S, et al. Consensus perspectives on prophylactic therapy for haemophilia: summary statement. *Haemophilia*. 2003;9(Suppl 1):1–4. doi:10.1046/j.1365-2516.9.s1.17.x
5. Ljung RC. Prophylactic infusion regimens in the management of hemophilia. *Thromb Haemost*. 1999;82(2):525–530. doi:10.1055/s-0037-1615875
6. Iorio A, Marchesini E, Marcucci M, et al. Clotting factor concentrates given to prevent bleeding and bleeding-related complications in people with hemophilia A or B. *Cochrane Database Syst Rev*. 2011;(9): CD003429. doi:10.1002/14651858.CD003429.pub4
7. Mannucci PM. The role of natural VWF/FVIII complex concentrates in contemporary haemophilia care: a guideline for the next decade. *Haemophilia*. 2012;18(Suppl 2):2–7. doi:10.1111/j.1365-2516.2012.02794.x
8. Calvez T, Chambost H, Claeyssens-Donadel S, et al. Recombinant factor VIII products and inhibitor development in previously untreated boys with severe hemophilia A. *Blood*. 2014;124 (23):3398–3408. doi:10.1182/blood-2014-07-586347
9. Srivastava A, Santagostino E, Dougall A, et al. WFH guidelines for the management of hemophilia. *Haemophilia*. 2020;26(Suppl 6):1–158. doi:10.1111/hae.14046
10. Ettingshausen CE, Kreuz W. Recombinant vs. plasma-derived products, especially those with intact VWF, regarding inhibitor development. *Haemophilia*. 2006;12(Suppl 6):102–106. doi:10.1111/j.1365-2516.2006.01373.x
11. Garagiola I, Palla R, Peyvandi F. Risk factors for inhibitor development in severe hemophilia A. *Thromb Res*. 2018;168:20–27. doi:10.1016/j.thromres.2018.05.027
12. Gringeri A. Factor VIII safety: plasma-derived versus recombinant products. *Blood Transfus*. 2011;9(4):366–370. doi:10.2450/2011.0092-10
13. Gouw SC, van der Bom JG, Ljung R, et al. Factor VIII products and inhibitor development in severe hemophilia A. *N Engl J Med*. 2013;368(3):231–239. doi:10.1056/NEJMoa1208024
14. Chalmers EA, Brown SA, Keeling D, et al. Early factor VIII exposure and subsequent inhibitor development in children with severe haemophilia A. *Haemophilia*. 2007;13(2):149–155. doi:10.1111/j.1365-2516.2006.01418.x
15. Goudemand J, Rothschild C, Demiguel V, et al. Influence of the type of factor VIII concentrate on the incidence of factor VIII inhibitors in previously untreated patients with severe hemophilia A. *Blood*. 2006;107(1):46–51. doi:10.1182/blood-2005-04-1371

16. Gringeri A, Monzini M, Tagariello G, et al. Occurrence of inhibitors in previously untreated or minimally treated patients with haemophilia A after exposure to a plasma-derived solvent-detergent factor VIII concentrate. *Haemophilia*. 2006;12(2):128–132. doi:10.1111/j.1365-2516.2006.01201.x
17. Mancuso ME, Mannucci PM, Rocino A, et al. Source and purity of factor VIII products as risk factors for inhibitor development in patients with hemophilia A. *J Thromb Haemost*. 2012;10(5):781–790. doi:10.1111/j.1538-7836.2012.04691.x
18. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia*. 2003;9(4):418–435. doi:10.1046/j.1365-2516.2003.00780.x
19. Ammollo CT, Semeraro F, Vitulli A, et al. FVIII/VWF complex displays a greater pro-haemostatic activity than FVIII preparations devoid of VWF: study in plasma and cell-based models. *Haemophilia*. 2020;26(4):e151–e160. doi:10.1111/hae.14008
20. Skotnicki A, Lissitchkov TJ, Mamonov V, et al. Efficacy, safety and pharmacokinetic profiles of a plasma-derived VWF/FVIII concentrate (VONCENTO®) in subjects with haemophilia A (SWIFT-HA study). *Thromb Res*. 2016;137:119–125. doi:10.1016/j.thromres.2015.10.014
21. Committee for Medicinal Products for Human Use (CHMP). Guideline on the clinical investigation of recombinant and human plasma-derived factor VIII products; 2019. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-clinical-investigation-recombinant-human-plasma-derived-factor-viii-products-revision-2_en.pdf. Accessed August 2, 2019.
22. Bjorkman S, Blanchette VS, Fischer K, et al. Comparative pharmacokinetics of plasma- and albumin-free recombinant factor VIII in children and adults: the influence of blood sampling schedule on observed age-related differences and implications for dose tailoring. *J Thromb Haemost*. 2010;8(4):730–736. doi:10.1111/j.1538-7836.2010.03757.x
23. Bjorkman S, Oh M, Spotts G, et al. Population pharmacokinetics of recombinant factor VIII: the relationships of pharmacokinetics to age and body weight. *Blood*. 2012;119(2):612–618. doi:10.1182/blood-2011-07-360594
24. Swystun LL, Ogiwara K, Rawley O, et al. Genetic determinants of VWF clearance and FVIII binding modify FVIII pharmacokinetics in pediatric hemophilia A patients. *Blood*. 2019;134(11):880–891. doi:10.1182/blood.2019000190
25. Klukowska A, Windyga J, Batorova A. Clinical efficacy of a novel VWF-containing FVIII concentrate, Wilate®, in the prophylaxis and treatment of bleeding episodes in previously treated haemophilia A patients. *Thromb Res*. 2011;127(3):247–253. doi:10.1016/j.thromres.2010.11.030
26. Valentino LA. Considerations in individualizing prophylaxis in patients with haemophilia A. *Haemophilia*. 2014;20(5):607–615. doi:10.1111/hae.12438
27. Petrini P, Valentino LA, Gringeri A, et al. Individualizing prophylaxis in hemophilia: a review. *Expert Rev Hematol*. 2015;8(2):237–246. doi:10.1586/17474086.2015.1002465
28. Giangrande PLF, Hermans C, O'Mahony B, et al. European principles of inhibitor management in patients with haemophilia. *Orphanet J Rare Dis*. 2018;13(1):66. doi:10.1186/s13023-018-0800-z
29. Oldenburg J, Schroder J, Brackmann HH, et al. Environmental and genetic factors influencing inhibitor development. *Semin Hematol*. 2004;41(1 Suppl 1):82–88. doi:10.1053/j.seminhematol.2003.11.016
30. Astermark J, Berntorp E, White GC, et al. The Malmo International Brother Study (MIBS): further support for genetic predisposition to inhibitor development in hemophilia patients. *Haemophilia*. 2001;7 (3):267–272. doi:10.1046/j.1365-2516.2001.00510.x
31. van der Bom JG, Mauser-Bunschoten EP, Fischer K, et al. Age at first treatment and immune tolerance to factor VIII in severe hemophilia. *Thromb Haemost*. 2003;89(3):475–479. doi:10.1055/s-0037-1613376

32. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL Cohort Study. *Blood.* 2007;109(11):4648–4654. doi:10.1182/blood-2006-11-056291

33. Peyvandi F, Garagiola I. Product type and other environmental risk factors for inhibitor development in severe hemophilia A. *Res Pract Thromb Haemost.* 2018;2(2):220–227. doi:10.1002/rth2.12094

Different Types of Minor Blood Group Incompatibility Causing Haemolytic Disease of Neonates in one of the National Children's Medical Centre in China

Mingchun Lin, Meixiu Liu, Shulian Zhang, Chao Chen & Jin Wang

Published online: 25 Jun 2021.

Submit your article to this journal ☑

Article views: 430

View related articles ☑

View Crossmark data ☑

Citing articles: 3 View citing articles ☑

CASE SERIES

Different Types of Minor Blood Group Incompatibility Causing Haemolytic Disease of Neonates in one of the National Children's Medical Centre in China

Mingchun Lin[1,2]
Meixiu Liu[3]
Shulian Zhang[1]
Chao Chen[1]
Jin Wang[1]

[1]Neonatal Department, Children's Hospital of Fudan University, Shanghai, 201102, People's Republic of China; [2]Neonatal Fellowship Training at Children's Hospital of Fudan University, Yueqing Maternal and Child Health Hospital, Wenzhou, Zhejiang Province, People's Republic of China; [3]Blood Bank, Children's Hospital of Fudan University, Shanghai, 201102, People's Republic of China

Purpose: To review the neonatal cases with different types of minor blood group incompatible haemolytic diseases in China, and to improve the clinical understanding and management.

Materials and Methods: Seven cases from January, 1st, 2013 to December 31st, 2019 were searched out and reviewed retrospectively. All clinical data and laboratory findings were collected.

Results: There were totally seven cases enrolled including three cases of MNS, three of Diego, and one of Kidd combined with Rh, anti-RhE incompatibility. Among the seven cases, two had intrauterine transfusion, two underwent exchange transfusion, five received intravenous immune globulin, five cases developed anaemia, and three of them had transfusion. But among them, only four were found to have positive antibody screening and three were confirmed HDN with antibody types antenatally.

Conclusion: The clinical presentation is diverse. Antibody screening followed by the technique of peak systolic velocity in the fetal middle cerebral artery (MCA-PSV) helps to filter out the severe cases.

Keywords: neonatal haemolytic disease, HDN, alloimmunization, MNS blood group, Diego blood group, Kidd blood group

Introduction

Haemolytic disease of the newborn (HDN) refers to foetal or neonatal alloimmune haemolysis caused by red blood cell antibodies due to incompatible maternal and foetal or neonatal blood types. The prevalence varies according to blood type.[1,2] ABO incompatibility is the most common cause of HDN;[3,5] Rh (D) antigen is the second most common, and Rh (C, c, E, e) antigen incompatibility occurs occasionally.[5–7] Several other alloantibodies have also been reported to be associated with haemolytic diseases, including MNS, Kidd, Diego, Duffy, Kell and Anti-Mur.[9–14] However, most literatures on these diseases were published as case reports. Therefore, the presentation of the haemolytic diseases related to different minor blood group incompatibilities is not well understood. Here, a retrospective analysis of neonates with minor blood type haemolytic disease admitted to one medical centre over the past 6 years was conducted to improve awareness and perinatal management for Chinese population.

Correspondence: Jin Wang
Neonatal Department, Children's Hospital of Fudan University, No. 399, Wanyuan Road, Shanghai, 201102, People's Republic of China
Tel +86-21-64932960
Email drwangjin@fudan.edu.cn

Materials and Methods

This research topic was approved by institutional research ethics board of Children's Hospital of Fudan University. All cases were identified from the electronic database and met the inclusion criteria: newborns admitted to the neonatal department of Children's Hospital of Fudan University with a diagnosis of neonatal alloimmune haemolytic disease due to minor blood type incompatibility between January 1st, 2013, and Dec 31st, 2019. All the electronic information of these cases was reviewed to rule out single ABO or Rh, including D, E, e, C, and c blood group incompatibility, as well as other reasons for haemolysis, such as G6PD and red blood cell membrane defects such as hereditary spherocytosis, as exclusion criteria. We obtained the written informed consents from the parents of all cases and got the permissions to have all the case details published.

The key diagnostic criteria were defined as serological tests showing positive Coombs' test and related blood group system antibodies detected in the serum of newborns and/or mothers. Other evidence of haemolysis, such as complete blood count, haemoglobin level, reticulocyte count, and serum bilirubin levels, were consistent with haemolytic disease. All the cases had the confirmed diagnostic tests done in Shanghai blood centre, and the blood samples were sent on admission or initially after birth. ABO and RhD blood typing tests were performed with the tube by standard methods for each sample, using monoclonal anti-A, anti-B and anti-D (Shanghai Blood Biomedicine Co., Ltd.). Other phenotypes of the red blood cells (RBCs) were determined using monoclonal anti-E, anti-C, anti-c, anti-e, anti-M, anti-N (Shanghai Blood Biomedicine Co., Ltd.), anti-Dia, anti-Dib, anti-JKa and anti-JKb antibodies (Sanquin Reagents B.V.) respectively. The spectrum cells used for blood group antibody identification were purchased from Shanghai Blood Biomedicine Co., Ltd. and Sanquin Reagents B.V. DAT (direct antiglobulin test) was performed with the tube using multispecific antiglobulin reagents, monospecific antibody-IgG, and monospecific antibody-C3d (Shanghai Blood Biomedicine Co., Ltd.). IAT (indirect anti-human globulin test, included free antibody test and antibody release test) was performed with the tube using multispecific antiglobulin reagents and column agglutination technique using gel cards (Bio-Rad Laboratories).

Results

Data Collection and Summary

A total of seven cases were finally enrolled, including three cases of MNS haemolytic disease, three cases of Diego incompatibility, and one case of Kidd combined with anti-RhE blood group incompatibility. All the electronic charts of the seven cases were reviewed and collected, including antenatal and postnatal information such as maternal history, birth history, gender, gestational age, laboratory tests including maternal and child blood types, blood cell count, haemoglobin level, reticulocytes, bilirubin, and Coombs test. Treatment data, including the use of phototherapy, IVIG, blood transfusion, or exchange transfusion, and outcomes were also collected. Phototherapy and exchange transfusion were managed referring to the criteria of the American Association of Paediatrics Clinical Practice Guideline for phototherapy and exchange transfusion in hospitalized infants of 35 or more weeks' gestation.[15]

Result

A total of three different minor blood types were found to be associated with haemolytic disease in our search, including the MNS, Diego, and Kidd blood groups. We summarized all the cases in separate categories as follows (Table 1).

Three Cases of MNS Haemolytic Disease

Case 1, a male born by caesarean section at 39^{+1} weeks with a birth weight of 3950 g to a healthy 32-year-old mother (G1P1), was admitted to our NICU on day 3 because of jaundice and anaemia from day 2 of life. Coombs' test was performed immediately according to the clinical presentation. DAT (direct agglutination test) was negative, but IAT (indirect antiglobulin test) was positive, and anti-M antibodies were detected in the newborn's serum. He received only phototherapy during the hospitalization period without blood transfusion because of no deterioration of haemoglobin afterward. This was the mother's first child, conceived through IVF, and the mother had an antibody screening result of anti-M and titres of 1:512 IgM and 1:256 IgG at 35 weeks gestation. All clinical characteristics are shown in Table 1.

Case 2, a male infant born by vaginal delivery at 37^{+6} weeks with a birth weight of 3000 g to a healthy 23-year-old mother (G2P2) was admitted to our NICU on day 4 because of jaundice from day 2 of life. Coombs' test was performed after admission to the patient with negative

Table 1 The Clinical Characters of Pregnant Women and Neonates with Minor Blood Group Haemolytic Disease

Case No.	GnPn	Sex	GA	DOA	Presentation		Laboratory							Neonatal Treatment					Outcome
					Onset Time	Other Hx	Blood Type (Mother)	Blood Type (Baby)	Coombs Test	Antibody	TSB on Ad (μmol/L)	Hb on Ad (g/L)	Ret (%)	Ex-Tx	Photo Tx	IVIG	Alb	RBC	
1	G1P1	M	39+1	Day3	Day 2	Antibody +	A+, NN	O+, MN	DAT-IAT+	Anti-M	235.5	93	5.4	N	Y	N	N	N	Survival
2	G2P2	M	37+6	Day4	Day 2	N/A	O+, NN	B+, MN	DAT-IAT+	Anti-M	259.5	159	1.3	N	Y	N	N	N	Survival
3	G5P1	M	34+1	Day27	Day 1	IUT	A+, NN	O+, MN	DAT-IAT+	Anti-M	137	49.2	0.1	N	Y	Y	N	Y	Survival
4	G5P2	M	37+4	Day4	Day 2	Twin A	A+, Di(a+b-)	O+, Di(a+b+)	DAT+IAT+	Anti-Dib	430	144	8.8	N	Y	Y	Y	N	Survival
5	G5P3	M	37+4	Day4	Day 2	Twin B	A+, Di(a+b-)	O+, Di(a+b+)	DAT+IAT+	Anti-Dib	356.7	207	6.2	N	Y	Y	Y	N	Survival
6	G4P2	M	32+1	Day1	<24 h	Hydrops IUT	A+, Di(a-b+)	O+, Di(a+b+)	DAT-IAT/+	Anti-Dia	223.5	57	1.2	Y	Y	Y	N	Y	Survival
7	G2P2	F	39+4	Day1	<24 h	Antibody +	A+, JK(a-b+) Ccee	A+, JK(a+b+) CcEe	DAT+IAT+	Anti-JKa Anti-E	444.2	117	4.3	Y	Y	Y	N	Y	Survival

Abbreviations: GnPn, Gravida number and para number; GA, gestational age; DOA, day of admission after birth; Hx, history; DAT, direct antibody test; IAT, indirect antibody test; TSB, total serum bilirubin; ad, admission; Hb, haemoglobin; Ret, reticular count; Ex-Tx, exchange transfusion; Photo, phototherapy; IVIG, intravenous immune globulin; Alb, albumin; RBC, red blood cell transfusion.

DAT and positive IAT, and anti-M antibodies were detected in the newborn's serum. He received phototherapy for treatment. This mother had received regular antenatal care during pregnancy, but no antibody screening test was performed. Her first child was a healthy four-year-old girl without a history of jaundice at birth.

Case 3, a male infant was admitted to our NICU on day 27 with a confirmed diagnosis of anti-M HDN at birth in a maternity hospital where he had positive IAT results and negative DAT results and received one blood transfusion, one IVIG and multiple phototherapies. Admission to our NICU was due to severe anaemia. Both DAT and IAT were negative, but the antibody screening test was positive for anti-M on day 27. A 2nd blood transfusion was given after this admission. This baby was a G5P1 preterm infant with a gestational age of 34^{+1} weeks and birth weight of 2345 g born by caesarean section due to MN-related HDFN in the foetus and elevated peak systolic velocity in the foetal middle cerebral artery (MCA-PSV) on ultrasound.[16] This 33-year-old mother had a very complicated maternal history with four previous failed pregnancies, including one intrauterine demise and three spontaneous abortions, two of which were confirmed as foetal hydrops. This patient was gravida 5, and positive antibody screening with anti-M was detected at 20 weeks gestation during this pregnancy. The foetus received intrauterine transfusion (IUT) twice with O RhD- and M-RBCs at 30 and 32^{+6} weeks gestation, respectively. Cordocentesis was performed during the first IUT, and allo-anti-M antibodies were eluted from the cord blood sample, which suggested MN-related HDFN in the foetus.

All three of these cases of MN blood type haemolytic disease have the same antibody type, which is anti-M. The MN blood types of the mothers and newborns were all the same, namely, MN for the mother and NN for the newborn. Jaundice occurred within the first two days in all three cases. The bilirubin levels were not very high because all the cases were sent from maternity hospitals, and phototherapy commenced as soon as jaundice was noted. Two of the newborns had different degrees of anaemia, and the haemoglobin trough values of cases 1 and 3 were as low as 93 g/L and 49.2 g/L, respectively. In case 3, the patient required two rounds of IUT and two rounds of RBC blood transfusion after birth, as well as IVIG, which is the most drastic therapy. Prenatal examination revealed positive antibody screening tests in two cases, and prenatal care follow-up of MCA-PSV helped to diagnose the most severe case (case 3).

Three Cases of Diego Haemolytic Disease

Case 4 and Case 5 were monochorionic diamniotic (MD) twins, born at a gestational age of 37^{+4} weeks, and both were admitted to our NICU on day 4. These two male newborns were G5P2 and G5P3 born by caesarean section with birth weights of 2700 g and 2200 g to a healthy 37-year-old mother. They were transferred because of severe jaundice from day 2 of life without anaemia and suspected haemolysis disease at a local hospital. G1P1 of this mother was a healthy girl without a history of jaundice during the newborn period, but the mother's other pregnancies were ended with induced labour twice and idiopathic spontaneous abortion once. She did not receive an antibody screening test during pregnancy. Coombs' tests were performed in the maternity hospital for the twins because of the early onset of jaundice. Both DAT tests were positive, but the laboratory could not confirm the antibody type. We sent all the blood samples of the mother and twins to the Shanghai Blood Centre to repeat the Coombs' tests, and positive results for both DAT and IAT were confirmed as anti-Dib. The mother's blood type was A+, Di(a+b-), and both twins had the same blood type, O+, Di(a+b+). Both twins received IVIG, albumin and phototherapy as treatment. The initial planned blood exchange transfusion was cancelled due to the lack of Di(b-) PRBC available on the first day, and the hyperbilirubinemia was well controlled afterwards.

Case 6, a preterm male newborn, was born to a 44-year-old mother (G4P2) at 32^{+1} weeks gestation by caesarean section because of suspecting foetal hydrops on ultrasound with a birth weight of 2380g. This foetus received intrauterine transfusion (IUT) six times from 24 weeks of gestational age until birth with O RhD-, Di(a-) RBCs after a confirmed maternal positive antibody test for anti-Dia and elevated peak systolic velocity in the foetal middle cerebral artery (MCA-PSV) on ultrasound. The mother was transferred from a local hospital because of positive antibody screening with unclear antibody type. The infant required CPAP support shortly after delivery due to neonatal respiratory distress syndrome (NRDS). He had no significant hydrops after full assessment at birth, but he had severe anaemia with a haemoglobin trough value as low as 57 g/L when he was admitted to our NICU within 24 hours of life, decreased from 116 g/L at birth. He was treated by blood exchange transfusion once after birth, three doses of IVIG, RBC transfusion once and multiple

phototherapies. The mother had one healthy child and two idiopathic spontaneous abortions prior to this pregnancy.

These three cases (No. 4, 5, and 6) were all considered haemolytic disease due to Diego blood type incompatibility. The twins (Case 4 and 5) presented moderate to severe jaundice from day 2 and had peak total bilirubin levels of 430 μmol/L and 356.7 μmol/L, respectively. However, just one of the twins had mild anaemia with a haemoglobin level of 144 g/L and a reticular count of 8.8%. In both cases, diagnosis was confirmed by positive DAT, IAT and anti-Dib antibody tests. The patients recovered with phototherapy, IVIG, and albumin transfusion. Case 6 was another case of haemolytic disease due to Diego incompatibility caused by anti-Dia antibody. This case presented severe HDFN from the beginning of the third trimester. Although the hyperbilirubinemia was not especially significant, with peak TSB at 223.5 μmol/L after birth, he had severe anaemia requiring exchange transfusion and red blood cell transfusion even after six IUTs.

One Case of Haemolytic Disease Due to Combination Kidd and Rh Blood Group Incompatibility

Case 7, A female was born at 38^{+4} weeks by vaginal delivery to a healthy 28-year-old mother (G2P2). The mother's first child was a healthy boy with no jaundice at birth and an uneventful pregnancy. However, during this pregnancy, she had a positive result in an antibody screen at local hospital, without identification of the antibody type. She was referred to a tertiary hospital for antenatal care and delivery. The newborn experienced severe hyperbilirubinemia within 24 hours after birth, with peak bilirubin 444.2 μmol/L and minimum haemoglobin value 117 g/l. Coombs' tests performed by the Shanghai Blood Centre were positive for DAT and IAT, with positive anti-E and anti-JKa antibody screening and DAT/IAT. The diagnosis of combined antibodies also appears in other literature.[17] Full treatments for HDN, including blood exchange, IVIG and phototherapy, were given.

All seven cases received formal Coombs tests for diagnosis at the Shanghai Blood Centre. The tests performed at hospital laboratories report positive DAT and/or IAT and positive antibody screening but do not confirm the blood type responsible for the haemolytic disease. Some of the mothers were transferred from local hospitals outside of Shanghai due to positive antibody screening in mother's serum with unclear antibody type (case 7). Antenatal diagnosis was performed in the Shanghai Blood Centre for

some cases (cases 1, 3 and 6) on the bases of the maternal blood antibody screening tests.

All the cases enrolled presented different degrees of jaundice, and the haemoglobin level ranged between 49.2 and 207 g/L, which may reflect the variability of the severity of haemolysis. The sickest of the three cases of MNS blood type had IUT and severe anaemia, but the other two cases had only mild anaemia and moderate jaundice requiring phototherapy. The same occurred in Diego blood type.

It was also noticed that the MNS blood type cases all had positive IAT and negative DAT. Two patients who underwent IUT both had negative DAT results. However, another two cases of Diego incompatibility had positive DAT and IAT.

All seven patients improved and were discharged. All of them underwent brain MRI and BAEP before discharge to assess potential brain damage due to early hyperbilirubinemia and anaemia. Case 7 had abnormal MRI signals on the right parietal lobe and right caudate nucleus, which were diagnosed as cerebral infarctions of unclear cause. Case 3 and case 6, as preterm infants, had characteristics of prematurity on brain MRI. None of the infants had significant signs of bilirubin encephalopathy on MRI. The results of brain stem auditory evoked potential (BAEP) showed that case 1 failed on the left side with a hearing threshold of 45 dB, and cases 6 and 7 failed on both sides with a mild degree of hearing loss. The remaining four infants passed this investigation before discharge. Cases 1, 6 and 7 passed the BAEP during follow-up. All seven cases were followed up at different ages. The oldest age of follow-up was three years for case 3; most of the other cases were followed up until two years of age. The shortest follow-up was for case 7, who was last seen at our clinic at 6 months of age. None of the patients required RBC transfusion after discharge, and some received iron supplementation. None of them presented long-term issues in hearing, motor development, or dental enamel, nor extrapyramidal symptoms or athetosis.

Discussion
Discussion

In China, HDN is most commonly caused by ABO blood group system incompatibility, followed by Rh blood group incompatibility. It has been reported that ABO HDN accounts for 85.3% and Rh accounts for much less than 14.6% of total cases in China.[18] Other blood group system

incompatibilities, such as MNS, Diego, and Kidd, are relatively rare. The prenatal discovery of haemolytic disease caused by minor blood group incompatibility is insufficient throughout China. The lack of confirmed antenatal diagnosis may present a challenge for neonatologists because the clinical severity of these entities varies. The clinical presentation and laboratory characteristics of ABO and Rh HDN are clear to most obstetricians and neonatologists,[19–21] but this is not the case for all of these minor blood type HDNs.[22] Consistent antenatal and postnatal management is important for both obstetricians and neonatologists. For early jaundice with positive Coombs test, either DAT or IAT, and unexplained antibody within the first few days, it is necessary to rule out all types of minor blood group haemolytic diseases.

We also noticed four cases with positive antibody screening during antenatal care in our cases. Antibody screening during pregnancy is encouraged for antenatal diagnosis. For pregnant women with positive results, further follow-up combined with determination of the peak systolic velocity of the foetal middle cerebral artery (MCA-PSV) on ultrasound is the protocol suggested to identify severe intrauterine haemolysis.[23] As cases 3 and 6 in our study both benefited from this screening, and the mothers in both cases had had more than one idiopathic spontaneous abortion before in previous pregnancies, foetal death due to severe haemolysis could be the reason for the previous abortions. Severe haemolysis symptoms were noticed after positive antibody screening during the case pregnancies, and intrauterine transfusions (IUTs) were used for rescue.[24] Even for cases without intrauterine interventions, antenatal screening is helpful for obstetricians and neonatologists to more carefully track the severity of haemolysis in the newborns, and early transfer or treatment could be performed, as in cases 1 and 7. Therefore, antenatal screening is necessary for identifying severe cases and following intrauterine intervention.[25]

Anti-M antibody is the second most common non-RhD antibody identified in pregnant women,[26] but it has not been considered an important cause of HDN, especially in Caucasian and black populations.[8,27] The reason is that anti-M antibodies have been identified as naturally occurring antibodies,[28] and both IgM and IgG anti-M antibodies have been reported in retrospective studies from American and Dutch populations, among others,[8,29] with no cases of mild or severe HDN. In addition, anti-M has been found in the serum of individuals who have not been exposed to human erythrocytes. However, an increasing number of

HDN cases caused by anti-M have been reported, especially in Japanese, Chinese, and other Asian populations.[30–32] Anti-M was the most common antibody in our study as well, and all cases were caused by the same anti-M antibody of the MNS blood group system. One case was the first pregnancy of the mother, and the other two cases were the second and fifth pregnancies, which is similar to the pattern observed for ABO HDN because anti-M antibodies can occur naturally.[26] The severity of this HDN is quite variable, which has also been reported in previous studies.[33,34] All cases were negative for DAT, which is different from other minor blood type and Rh HDNs. This is an important point to help clinicians to identify anti-M HDN, because most cases of HDN have positive DAT, except ABO HDNs.[4] When the DAT is negative, most clinicians will rule out minor blood group haemolysis disease, and IAT of ABO will be conducted if the patient presents haemolysis, which may cause the misdiagnosis of anti-M haemolysis.

Therefore, we suggest that both DAT and IAT tests should be performed for all patients with suspected haemolysis disease. For the cases with positive DAT and IAT, it is better to identify the type of RBC antibody. As minor blood group haemolysis diseases are rare in the population, this full laboratory test with all blood type standard red blood cells is not economical to perform in all local hospitals, but it is necessary for one lab in an area to have the capability of identifying these antibodies so that rare samples can be sent for diagnosis. Another solution is to organize central blood centres equipped with laboratory technology for the determination of most minor blood group antibodies and a few tertiary medical centres that can perform antepartum treatments, such as intrauterine transfusion (IUT). Then, the pregnant woman would be able to be transferred upon suspicion of minor blood group incompatibility, as in some of our cases.

The remaining types, Diego[35,36] and Kidd,[37,38] presented typically with jaundice, anaemia after birth, and positive DAT and IAT, which helped make the confirmed diagnosis. The only case with negative DAT had multiple IUTs, which probably indicates that the laboratory results of foetal and neonatal blood type, DAT and IAT may be changed after IUTs.

Complex anti-E and JKa antibodies occurred in case 7, and the patient presented severe haemolysis symptoms requiring either exchange transfusion or red blood cell transfusion. In the laboratory results, the titres of the two types of antibodies were similar, and it is therefore

difficult to identify which is the major cause. However, mixed antibodies from different blood types may make haemolysis symptoms more serious.[17]

The overall management for all seven cases in our project was routine clinical care, including bilirubin monitoring, phototherapy, exchange transfusion, IVIG, red blood cell transfusion, and evaluation of the central nervous system.[15] Although the time of anaemia and hyporegeneration of erythroblasts varies, the severity of haemolysis could vary by type, and close monitoring and follow-up are necessary. All seven patients in this case series survived. This indicates that the management and follow-up for HDN is currently well performed in China, but early clarification of the diagnosis affects the management, which may help prevent early delivery or abortion and hydrops foetalis.

Conclusion

There are more than 30 types of minor blood type haemolysis disease, and we had 7 such cases, including three different blood types in one centre. The characteristics of these three types are variant, and we highlight that the antenatal antibody screening test is a simple first-line test for pregnant women. The examination of peak systolic velocity in the foetal middle cerebral artery (MCA-PSV) on ultrasound helps to identify severe cases. Full Coombs' test including both DAT and IAT for suspected neonates is necessary. It is not reliable to only check the DAT of Coombs' test for the diagnosis of minor blood group HDN since the DAT may be affected by the intrauterine intervention and negative for MNS haemolysis. Access to an unhindered regional transfer system for blood samples and patients in serious cases is necessary for sites without full diagnostic capability. Clear alloantibody typing results are important for preparing exchange transfusions and/or red blood cell (RBC) transfusions in serious cases and follow-up.

Author Contributions

Lin Mingchun: acquisition and analysis of data, drafting the article.

Liu Meixiu: data curation and analysis.

Zhang Shulian: data curation, interpretation of data.

Chen Chao: final approval of the version.

Wang Jin: design of the study, revising and final approval of the draft.

All authors contributed to the article have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

Disclosure

All the authors have disclosed no conflicts of interest.

References

1. de Haas M, Thurik FF, Koelewijn JM, van der Schoot CE. Haemolytic disease of the fetus and newborn. *Vox Sang*. 2015;109 (2):99–113. doi:10.1111/vox.12265
2. Ma X, Chen FZ, Hong Q. [Analysis of screening results in 501 newborns with hemolytic disease]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2019;27(1):192–196. Chinese. doi:10.7534/j.issn.1009-2137.2019.01.031
3. Doyle B, Quigley J, Lambert M, et al. A correlation between severe haemolytic disease of the fetus and newborn and maternal ABO blood group. *Transfus Med*. 2014;24(4):239–243. doi:10.1111/tme.12132
4. Herschel M, Karrison T, Wen M, Caldarelli L, Baron B. Isoimmunization is unlikely to be the cause of hemolysis in ABO-incompatible but direct antiglobulin test-negative neonates. *Pediatrics*. 2002;110(1 Pt 1):127–130.
5. Rath ME, Smits-Wintjens VE, Lindenburg IT, et al. Postnatal outcome in neonates with severe Rhesus c compared to rhesus D hemolytic disease. *Transfusion*. 2013;53(7):1580–1585. doi:10.1111/j.1537-2995.2012.03937.x
6. Tewari VV, Kumar A, Singhal A, et al. Evaluation of Rh-Hemolytic disease in neonates and management with early intensive phototherapy in the neonatal intensive care unit. *J Trop Pediatr*. 2020;66 (1):75–84. doi:10.1093/tropej/fmz033
7. Altunyurt S, Okyay E, Saatli B, Canbahishov T, Demir N, Ozkan H. Neonatal outcome of fetuses receiving intrauterine transfusion for severe hydrops complicated by Rhesus hemolytic disease. *Int J Gynaecol Obstet*. 2012;117(2):153–156. doi:10.1016/j.ijgo.2011.12.013
8. Koelewijn JM, Vrijkotte TG, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. *Transfusion*. 2008;48 (5):941–952.
9. Katsuragi S, Ohto H, Yoshida A, et al. Anemic disease of the newborn with little increase in hemolysis and erythropoiesis due to maternal anti-Jr(a): a Case Study and Review of the Literature. *TRANSFUS MED REV*. 2019;33(3):183–188. doi:10.1016/j.tmrv.2019.03.002
10. Gu S, Wang HX, Yang CY, et al. [Clinical analysis of seven cases of rare hemolytic disease of the newborn]. *Zhonghua Er Ke Za Zhi*. 2018;56(5):369–372. Chinese. doi:10.3760/cma.j.issn.0578-1310.2018.05.012
11. Wei CT, Al-Hassan FM, Naim N, Knight A, Joshi SR. Prevalence of Diego blood group antigen and the antibody in three ethnic population groups in Klang valley of Malaysia. *Asian J Transfus Sci*. 2013;7 (1):26–28. doi:10.4103/0973-6247.106725
12. Bakhtary S, Gikas A, Glader B, Andrews J. Anti-Mur as the most likely cause of mild hemolytic disease of the newborn. *Transfusion*. 2016;56(5):1182–1184. doi:10.1111/trf.13552
13. Weiner CP, Widness JA. Decreased fetal erythropoiesis and hemolysis in Kell hemolytic anemia. *Am J Obstet Gynecol*. 1996;174 (2):547–551. doi:10.1016/S0002-9378(96)70425-8

14. Management of hyperbilirubinemia in the newborn infant. 35 or more weeks of gestation. *Pediatrics*. 2004;114(1):297–316. doi:10.1542/peds.114.1.297

15. Mari G, Deter RL, Carpenter RL, et al. Noninvasive diagnosis by Doppler ultrasonography of fetal anemia due to maternal red-cell alloimmunization. Collaborative group for Doppler assessment of the blood velocity in anemic fetuses. *N Engl J Med*. 2000;342(1):9–14. doi:10.1056/NEJM200001063420102

16. Markham KB, Rossi KQ, Nagaraja HN, O'Shaughnessy RW. Hemolytic disease of the fetus and newborn due to multiple maternal antibodies. *Am J Obstet Gynecol*. 2015;213(1):61–68. doi:10.1016/j.ajog.2015.01.049

17. Yang ZYST. The value of antibody screening test during antenatal clinic to prevent HDN. Attachment of one case with anti-M neonatal hemolysis disease. *Chin J Immunol*. 2017;2(33):278–279.

18. Christensen RD, Baer VL, MacQueen BC, O'Brien EA, Ilstrup SJ. ABO hemolytic disease of the fetus and newborn: thirteen years of data after implementing a universal bilirubin screening and management program. *J PERINATOL*. 2018;38(5):517–525. doi:10.1038/s41372-018-0048-4

19. Louis D, More K, Oberoi S, Shah PS. Intravenous immunoglobulin in isoimmune haemolytic disease of newborn: an updated systematic review and meta-analysis. *Arch Dis Child Fetal Neonatal Ed*. 2014;99(4):F325–31. doi:10.1136/archdischild-2013-304878

20. Christensen RD, Yaish HM. Hemolysis in preterm neonates. *Clin Perinatol*. 2016;43(2):233–240. doi:10.1016/j.clp.2016.01.002

21. Moise KJ. Non-anti-D antibodies in red-cell alloimmunization. *Eur J Obstet Gynecol Reprod Biol*. 2000;92(1):75–81. doi:10.1016/S0301-2115(00)00428-0

22. Lee CK, Ma ES, Tang M, Lam CC, Lin CK, Chan LC. Prevalence and specificity of clinically significant red cell alloantibodies in Chinese women during pregnancy–a review of cases from 1997 to 2001. *Transfus Med*. 2003;13(4):227–231. doi:10.1046/j.1365-3148.2003.00445.x

23. Castilho L. An update on the MNS blood group system. *Immunohematology*. 2019;35(2):61–62. doi:10.21307/immunohematology-2020-014

24. Slootweg YM, Walg C, Koelewijn JM, Van Kamp IL, De Haas M. Knowledge, attitude and practices of obstetric care providers towards maternal red-blood-cell immunization during pregnancy. *Vox Sang*. 2020;115(3):211–220. doi:10.1111/vox.12883

25. Reid ME. MNS blood group system: a review. *Immunohematology*. 2009;25(3):95–101.

26. De Young-owens A, Kennedy M, Rose RL, Boyle J, O'Shaughnessy R. Anti-M isoimmunization: management and outcome at the Ohio State University from 1969 to 1995. *Obstet Gynecol*. 1997;90(6):962–966. doi:10.1016/S0029-7844(97)00476-6

27. Hassan MN, Mohd NN, Johan NS, Sukri SA, Mustafa R, Luc AH. Hemolytic disease of fetus and newborn due to maternal red blood cell alloantibodies in the Malay population. *Asian J Transfus Sci*. 2014;8(2):113–117. doi:10.4103/0973-6247.137449

28. Li S, Mo C, Huang L, et al. Hemolytic disease of the fetus and newborn due to alloanti-M: three Chinese case reports and a review of the literature. *Transfusion*. 2019;59(1):385–395. doi:10.1111/trf.15054

29. Stetson B, Scrape S, Markham KB. Anti-M alloimmunization: management and outcome at a single institution. *AJP Rep*. 2017;7(4):e205–10. doi:10.1055/s-0037-1607028

30. Mohd NH, Noor HM, Shafini MY, Noor SAA, Rapiaah M, Wan ZA. Anti-M induced severe haemolytic disease of foetus and newborn in a Malay woman with recurrent pregnancy loss. *Malays J Pathol*. 2017;39(1):73–76.

31. Yasuda H, Ohto H, Nollet KE, et al. Hemolytic disease of the fetus and newborn with late-onset anemia due to anti-M: a case report and review of the Japanese literature. *Transfus Med Rev*. 2014;28(1):1–6. doi:10.1016/j.tmrv.2013.10.002

32. Gao XY, Huang H, Li LD. [Hemolytic disease of neonates due to anti-M: report of one case and review of reports of 21 cases]. *Zhonghua Er Ke Za Zhi*. 2009;47(9):648–652. Chinese.

33. Arora S, Doda V, Maria A, Kotwal U, Goyal S. Maternal anti-M induced hemolytic disease of newborn followed by prolonged anemia in newborn twins. *Asian J Transfus Sci*. 2015;9(1):98–101. doi:10.4103/0973-6247.150968

34. Wikman A, Edner A, Gryfelt G, Jonsson B, Henter JI. Fetal hemolytic anemia and intrauterine death caused by anti-M immunization. *Transfusion*. 2007;47(5):911–917. doi:10.1111/j.1537-2995.2007.01209.x

35. Lenkiewicz B, Zupanska B. The first example of anti-Diego(b) found in a Polish woman with the Di(a+b-) phenotype and haemolytic disease of the newborn not requiring treatment. *Transfus Med*. 2003;13(3):161–163. doi:10.1046/j.1365-3148.2003.00437.x

36. Oh EJ, Jekarl DW, Jang HS, et al. Severe hemolytic disease of the newborn due to anti-Di b treated with phototherapy and intravenous immunoglobulin. *Ann Clin Lab Sci*. 2008;38(1):80–82.

37. Hamilton JR. Kidd blood group system: a review. *Immunohematology*. 2015;31(1):29–35.

38. Lawicki S, Covin RB, Powers AA. The Kidd (JK) Blood Group System. *Transfus Med Rev*. 2017;31(3):165–172. doi:10.1016/j.tmrv.2016.10.003

Can Hematological Ratios Predict Outcome of COVID-19 Patients? A Multicentric Study

Mai M Aly, Taghreed S Meshref, Marwa A Abdelhameid, Shimaa A Ahmed, Asmaa S Shaltout, Alaa Eldin Abdel-Moniem & Dina A Hamad

Published online: 29 Jun 2021.

Submit your article to this journal

Article views: 228

View related articles

View Crossmark data

Citing articles: 23 View citing articles

ORIGINAL RESEARCH

Can Hematological Ratios Predict Outcome of COVID-19 Patients? A Multicentric Study

Mai M Aly [iD] [1,*]
Taghreed S Meshref [2,*]
Marwa A Abdelhameid [3]
Shimaa A Ahmed [4]
Asmaa S Shaltout [5]
Alaa Eldin Abdel-Moniem [iD] [2]
Dina A Hamad [2]

[1]Clinical Hematology Unit, Department of Internal Medicine, Faculty of Medicine, Assiut University, Assiut, Egypt; [2]Critical Care Unit, Department of Internal Medicine, Faculty of Medicine, Assiut University, Assiut, Egypt; [3]Department of Internal Medicine, Faculty of Medicine, Aswan University, Aswan, Egypt; [4]Department of Internal Medicine, Faculty of Medicine, Qena University, Qena, Egypt; [5]Department of Medical Microbiology & Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt

*These authors contributed equally to this work

Correspondence: Mai M Aly
Clinical Hematology Unit, Department of Internal Medicine, Faculty of Medicine, Assiut University, Assiut, Egypt
Tel +201223971678
Email mai_heamatology@aun.edu.eg

Introduction: Coronaviruses belong to a large family that leads to respiratory infection of various severity. Hematological ratios are indicators of inflammatory response widely used in viral pneumonia with affordability in developing countries.

Purpose: Study the role of the neutrophil lymphocyte ratio (NLR), derived NLR ratio (d-NLR), platelet lymphocyte ratio (PLR), and lymphocyte monocyte ratio (LMR) in predicting the outcome of COVID-19 Egyptian patients.

Methods: A retrospective study on 496 COVID-19 Egyptian patients, managed in four tertiary centers, grouped into non-severe, severe, and critical. Patients' laboratory assessment including total leucocyte count (TLC), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute monocyte count (AMC), NLR, d-NLR, LMR and, PLR were reported as well as C reactive protein (CRP), D-dimer and serum ferritin.

Results: TLC, ANC, AMC, NLR, d-NLR and, PLR were highest in the critical group ($p<0.001$ for all except AMC $p=0.033$), while this group had the least ALC and LMR ($p=0.049$ and <0.001, respectively). Higher CRP and d-dimer levels were reported in the critical group ($p<0.001$). At the same time, higher ferritin was found in the severe group more than the critical and non-severe groups ($p<0.001$, $p=0.005$, respectively). We calculated the optimal cut-off values of the hematological ratio; NLR (3.5), d-NLR (2.86), PLR (192), and LMR (3). D-NLR had the highest specificity (89.19%), while NLR had the highest sensitivity (71.38%). By univariate logistic regression, age, DM, HTN, cardiovascular diseases, COPD, NLR, d-NLR, LMR and PLR, CRP, steroid, oxygen aids, and mechanical ventilation were associated with the severity of COVID-19. Still, only age, NLR, CRP, and oxygen aid were independent predictors in multivariate logistic regression.

Conclusion: NLR is a predictor for severity in COVID-19. LMR, d-NLR, and PLR may assist in risk stratification.

Keywords: COVID-19, NLR, d-NLR, LMR, PLR, Egypt

Introduction

COVID-19 virus, a single-chain enveloped RNA virus,[1] causes multisystemic infections in animals and humans, mainly leads to respiratory tract infection.[2] Classically patients exhibit mild symptoms such as fever, sore throat, and upper respiratory tract infections.[3] Severe respiratory tract infection that contributes to the syndrome of adult respiratory distress, multiple organ failure, and even death is reported, especially in the elderly and in patients with comorbidities.[4,5] Declaring COVID-19 as a pandemic, serious morbidity, and mortality; created an urgency to study diagnostics, treatment, and prognostic markers.[4]

Inflammatory biomarkers representing the immune status are possible predictors of prognosis for COVID-19.[5] Hematological ratio such as neutrophil to lymphocyte

505

ratio (NLR), derived NLR ratio (d-NLR) (calculated by neutrophil count divided by the result of WBC count minus neutrophil count), platelet to lymphocyte ratio (PLR), and lymphocyte to monocyte ratio (LMR) are systemic inflammatory markers that have already been extensively investigated as a potential predictor of viral pneumonia.[6] Such ratios are useful, inexpensive prognostic indicators that can be widely tested in developing countries with respectable significance in viral pneumonia, including COVID-19.[6–8]

The diagnostic use of NLR in bacteria[9] and viral pneumonia[6] as well as in COVID-19 positive patients has been documented; the NLR is substantially greater in COVID-19 cases vs controls.[8] NLR can be easily used as a rapid, inexpensive, available prognostic indicator.[6,8] Both PLR and LMR are considered indicators of the systematic inflammatory response and useful predictors for the prognosis in viral pneumonia.[10] More studies are demanded to decide the applicable threshold of d-NLR, PLR, and LMR and decide their predictive value.

This retrospective, multi-centric study was conducted to investigate the complete blood count parameters (NLR, d-NLR, PLR, and LMR) as inflammatory biomarkers and prognostic indicators in 496 COVID-19 positive cases.

Methods
Study Design
The study was a retrospective study, including 496 COVID 19 patients, diagnosed based on guidance from WHO with Positive SARS-CoV-2 RNA identification by real-time PCR methods in throat swab specimens.[11] The study included patients admitted to four major tertiary centers in Upper Egypt (Assiut University Hospital, El Rajhi Hospital, Aswan University Hospital, South Valley University Hospital) through June 2020.

Data of the Patients
Data of the patients were gathered from the hospital reports following the authorization of the local research ethics committee (IRB number: 17300513) according to the Declaration of Helsinki. It included: age, sex, history of smoking, diabetes, hypertension, chronic chest disease and cardiovascular disease, duration of hospital admission and outcome, treatment included steroid and oxygen supply either by low flow oxygen supplementation (nasal cannula, facial masks, or non-rebreather facial masks) or high flow oxygen supplementation (high flow nasal cannula, continuous positive ventilation pressure (CPAP) or mechanical ventilation).

Investigations Included
Complete blood count; total leukocyte count (TLC), red blood cells, hemoglobin level, absolute lymphocyte count (ALC), absolute monocyte count (AMC), absolute neutrophil count (ANC), NLR (ANC/ALC), derived NLR ratio (d-NLR; calculated by ANC divided by the result of TLC count minus ANC), PLR (Platelet count/ALC) and LMR (ALC/AMC). Inflammatory markers; CRP (C-reactive protein) and serum ferritin and coagulation factor; D-dimer. All collected laboratory results are at the peak of the disease. Definition as the beginning of the symptoms that meet the categories of severity of illness as determined by WHO.[12,13]

Computed tomography (CT) of the chest of the patients was classified into specific findings suggestive to COVID19 infection as bilateral or unilateral multifocal ground-glass opacities that classically predominate in the peripheral, posterior, and basal part of the lungs or other less specific findings. CORADS classification was scored from very low or CO-RADS 1 to very high or CO-RADS 6 based on the CT findings.

Patients were classified into the following categories of severity of illness:[14]

1) Critical: patients with any of the following criteria: respiratory failure that requires artificial ventilation, shock, or other organ damage that requires intensive care unit monitoring and treatment.

2) Severe: patients with a ratio of arterial partial oxygen pressure to inspired oxygen fraction (PaO2/FiO2) <300 mmHg, respiratory rate >30 breaths per minute, lung infiltrates >50%, or patients with SpO2 <94% in room air.

3) Non-severe: patients who did not meet the criteria of critical or severe illness.

All patients were treated according to the World Health Organization's provisional guidelines (WHO). All patients received steroids, and none of the patients received immunomodulatory drugs.[2,11]

Statistical Analysis
SPSS version 25.0 was used for data management and data analysis. Quantitative variables were first subjected to the normality test (Kolmogorov v Smirnov). Continuous variables were presented median (interquartile range), and the Kruskal–Wallis test assessed their differences. Categorical variables were described as numbers (percentage) and were compared by the chi-square test and fisher's exact test. The

optimal cut-off values of the continuous NLR, d-NLR, PLR, and LMR were calculated using the receiver operating curve (ROC) analysis. The Kaplan–Meier survival analysis and COX regression analysis were used to investigate the independent adverse factors that might check patients' recovery and discharge with COVID-19. The demographics and laboratory data with significant differences between the three groups were assessed by univariate and multivariate logistic regression analysis to discover the independent early predictors and risk factors associated with the disease severity of COVID-19. A two-sided P<0.05 was considered statistically significant.

Results

Characteristics of the Studied Population

The studied population were divided into 3 groups, non-severe (n=185; 37.3%), severe (n= 165; 33.3%) and, critical (n=146; 29.4%); older patients were found in the critical group (median age =61) then the severe group (median age=55) while, the youngest were among the non-severe group (median age =33.5) with a significant difference between them (p <0.001). Most of the patients were males in all groups.

In comparing associated co-morbidities, the critical group was more associated with diabetes mellitus (DM), hypertension (HTN), cardiovascular diseases and, chronic obstructive pulmonary diseases (COPD) versus the non-severe and severe groups (p <0.001).

Regarding hematological parameters, the hemoglobin level was least in the severe group [median (IQR); 12 (10–13)], and highest in the non-severe group [Median (IQR);12 (11–14) p =0.04]. Surprisingly, platelets were lowest in the non-severe group, with a significant difference between the three groups (p<0.001). TLC, ANC, AMC, NLR, d-NLR and, PLR were highest in the critical group, followed by the severe group and least in the non-severe group with a significant difference (p<0.001 for all except AMC p-value=0.033). ALC and LMR were inversely proportionate to severity (p = 0.049 and <0.001, respectively).

Higher CRP and d-dimer levels were reported in the critical group (p <0.001). At the same time, higher ferritin was found in the severe group more than the critical and non-severe groups (p <0.001, p=0.005 respectively).

The critical group patients had multi-slice computed tomography (MSCT) imaging, which showed more extensive lesions (either bilateral lesions or multiple unilateral lesions) than in the non-severe and severe groups (p <0.001). We used CORAD system to assess the probability of COVID-19 infection in our studied cohort; most of them were CORAD 6. CORAD 1, 2 were reported in the non-severe group, while only three patients were in the severe group.

In comparing the oxygen supplementation given to the patients, most of the severe group patients (83%) received oxygen aid, and only 10% were mechanically ventilated. In comparison, 92.5% received oxygen aids in the critical group, and 72.4% required mechanical ventilation.

Most of the non-severe group recovered (93%) and discharged home. The death was more in the critical (65.1%) and severe (15.2%) groups. Demographic and baseline data of the studied cohort are shown in (Table 1).

ROC Curve to Detect Optimal Cut-off Values of the Hematological Ratios

We analyzed the optimal cut-off values of NRL, d-NLR, LMR, and PLR calculated by the ROC analysis and presented in (Figure 1). Areas under the curve (AUC) of NLR, d-NLR, PLR and LMR were 0.838, 0.817, 0.643, and 0.285, respectively. LMR could not be used as a potential diagnostic biomarker for subsequent analysis because its AUC was less than 0.50. The optimal cut-off values were NLR (3.5), d-NLR (2.86), and PLR (192). D-NLR had the highest specificity (89.19%), followed by NLR (87%) then PLR (77.62%). The most heightened sensitivity was in favor of NLR (71.38%), then the d-NLR (67.2%), and the PLR (50%; Table 2).

Survival Analysis with Kaplan–Meier Curves

The estimated mean time until death was 21.3 days for non-severe vs 19.1 days for severe vs 16.2 days for critical patients (p<0.0001). The survival probability is lowest in critical patients at all-time points, so they are less likely to survive (Figure 2A).

We estimated the mean survival time according to NLR (estimated mean time until death is 20.5 days for NLR <3.5 and 17.7 days for NLR >3.5; p<0.001), d-NLR (The estimated mean time until death is 20.2 days for d-NLR <2.5 and 17.5 days for d-NLR >2.5; p<0.001), and PLR (the estimated mean time until death is 19.3 days for PLR <192 and 18.8 days for PLR>192; p=0.04). So, it is obvious that those patients with NLR >3.5, d-NLR >2.5, or PLR> 192 are less likely to survive (Figure 2B–D).

Table I Characteristics of the Studied Population

Variant	Group			P-value
	Non-Severe n=185 (37.3%)	**Severe n=165 (33.3%)**	**Critical n=146 (29.4%)**	
Age/years	33.5 (26–54)	55(47–62)	61(50–69)	**<0.001***
Sex/males n (%)	92 (49.5)	94 (57)	87 (60)	0.167**
Smoking n (%)	41 (22%)	36 (21.8%)	29 (20)	0.867**
Co-morbidities				
• **DM n (%)**	16 (8.6)	39 (23.6)	42 (29)	**<0.001****
• **HTN n (%)**	19 (10.3)	40 (24.2)	73 (50.3)	**<0.001****
• **Cardiovascular diseases n (%)**	10 (5.4)	31 (18.8)	54 (37)	**<0.001****
• **COPD n (%)**	4 (2.2)	15 (9.1)	20 (13.7)	**<0.001*****
Laboratory data				
CBC median (range)				
HB (g/dL)	12 (11–14)	12 (10–13)	12 (11–13)	**0.043***
Platelets (×10^9/L)	204 (136–264)	234 (162–325)	231 (167–328)	**<0.001***
TLC (×10^9/L)	4 (3.1–6)	8 (5–12)	11 (9–15)	**<0.001***
ANC (×10^9/L)	2 (1.4–4)	6 (3–9.5)	9 (7–12)	**<0.001***
ALC (×10^9/L)	1.53 (1–2)	1 (1–2)	1 (1–2)	**0.049***
AMC (×10^9/L)	0.2 (0–1)	1 (0–1)	1 (0–1)	**0.022***
NLR	1.55 (1–3)	4 (2–8)	8 (5–11)	**<0.001***
LMR	4 (3–6)	3 (2–5)	2 (1–3)	**<0.001***
d-NLR	1 (1–2)	3 (1–5)	5 (3–7)	**<0.001***
PLR	131 (91–188)	172 (103–286)	203 (107–324)	**0.001***
Inflammatory markers				
CRP (mg/l)	20 (4.5–54)	54 (30.1–82)	79 (45–85)	**<0.001***
Ferritin (mcg/mL)	189 (68–408)	191(98–450)	145 (40.5–202)	**0.001**
D- dimer (mcg/mL)	0.81 (0.26–9.2)	3 (0.69–17)	8 (1.5–20)	**<0.001**
MSCT chest				
• Bilateral affection or multiple unilateral affection n (%)	172(93)	164(99.4)	146(100)	**<0.001*****
Oxygen support				
• Any oxygen aids other than mechanical ventilation n (%)	–	137(83.0)	135(92.5)	**<0.001****
• Mechanical ventilation n (%)	–	17(10)	105(72.4)	**<0.001****
Outcome				
• Recovery n (%)	172(93)	140(84.8)	51(34.9)	**<0.001****
• Death n (%)	13(7)	25(15.2)	95(65.1)	**<0.001****

Notes: *Kruskal–Wallis test. **Chi square test. ***Fisher's Exact test. Bold is significant p value; P value is significant if <0.05.
Abbreviations: ALC, absolute lymphocyte count; AMC, absolute monocyte count; ANC, absolute neutrophil count; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; DM, diabetes mellitus; d-NLR, derived neutrophil to lymphocyte ratio; HB, hemoglobin; HTN, hypertension; LMR, lymphocyte to monocyte ratio; MSCT, multi-slice computed tomography; n, number; NLR, neutrophil to lymphocyte ratio; PLR, platelets to lymphocyte ratio; TLC, total leukocyte count.
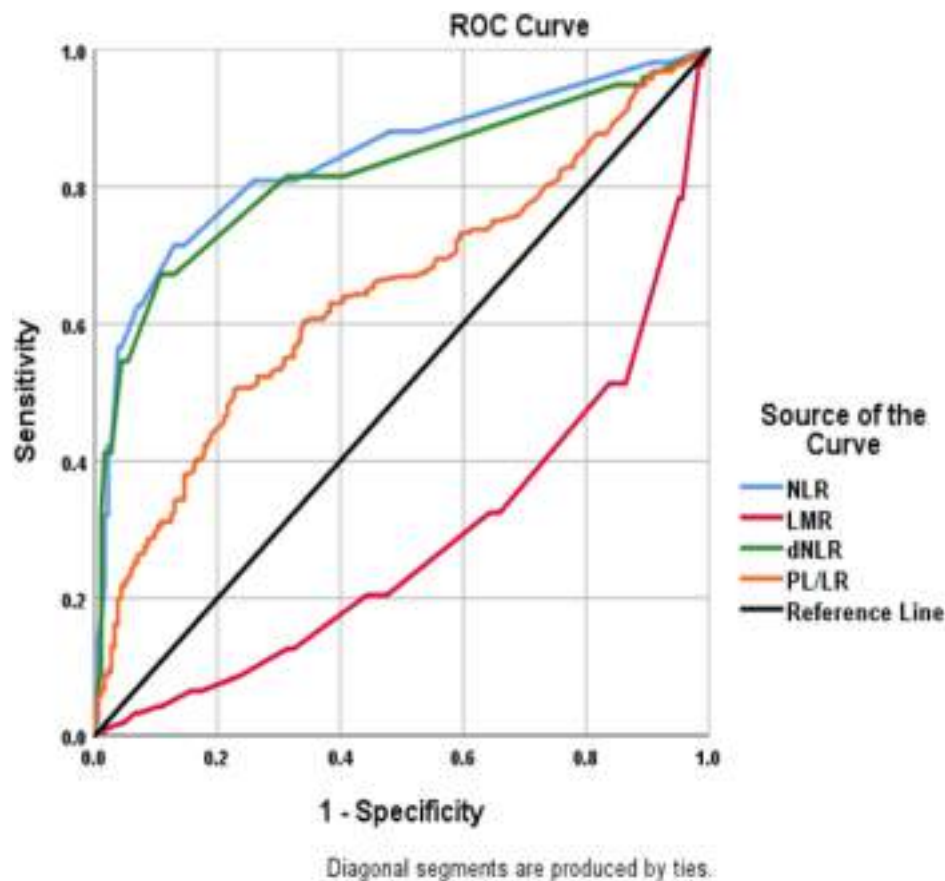
Figure 1 ROC curve was used to study the optimal cut off values of different hematological ratio; NLR (3.5), d-NLR (2.86), and PLR (192). D-NLR specificity (89.19%) NLR (87%) and PL/LR (77.62%). The sensitivity of NLR (71.38%), d-NLR (67.2%) and the PL/LR (50%).
Abbreviations: D-NLR, derived neutrophil lymphocyte ratio; LMR, lymphocyte monocyte ratio; NLR, neutrophil lymphocyte ratio; PLR, platelets lymphocyte ratio.

Identification of Possible Predictors of Severity in COVID-19 Patients

To determine the effect of hematological ratios on the prognosis of COVID-19 patients, we also performed Kaplan–Meier survival analysis and COX regression analysis to explore the possible independent predictors for severe COVID-19. Further univariate and multivariate analysis of

COX regression showed that the estimated NLR (HR 1.056, 95% CI 1.0.37–1.075), d-NLR (HR 1.075, 95% CI 1.045–1.104), and PLR (HR 1.001, 95% CI 1–1.002) identified by univariate Cox regression but in multivariate Cox regression, only elevated NLR (HR 1.046, 95% CI 1.024–1.069) was the independent adverse factor affecting recovery and discharge of patients with COVID-19.

Table 2 ROC Curve Analysis of All Studied Population to Detect Optimal Cut-off Values, Sensitivity and Specificity of the Hematological Ratios

	AUC	*P* value	95% CI		Cut off Point	Sensitivity	Specificity	+LR	−LR	+PV	−PV	Youden's Index
			Lower	Upper								
NLR	0.838	<0.001	0.803	0.874	>3.5	71.38%	87.03%	5.50	0.33	90.2	64.4	0.5841
d-NLR	0.817	<0.001	0.780	0.854	>2.86	67.20%	89.19%	6.22	0.37	91.3	61.8	0.5639
PLR	0.643	<0.001	0.595	0.692	>192	50.00%	77.62%	2.20	0.65	78.7	48.0	0.2762
LMR	0.285	<0.001	0.239	0.331	≤3	67.64%	65.95%	1.99	0.49	76.8	55.0	0.3395

Notes: The test variable(s): NLR, d-NLR, PLR and LMR has at least one tie between the positive actual state group and the negative actual state group. *P value* is significant if *<0.05*.
Abbreviations: AUC, area under curve; CI, confidence interval; d-NLR, derived neutrophil lymphocyte ratio; LMR, lymphocyte monocyte ratio; LR, Likelihood ratio; NLR, neutrophil lymphocyte ratio; PLR, platelet lymphocyte ratio; PV, predictive value; ROC, receiver operating characteristics.
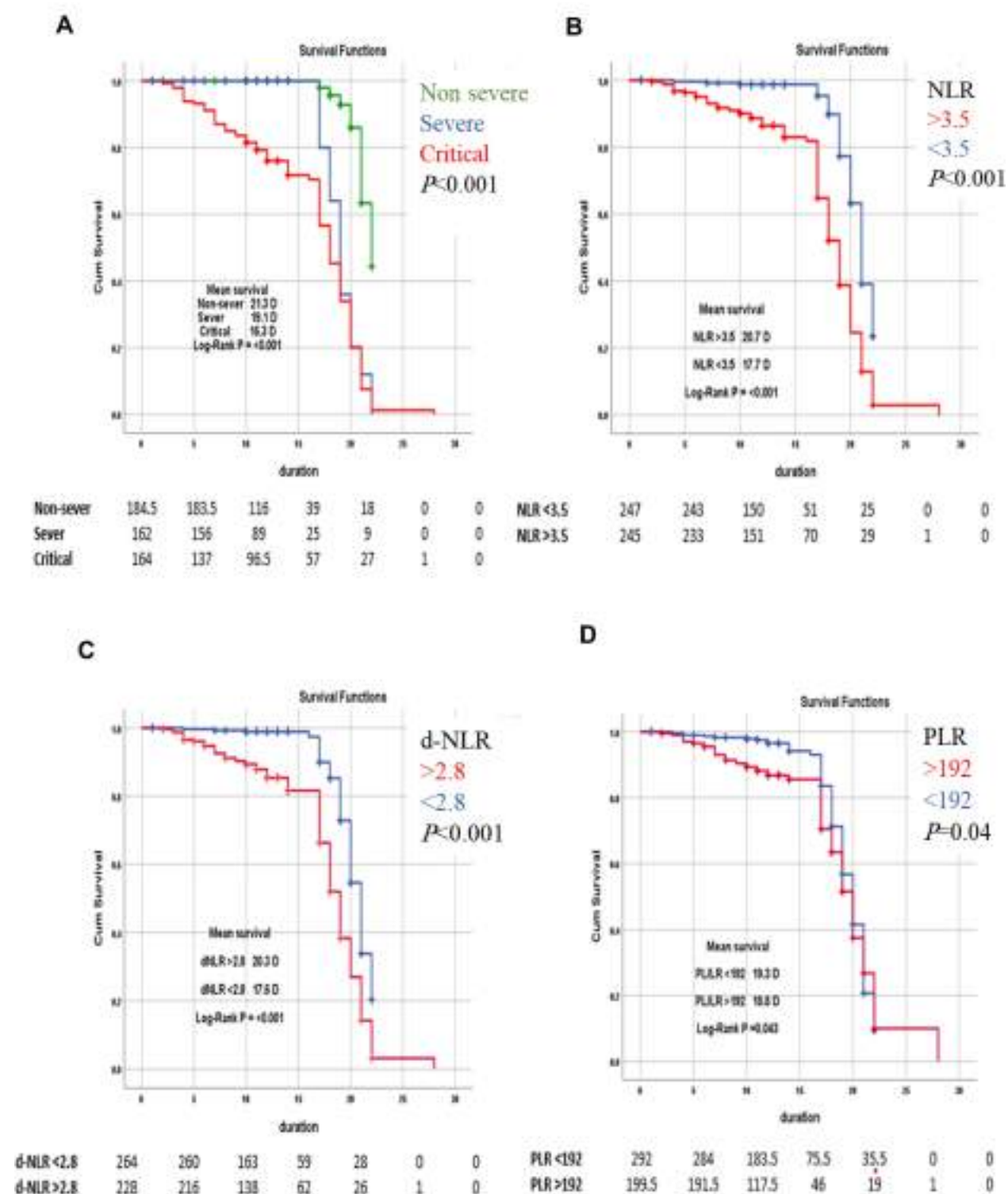
Figure 2 Survival analysis using Kaplan–Meier curve (**A**) Kaplan–Meier curves showing survival in non-severe, severe and critical group. (**B**) Kaplan–Meier curve according to NLR (Neutrophil lymphocyte ratio) of COVID-19 patients. (**C**) Kaplan–Meier curve according to d-NLR (derived neutrophil lymphocyte ratio) of COVID-19 patients. (**D**) Kaplan–Meier curve according to PLR (platelets lymphocyte ratio) of COVID-19 patients. Numbers below each cure show number at risk.

Table 3 Identification of Possible Predictors of Severity in COVID-19 Patients

	Univariable Odds Ratio	95% C. I		P value	Multivariable Odds Ratio	95% C.I		P value
		Lower	Upper			Lower	Upper	
Age	1.069	1.055	1.084	**<0.001**	1.056	1.009	1.098	**0.018**
DM (yes/no)	3.698	2.087	6.551	**<0.001**	1.852	0.790	4.338	0.156
HTN (yes/no)	4.956	2.923	8.404	**<0.001**	0.823	0.345	1.963	0.661
Cardiovascular diseases (yes/no)	6.582	3.320	13.048	**<0.001**	1.796	0.618	5.215	0.282
COPD (yes/ no)	5.759	2.013	16.480	**0.001**	4.058	0.921	17.878	0.064
NLR	1.602	1.450	1.771	**<0.001**	1.453	1.123	1.765	**0.003**
d-NLR	1.913	1.654	2.212	**<0.001**	1.002	0.827	1.204	0.983
PLR	1.004	1.002	1.006	**<0.001**	1.000	0.998	1.003	0.681
LMR	0.784	0.726	0.847	**<0.001**	1.020	0.900	1.156	0.757
CRP	1.013	1.008	1.018	**<0.001**	1.006	1.003	1.009	**0.043**
Ferritin	1.001	1.000	1.002	0.154				
D- dimer	23.362	12.617	43.259	**<0.001**	46.957	15.689	140.547	**<0.001**
Steroid (yes/no)	3.832	1.532	9.583	**0.004**	1.160	0.315	4.270	0.823
Oxygen aids (yes/no)	40.813	24.061	69.228	**<0.001**	22.031	11.733	38.621	**<0.001**
Mechanical (yes/no)	12.452	5.910	26.238	**<0.001**	1.852	0.736	4.662	0.191

Notes: Bold is significant *p* value; *P* value is significant if <0.05.
Abbreviations: COPD, chronic obstructive pulmonary disease; CI, Confidence interval; CRP, C-reactive protein; DM, diabetes mellitus; d-NLR, derived neutrophil to lymphocyte ratio; HTN, hypertension; LMR, lymphocyte to monocyte ratio; NLR, neutrophil to lymphocyte ratio; PLR, platelets to lymphocyte ratio.

The univariate logistic regression analysis showed age, DM, HTN, cardiovascular diseases, COPD, NLR, d-NLR, LMR, PLR, CRP, d-dimer, steroid, oxygen aids, and mechanical ventilation were associated with increased severity of COVID-19. Subsequently, all the above univariate analysis parameters with statistical significance were integrated for in-depth analysis into the multivariate logistic regression model. In the multivariate logistic regression model, considering the likelihood of overfitting, we assumed a stepwise forward method for logistic regression analysis to decrease the number of independent variables entering the model to reduce the probability of overfitting the model.

The results showed that the early independent predictors for severe COVID-19 were age, NLR, CRP, d-dimer, and oxygen aid upon entry, while the role of d-NLR, LMR, and PLR is unclear (Table 3).

Discussion

A cluster of cases of pneumonia of unknown etiology started to emerge in Wuhan, China, at the end of 2019, a striking event that terrifies the whole world. A rapid spread of that infection took place with a relatively low mortality rate.[3] At the end date of the collection of data (2020-12-5), more than one and a - half million deaths were reported to WHO. Mortality rates vary from one region to another; it was less than 2.5% in CDC, China, but in Egypt, the mortality rate was higher, reaching up to 6.7%. Lack of accurate recording and delayed diagnosis may play a significant role in explaining such a high mortality rate. This issue pushed us to investigate possible cheap, rapid, and simple predictors that may force attention to more risky victims.

Not enough data supporting increased liability of COVID-19 infection to patients with chronic diseases but, many previous researchers –in line with our results- confirmed the increased severity in older people, especially those suffering from diabetes mellitus (DM),[15] hypertension (HTN)[3] cardiovascular diseases;[16] also, we reported increased severity in chronic obstructive pulmonary disease (COPD) patients which appears logic as the virus affects mainly the respiratory system and it is well known that serious adverse effects are

associated with respiratory viral infections in those with COPD;[17] chronic pulmonary diseases, however, are under-reported, according to the first epidemiological studies and detected only in 0.3–2.5% without a significant increase in risk as it was expected.[18]

C-reactive protein (CRP), d-dimer and ferritin, are markers that are extensively studied in COVID-19 infected victims;[19–21] CRP levels correlate with the degree of inflammation. Interestingly, it was found to increase parallel to the increase in the diameter of the largest pneumonia lesion in COVID-19 patients;[19] this supports our findings in which CRP is least in the non-severe group. D-dimer >2 was found in an early study to be the only factor associated with mortality in COVID-19 patients;[20] this is also observed in our results, where it was highest in the critical group. Hyperferritinemia in a large meta-analysis found to be associated with severe conditions and adult respiratory distress syndrome (ARDS).[21] However, Wu et al found that ferritin was neither associated with ARDS nor severe cases of COVID-19.[22] Surprisingly, in ours, mean ferritin levels favored the severe group, not the critical one.

The complete blood count is the test used to approach varieties of infections, with the advantages of being available and highly informative. In the Chinese population, leucopenia was reported in COVID-19 patients at the expense of lymphopenia without investigating its relation to disease severity.[23] In our research, critical group patients suffered from the lowest lymphocyte count and the highest leukocyte count mostly due to neutrophilia, which could be explained by secondary bacterial infection occurred in most critical cases; this was also approved previously by a large meta-analysis;[24] Recently, an autopsy report on a 50-year-old COVID-19 patient with ARDS revealed that although the peripheral blood lymphocyte count was dramatically decreased, there was inflammatory lymphocyte infiltration in both lungs and immune hyperactivation.[25] Lymphopenia in COVID-19 patients can be explained by varieties of theories, as the ability of the virus to infect lymphocytes,[26] to destroy lymphatic organs like the thymus and spleen, to disturb the levels of interleukin (IL)-6 and other inflammatory cytokines may result in lymphocyte apoptosis,[27] and the inhibition of lymphocytes proliferation by metabolic molecules as the lactic acid.[28] High monocyte count is another hematological parameter found more in critical population, representing the main generators of inflammation in COVID-19 and predisposing to serious outcomes mostly due to "a dysregulation syndrome";[29] in contrary, the difference in monocyte count between healthy and COVID-19 patients was not significant in another study.[30]

In the present study, we concentrated on the value of the hematological ratios as NLR, d-NLR, LMR and, PLR; our results supported a previous study that indicated that elevated NLR predicts poor outcome in COVID-19 patients so that those with high NLR attract more attention than those with low NLR.[6,7,30] It is well established that NLR tends to increase with severe infection or systemic inflammation in line with the magnitude of clinical status and outcome.[31] NLR, as well as, age were independent factors for poor clinical outcome of COVID-19 in Chinese cohort. NLR was one of the earliest studied markers that were correlated with severity.[4,32,33] The following are potential explanations for these findings in COVID-19 patients; on the one hand, neutrophil releases large amounts of reactive oxygen species stimulates the antibody-dependent cell-mediated cell (ADCC) that can directly kill the virus, expose virus antigen, and activate cell-specific and humoral immunities.[34] Besides, neutrophil produces numerous cytokines, such as circulating vascular endothelial growth factor (VEGF), which is profoundly expressed in COVID-19 patients.[35] Moreover, several inflammatory factors, including interleukin-6, tumor necrosis factor-alpha, granulocyte colony-stimulating factor, and interferon-gamma factor, can trigger neutrophils.[36–38] On the other hand, viral infections mostly affect lymphocytes,[39] as systematic inflammation causes marked suppression of CD4+ T lymphocytes and increases CD8+ suppressor T lymphocyte.[40] Thus, the critical group had higher NLR in comparison to severe and non-severe groups.

We studied a unique ratio derived from the NLR and named the d-NLR; this ratio was previously correlated with severity in chronic and neoplastic conditions.[41] According to our knowledge, few studies had investigated its role in Covid-19 cases.[4] Surprisingly, d-NLR carried the highest specificity among all studied factors with an optimal cut-off value of 2.86.

Another attractive ratio is LMR, which was signed in its lower values in critical patients of COVID-19. This finding was previously detected but concerning community-acquired pneumonia without identification of the causative pathogen.[32,42,43] In another study, 190 COVID-19 patients were enrolled, and LMR was significantly lower than the healthy group, with a significant negative correlation with body temperature.[30]

PLR is a new inflammatory index of interest, which is more valuable than the simple platelet or lymphocyte count. It mostly reflects changes in platelets, which are

considered to have a unique role in inflammation and immune response,[32,44] and higher in the critical group in our study. Previous investigators proved that peak PLR is more elevated in severe populations than in non-severe.[45] In addition to a significant correlation with a poor prognosis on COVID-19,[45] while others failed to detect any value to such index in COVID-19 patients.[46]

The optimal thresholds for NLR, d-NLR, PLR, and LMR were estimated using the ROC curve. The NLR yielded the highest AUC value than the other ratios, and its optimal cut-off value was 3.5, with 87.03% specificity and 71.38% sensitivity. In line with ours, a previous study showed that NLR had the highest AUC value with a relative cut-off value (3.3) with the highest specificity and sensitivity (63.6% and 88%, respectively).[4] In a comparable study, the monocyte to lymphocyte ratio (MLR) had the highest AUC value with an optimal cut-off value of 0.23, with 90.00% specificity and 75.79% sensitivity (9). Some studies have shown that a surrogate predictor for influenza A is an LMR value <2.[47]

To predict severity in COVID-19 patients, regression analysis found that age, NLR, CRP, d-dimer, and oxygen aid on admission were reasonable predictors. In agreement with ours, previous research confirmed the prediction role of NLR in COVID-19 patients.[4] Another one found that PLR value was an independent determining factor in severe patients.[45]

There are some limitations to this study. First, the research was retrospective. The data were recruited from hospital medical records after a set of criteria were met, which carry the risk of selection bias. Second, because of data limitation, we only used one measure in time rather than a longitudinal measure. Third, despite trials to exclude confounding factors, some confounders might affect the value of the hematological ratios.

In conclusion, NLR is a predictor for severity in COVID-19. LMR, d-NLR, and PLR may assist in risk stratification. According to our observations in this study, NLR can improve risk stratification of COVID-19 severity, LMR, d-NLR, and PLR can be quick, cost-effective, and interesting potential markers.

Ethical Approval

Authorization of the local research ethics committee of Assiut University.

Consent to Participate

Authors consented to participate in this research article.

Encrypted patients' data was collected from hospital reports without a written consent; the reason for the written consent wavier was that the research presents no risk of harm to participants and involves no procedure for which written consent is normally required outside of the research context (retrospective survey) as well as participant verbally approved documentation and reporting of their medical and laboratory information. Approval of the local research ethics committee (IRB number: 17300513) according to the Declaration of Helsinki was provided. Patient data confidentiality was maintained.

Consent for Publication

Authors consented for publication.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

References

1. Zhou Y, Yang Y, Huang J, Jiang S, Du L. Advances in MERS-CoV vaccines and therapeutics based on the receptor-binding domain. *Viruses*. 2019;11(1):60. doi:10.3390/v11010060
2. World Health Organization. *Clinical Management of Severe Acute Respiratory Infection When Novel Coronavirus (nCoV) Infection is Suspected: Interim Guidance, 25 January 2020*. World Health Organization; 2020.
3. Guan W-J, Liang W-H, Zhao Y, et al. Comorbidity and its impact on 1590 patients with Covid-19 in China: a nationwide analysis. *Eur Respir J*. 2020;55(5):2000547. doi:10.1183/13993003.00547-2020
4. Yang A-P, Liu J, Tao W, Li H-M. The diagnostic and predictive role of NLR, d-NLR and PLR in COVID-19 patients. *Int Immunopharmacol*. 2020;106504.
5. Zhu N, Zhang D, Wang W, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med*. 2020;382(8):727–733. doi:10.1056/NEJMoa2001017
6. Qin C, Zhou L, Hu Z, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis*. 2020;71 (15):762–768. doi:10.1093/cid/ciaa248
7. Zahorec R, Hulin I, Zahorec P. Rationale use of neutrophil-to-lymphocyte ratio for early diagnosis and stratification of COVID-19. *Bratisl Lek Listy*. 2020;121(7):466–470. doi:10.4149/BLL_2020_077
8. Feng G, Zheng KI, Yan -Q-Q, et al. COVID-19 and liver dysfunction: current insights and emergent therapeutic strategies. *J Clin Transl Hepatol*. 2020;8(1):18. doi:10.14218/JCTH.2020.00018

9. Um S-J, Huh J-H, Choi IS, Lee S-K, Yoon N-B, Son C. Role of the neutrophil-lymphocyte count ratio in the differential diagnosis of pulmonary tuberculosis and bacterial community acquired pneumonia. In: *C61. Immunodiagnostics for Latent Tuberculosis Infection and Tuberculosis*. American Thoracic Society; 2012:A4737–A4737.

10. Ying H-Q, Deng Q-W, He B-S, et al. The prognostic value of preoperative NLR, d-NLR, PLR and LMR for predicting clinical outcome in surgical colorectal cancer patients. *Med Oncol*. 2014;31 (12):305. doi:10.1007/s12032-014-0305-0

11. World Health Organization. *Clinical Management of Severe Acute Respiratory Infection When Middle East Respiratory Syndrome Coronavirus (MERS-Cov) Infection is Suspected: Interim Guidance*. World Health Organization; 2019.

12. Tian L, Li X, Qi F, et al. Harnessing peak transmission around symptom onset for non-pharmaceutical intervention and containment of the COVID-19 pandemic. *Nat Commun*. 2021;12(1):1–12. doi:10.1038/s41467-021-21385-z

13. Commission NH. Translation: diagnosis and treatment protocol for novel coronavirus pneumonia (trial version 7). *Infect Microb Dis*. 2020;2(2):48–54. doi:10.1097/IM9.0000000000000022

14. Wang M, Guo L, Chen Q, Xia G, Wang B. Typical radiological progression and clinical features of patients with coronavirus disease 2019. *Aging (Albany NY)*. 2020;12(9):7652–7659. doi:10.18632/aging.103170

15. Ugwueze CV, Ezeokpo BC, Nnolim BI, Agim EA, Anikpo NC, Onyekachi KE. COVID-19 and diabetes mellitus: the link and clinical implications. *Dubai Diabetes Endocrinol J*. 2020;26(2):69–77. doi:10.1159/000511354

16. Nishiga M, Wang DW, Han Y, Lewis DB, Wu JC. COVID-19 and cardiovascular disease: from basic mechanisms to clinical perspectives. *Nat Rev Cardiol*. 2020;17(9):543–558. doi:10.1038/s41569-020-0413-9

17. Busse WW, Lemanske RF Jr, Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet*. 2010;376 (9743):826–834. doi:10.1016/S0140-6736(10)61380-3

18. Bernheim A, Mei X, Huang M, et al. Chest CT findings in coronavirus disease-19 (COVID-19): relationship to duration of infection. *Radiology*. 2020;295(3):200463. doi:10.1148/radiol.2020200463

19. Wang L. C-reactive protein levels in the early stage of COVID-19. *Med Mal Infect*. 2020;50(4):332–334. doi:10.1016/j.medmal.2020.03.007

20. Yao Y, Cao J, Wang Q, et al. D-dimer as a biomarker for disease severity and mortality in COVID-19 patients: a Case Control Study. *J Intensive Care*. 2020;8(1):49. doi:10.1186/s40560-020-00466-z

21. Cheng L, Li H, Li L, et al. Ferritin in the coronavirus disease 2019 (COVID-19): a systematic review and meta-analysis. *J Clin Lab Anal*. 2020;34(10):e23618. doi:10.1002/jcla.23618

22. Wu C, Chen X, Cai Y, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. *JAMA Intern Med*. 2020;180(7):934. doi:10.1001/jamainternmed.2020.0994

23. Gopaul C, Ventour D, Thomas D. *Laboratory Predictors for COVID-19 ICU Admissions in a Caribbean Territory*. 2020.

24. Xu X, Yu C, Qu J, et al. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2. *Eur J Nucl Med Mol Imaging*. 2020;47(5):1275–1280. doi:10.1007/s00259-020-04735-9

25. Xu Z, Shi L, Wang Y, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med*. 2020;8(4):420–422. doi:10.1016/S2213-2600(20) 30076-X

26. Xu H, Zhong L, Deng J, et al. High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *Int J Oral Sci*. 2020;12(1):1–5. doi:10.1038/s41368-020-0074-x

27. Liao Y-C, Liang W-G, Chen F-W, Hsu J-H, Yang -J-J, Chang M-S. IL-19 induces production of IL-6 and TNF-α and results in cell apoptosis through TNF-α. *J Immunol*. 2002;169(8):4288–4297. doi:10.4049/jimmunol.169.8.4288

28. Fischer K, Hoffmann P, Voelkl S, et al. Inhibitory effect of tumor cell–derived lactic acid on human T cells. *Blood*. 2007;109 (9):3812–3819. doi:10.1182/blood-2006-07-035972

29. Mangalmurti N, Hunter CA. Cytokine storms: understanding COVID-19. *Immunity*. 2020;53(1):19–25. doi:10.1016/j.immuni.2020.06.017

30. Peng J, Qi D, Yuan G, et al. Diagnostic value of peripheral hematologic markers for coronavirus disease 2019 (COVID-19): a Multicenter, Cross-Sectional Study. *J Clin Lab Anal*. 2020;34(10): e23475. doi:10.1002/jcla.23475

31. Curbelo J, Rajas O, Arnalich B, et al. Neutrophil count percentage and neutrophil–lymphocyte ratio as prognostic markers in patients hospitalized for community-acquired pneumonia. *Arch Bronconeumol*. 2019;55(9):472–477. doi:10.1016/j.arbr.2019.07.002

32. Asghar MS, Khan NA, Haider Kazmi SJ, et al. Hematological parameters predicting severity and mortality in COVID-19 patients of Pakistan: a retrospective comparative analysis. *J Community Hosp Intern Med Perspect*. 2020;10(6):514–520. doi:10.1080/20009666.2020.1816276

33. Asghar MS, Kazmi SJH, Khan NA, et al. Clinical profiles, characteristics, and outcomes of the first 100 admitted COVID-19 patients in Pakistan: a single-center retrospective study in a tertiary care hospital of Karachi. *Cureus*. 2020;12(6).

34. Kusumanto YH, Dam WA, Hospers GA, Meijer C, Mulder NH. Platelets and granulocytes, in particular the neutrophils, form important compartments for circulating vascular endothelial growth factor. *Angiogenesis*. 2003;6(4):283–287. doi:10.1023/B:AGEN.0000029415.62384.ba

35. Kim S-L, Lee S-T, Trang KTT, et al. Parthenolide exerts inhibitory effects on angiogenesis through the downregulation of VEGF/VEGFRs in colorectal cancer. *Int J Mol Med*. 2014;33 (5):1261–1267. doi:10.3892/ijmm.2014.1669

36. Blaser MJ, Chyou P, Nomura A. Age at establishment of Helicobacter pylori infection and gastric carcinoma, gastric ulcer, and duodenal ulcer risk. *Cancer Res*. 1995;55(3):562–565.

37. Scholl S, Pallud C, Beuvon F, et al. Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *J Natl Cancer Inst*. 1994;86(2):120–126. doi:10.1093/jnci/86.2.120

38. Gupta SC, Hevia D, Patchva S, Park B, Koh W, Aggarwal BB. Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. *Antioxid Redox Signal*. 2012;16(11):1295–1322. doi:10.1089/ars.2011.4414

39. Rabinowich H, Cohen R, Bruderman I, Steiner Z, Klajman A. Functional analysis of mononuclear cells infiltrating into tumors: lysis of autologous human tumor cells by cultured infiltrating lymphocytes. *Cancer Res*. 1987;47(1):173–177.

40. Menges T, Engel J, Welters I, et al. Changes in blood lymphocyte populations after multiple trauma: association with posttraumatic complications. *Crit Care Med*. 1999;27(4):733–740. doi:10.1097/00003246-199904000-00026

41. Putzu C, Cortinovis DL, Colonese F, et al. Blood cell count indexes as predictors of outcomes in advanced non-small-cell lung cancer patients treated with Nivolumab. *Cancer Immunol Immunother*. 2018;67(9):1349–1353. doi:10.1007/s00262-018-2182-4

42. Huang Y, Liu A, Liang L, et al. Diagnostic value of blood parameters for community-acquired pneumonia. *Int Immunopharmacol*. 2018;64:10–15. doi:10.1016/j.intimp.2018.08.022

43. Lissoni P, Rovelli F, Monzon A, Privitera C, Messina G, Porro G. Evidence of abnormally low lymphocyte-to-monocyte ratio in covid-19-induced severe acute respiratory syndrome. *J Immunol Allergy*. 2020;1(2):1–6.

44. Nicolai L, Massberg S. Platelets as key players in inflammation and infection. *Curr Opin Hematol*. 2020;27(1):34–40. doi:10.1097/MOH.0000000000000551

45. Mazza MG, De Lorenzo R, Conte C, et al. Anxiety and depression in COVID-19 survivors: role of inflammatory and clinical predictors. *Brain Behav Immun*. 2020;89:594–600. doi:10.1016/j.bbi.2020.07.037

46. Wang X, Li X, Shang Y, et al. Ratios of neutrophil-to-lymphocyte and platelet-to-lymphocyte predict all-cause mortality in inpatients with coronavirus disease 2019 (COVID-19): a Retrospective Cohort Study in a single medical centre. *Epidemiol Infect.* 2020;148.

47. Merekoulias G, Alexopoulos EC, Belezos T, Panagiotopoulou E, Jelastopulu E. Lymphocyte to monocyte ratio as a screening tool for influenza. *PLoS Curr.* 2010;2:RRN1154. doi:10.1371/currents. RRN1154

3D Multicellular Spheroid for the Study of Human Hematopoietic Stem Cells: Synergistic Effect Between Oxygen Levels, Mesenchymal Stromal Cells and Endothelial Cells

Emilia Barreto-Duran, Claudia Camila Mejia-Cruz, Luis Fernando Jaramillo-Garcia, Efrain Leal-Garcia, Alfonso Barreto-Prieto & Viviana Marcela Rodriguez-Pardo

Submit your article to this journal ☑

Article views: 188

View related articles ☑

View Crossmark data ☑

Citing articles: 6 View citing articles ☑

ORIGINAL RESEARCH

3D Multicellular Spheroid for the Study of Human Hematopoietic Stem Cells: Synergistic Effect Between Oxygen Levels, Mesenchymal Stromal Cells and Endothelial Cells

Emilia Barreto-Duran [1]
Claudia Camila Mejia-Cruz [1]
Luis Fernando Jaramillo-Garcia [2]
Efrain Leal-Garcia [3]
Alfonso Barreto-Prieto [1]
Viviana Marcela Rodriguez-Pardo [1]

[1]Grupo de Inmunobiología y Biología Celular, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá D.C., Colombia (South America); [2]Departamento de Patología, Facultad de Medicina, Pontificia Universidad Javeriana., Hospital Universitario San Ignacio, Bogotá D.C., Colombia (South America); [3]Departamento de Ortopedia y Traumatología, Facultad de Medicina, Pontificia Universidad Javeriana., Hospital Universitario San Ignacio, Bogotá D.C., Colombia (South America)

Introduction: The human bone marrow microenvironment is composed of biological, chemical and physical factors that act in a synergistic way to modulate hematopoietic stem cell biology, such as mesenchymal stromal cells (MSCs), endothelial cells (ECs) and low oxygen levels; however, it is difficult to mimic this human microenvironment in vitro.

Methods: In this work, we developed 3D multicellular spheroid (3D-MS) for the study of human hematopoietic stem cells (HSCs) with some components of perivascular niche. HSCs were isolated from umbilical cord blood, MSCs were isolated from human bone marrow and a microvasculature EC line (CC-2811, Lonza®) was used. For the formation of a 3D structure, a magnetic levitation culture system was used. Cultures were maintained in 21%, 3% and 1% O_2 for 15 days. Culture volume, sphericity index and cell viability were determined. Also, human HSC proliferation, phenotype and production of reactive oxygen species were evaluated.

Results: After 15 days, 3D-MS exhibited viability greater than 80%. Histology results showed structures without necrotic centers, and higher cellular proliferation with 3% O_2. An increase in the expression of the CD34 antigen and other hematopoietic antigens were observed to 1% O_2 with MSCs plus ECs and low ROS levels.

Conclusion: These findings suggest that 3D-MS formed by MSCs, ECs and HSCs exposed to low concentrations of oxygen (1–3% O_2) modulate human HSC behavior and mimics some features of the perivascular niche, which could reduce the use of animal models and deepen the relationship between the microenvironment of HSC and human hematological diseases development.

Keywords: hematopoietic stem cells, niche, bone marrow, microenvironment, 3D culture

Introduction

Hematopoiesis is a very active and efficient process that generates approximately $4–5\times10^{11}$ hematopoietic cells per day.[1] This complex system is regulated by the interaction between hematopoietic stem cells (HSCs) and other cell populations that reside in the bone marrow (BM), soluble factors and physicochemical conditions such as oxygen perfusion.[2] HSCs are distributed in endosteal and perivascular niches[3–5]; however, recent evidence suggests that HSCs have a preferential location in the perivascular niche,[6–8] which are enriched with different populations of mesenchymal stromal cells (MSCs) and endothelial cells (ECs).[9–12]

Correspondence: Viviana Marcela Rodriguez-Pardo
Email vivianar@javeriana.edu.co

Several studies have shown the close relationship between HSCs, MSCs and ECs and the influence of these relationships on hematopoiesis.[13] MSCs are the major source of SCF and SDF-1 (CXCL12)[14] in the bone marrow, which once deleted, greatly affect the quantity and engrafting capacity of HSCs.[11,14,15] It has also been shown that ECs regulate self-renewal, proliferation and phenotype modulation of HSCs through direct contact or soluble factors.[16–20]

Contrary to the findings about the murine HSC niche, information about the microenvironment of human HSCs is limited. It has been shown that 86% of human primitive CD34+ HSCs are located in the perivascular region close to CD271+/CD146+ MSCs,[21,22] which secrete factors that promote the maintenance of human HSCs and increase their ability to repopulate in vivo.[23–25] Furthermore, HSCs are particularly sensitive to low oxygen levels that induce intracellular signals that promote the maintenance of their self-renewal, stemness, phenotype modulation, glycolytic metabolism and low production of reactive oxygen species (ROS).[7,26,27] Recent evidence shows that the most primitive HSCs are located in the perivascular niches in close relationship with MSCs, ECs and low levels of oxygen.[27,28]

To establish an in vitro system that mimics the microenvironment of human HSCs, we previously established a 3D culture system using magnetic levitation for the study of the human HSC microenvironment.[29] In this work, we evaluated the synergistic effect between MSCs, endothelial cells and different levels of oxygen levels on the viability, proliferation, phenotype modulation and production of reactive oxygen species of human HSCs using 3D multicellular spheroid (3D-MS).

Table 1 Umbilical Cord Blood (UCB) Donors for HSC Isolation

No.	Gestation Time (Weeks)	Volume UCB (mL)	CD34+ Cells (Total Counts)	CD34+ (Purity %)
1	38	34	6.00×10^5	82
2	38	76	3.20×10^6	97
3	39	16	1.40×10^6	71
4	39	25	6.00×10^5	60
5	37	31	4.00×10^5	86
6	38	40	1.00×10^6	96
7	38	36	1.80×10^6	90
8	40	44	2.00×10^6	97
9	40	52	8.00×10^5	73
10	39	60	1.30×10^6	71
11	38	61	1.90×10^6	87
12	39	31	1.10×10^6	94
13	38	64	1.66×10^6	97
14	37	42	4.00×10^6	93
15	38	17	5.00×10^6	95
16	39	45	1.10×10^6	98
17	40	55	2.00×10^6	79
18	38	47	9.00×10^5	85
19	39	75	1.00×10^6	90
20	39	24	8.00×10^5	89

Table 2 Bone Marrow Donors for MSC Isolation and Expansion

No.	Age Donors (Years)	Gender (Female / Male)	Volume BM (mL)	MNC (Total Counts)
1	56	F	27	1.16×10^8
2	59	F	40	4.81×10^7
3	53	F	20	4.58×10^7
4	75	F	30	2.18×10^7
5	35	F	41	3.10×10^7
6	60	F	20	9.40×10^6

Methods
Generation of 3D Multicellular Spheroids

HSCs (CD34+) were isolated from umbilical cord blood (UCB) (Table 1), and MSCs were isolated from human bone marrow (hBM) (Table 2), after accepting the informed consent for the voluntary donation of UCB and hBM was conducted in accordance with the Declaration of Helsinki and approval of the ethics committee of Hospital Universitario San Ignacio, Act No 18 (2014/154). HSC and MSC isolation, expansion and characterization have been previously demonstrated by our group.[29–34] HMVEC-dBlAd (CC-2811 Lonza®) cell line was expanded in endothelial cell growth medium (EGMTM-2 BulletKit™, Lonza®). HpDLF (fibroblast, CC-7049 Lonza®) cell line was expanded in modified Eagle's medium (Merck®), and this was used as a negative control. The 3D structures were generated with a magnetic levitation system containing MSCs, HSCs and ECs (ratio 1:2:2).[29] 3D structure control groups were generated: fibroblasts, HSCs and ECs (Fb-HSC-ECs), and MSCs-HSCs. 3D-MS were cultured at three O_2 concentrations (1%, 3% and 21%) for 15 days. For hypoxic conditions, 3D-MS were cultured in a hypoxia chamber (chamber C-Chamber Incubator Subchamber, BioSpherix ©) using a mixture of gases (O_2, N_2 and CO_2).

518

Journal of Blood Medicine 2021:12

Morphological Evaluation

Volume and sphericity index (SI) were performed according to Kelm et al,[35] using ImageJ program (Fiji, version 1.0) to calculate the area and perimeter from the photographs obtained from each of the 3D structures on day 5 (n = 88), 10 (n = 72) and 15 (n = 48).

Histological and Immunohistochemical (IHQ) Analysis

Pathology Department at the Hospital Universitario San Ignacio (HUSI) (Bogotá, Colombia) supported the development of histochemical tests. 3D-MS were fixed with formaldehyde, dehydrated with ethanol, cleared with xylenes (Thermo Scientific™ Excelsior™ AS Tissue Processor), embedded in paraffin (Thermo Scientific™ HistoStar™ Embedding Workstation) and sectioned into 3 μm thick sections with a microtome (Leica® RM2125 RTS). Serial slides of 3D structures sections were then processed with hematoxylin and eosin (H&E), anti-Ki67 (Flex Ki-67 antigen, MxH/MIB-1, Dako®) and anti-vimentin (Flex vimentinMx/V9, Dako®).

Cell Viability and Hematopoietic Antigens Evaluation

LIVE/DEAD® Cell Imaging Kit (R37601 Thermo Scientific®) was used following the manufacturer's instructions. This viability staining was performed on the complete and living 3D structure. For hematopoietic antigens, 3D-MS serial slides were incubated at room temperature with primary antibodies (Anti-CD34 APC clone 581 Thermo Scientific®, anti-Ki67 clone 4A1 Thermo Scientific®, anti-CD133 clone AC133 Miltenyi Biotec®, anti-CD33 clone WM53ThermoScientific® and anti-CD7 clone MEM-186, Thermo Scientific®). Alexa Fluor 514 goat anti-mouse IgG (Thermo Scientific®) was used as a secondary antibody for CD133, CD33 or CD7 detection. Anti-α-tubulin (Alexa Fluor 488 clone B512, Invitrogen®) was used for cytoskeletal labeling, and DAPI (4′,6-diamidino-2-phenylindole, ref D1306, Invitrogen®) was used for nuclei identification. Slide-mounted 3D structure sections were sealed with 10 μL of ProLong Gold antifade reagent (Invitrogen®) and covered with coverslips.

Reactive Oxygen Species (ROS) Evaluation

3D structure sections were treated with CellROX™ Deep Red Reagent (Thermo Scientific®) at room temperature.

The cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with anti-α-tubulin and DAPI following the protocol mentioned above.

Image Acquisition and Analysis Strategy for Confocal Microscopy

An Olympus FV1000 confocal microscope with 488-, 543-, 635- and 405-nm lasers was used to collect images of sphere and sphere sections. Viability imaging and ROS imaging was performed using a UPLFLN 20X objective with a numerical aperture (NA) of 0.50 every 5μm to generate reconstructions in XYZ planes. Between 25 and 30 images were obtained to assess viability (complete spheres), and 15 to 20 images were obtained to assess ROS (sphere sections). For proliferation and hematopoietic phenotype modulation, Z stack images of sphere sections were collected with UPLSAPO 60X and UPLFLN 20X objectives using an NA of 1.35 in the XY focal plane. 3D reconstruction and data analysis were performed with FlowView (Olympus®) and Fiji 1.0, respectively. For the evaluation of viability, the percentage of green area (living cells) and red area (dead cells) was calculated in separate channels using the "threshold" and "analyze particles" tools to select the estimated area for each color. Hematopoietic antigens and ROS were calculated using the following equation: antigen expression area $\% =$ antigen expression area$(\mu m^2) \times 100\%/\alpha-$tubulin expression area$(\mu m^2)$

Statistical Analysis

Shapiro–Wilk tests were applied to determine the normality of the data. The comparisons between two samples were made using Wilcoxon tests. For the comparison of more than three groups of data, Kruskal–Wallis (KW) tests, Friedman test with Dunn's test was used. All the data were analyzed and plotted with GraphPad Prism 6™ software. * p = 0.05, ** p = 0.01, and *** p = 0.001.

Results

Structure and Viability of 3D Cultures

After 15 days in culture, homogeneous 3D structures were obtained under the three oxygen conditions; mesenchymal cells control formed by fibroblasts, hematopoietic cells and endothelial cells reached a larger size (Fb-HSC-EC) (Figure 1). The volumes of MSC-HSC-EC were between 0.1 and 0.5 mm³ in all culture conditions and the sphericity index (SI) was between 0.6 and 0.8 during 15 days. Fb-HSC-EC 3D
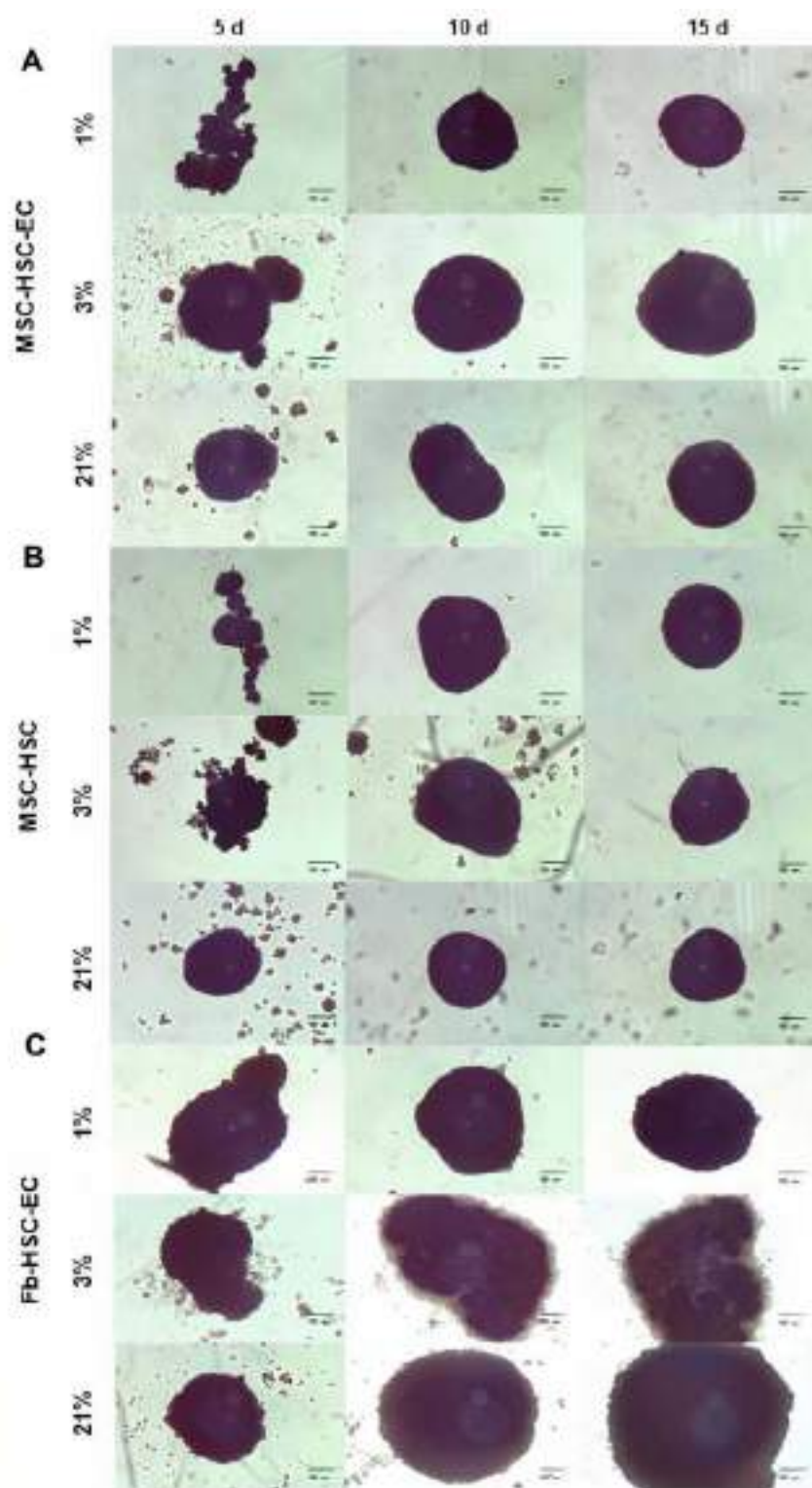
Figure 1 Structure of 3D cultures. Structures at days 5, 10 and 15 in three O_2 conditions (1%, 3% and 21% O_2 levels). (**A**) Experimental condition: MSC-HSC-EC, (**B**) Endothelial cells control: MSC-HSC, (**C**) Mesenchymal cells control: Fb-HSC-EC. (Olympus® CKX31, 10×) (scale bar: 100 μm).

520

Journal of Blood Medicine 2021:12

structures had an approximate volume between 1.7 and 7.5 mm^3 at day 10 of culture and between 3.0 and 10.5 mm^3 after 15 days, with a sphericity between 0.4 and 0.8. Some variations in size and sphericity were observed between oxygen culture concentrations, although significant differences were observed in the volume of the 3D structure between the MSC-HSC-EC condition versus controls at day 5 (Figure 2). All evaluated 3D structures had a viability >80%, and no significant differences were found in cell viability between experimental organoid prototype and controls (p > 0.9999) (Figure 3).

Histology and Immunohistochemistry

H&E staining demonstrated that all evaluated 3D-MS showed a cellular organization similar to that found in tissue (Figure 4A). Brown areas were accumulations of metallic nanoparticles in the extracellular matrix of the structure. There was no evidence of a necrotic center in 3D structures (Figure 4A). Vimentin expression was strongly positive compared to controls (Figure 4B–D).

Evaluation of Proliferation and Phenotype Modulation of Human HSC in 3D Structures

A lower expression of Ki67 was observed in cultures with 1% O$_2$ but Ki67 expression by immunochemistry was not conclusive because the nanoparticles did not allow the protein to be visualized in the nucleus (Figure 5A–D). Global expression of Ki67 by confocal microscopy allowed to demonstrate significant differences between the oxygen conditions, especially in MSC-HSC-EC structure exposed to the lowest levels (1–3%) (Figure 5E and F). After 15 days, MSC-HSC-EC structure showed a higher expression of CD34 antigen with 1% O$_2$ in comparison with 21% O$_2$ (Figure 6A and B). For the CD133 antigen there was a higher expression at 3% and 1% O$_2$ than there was at 21%, and it was generally shown that there was higher expression in experimental MSC-HSC-EC than there was in the controls (Figure 6C and D). Regarding the expression of CD33, there was a statistically significant difference in the MSC-HSC-EC structures maintained at 21% and 1% O$_2$ (Figure 6E and
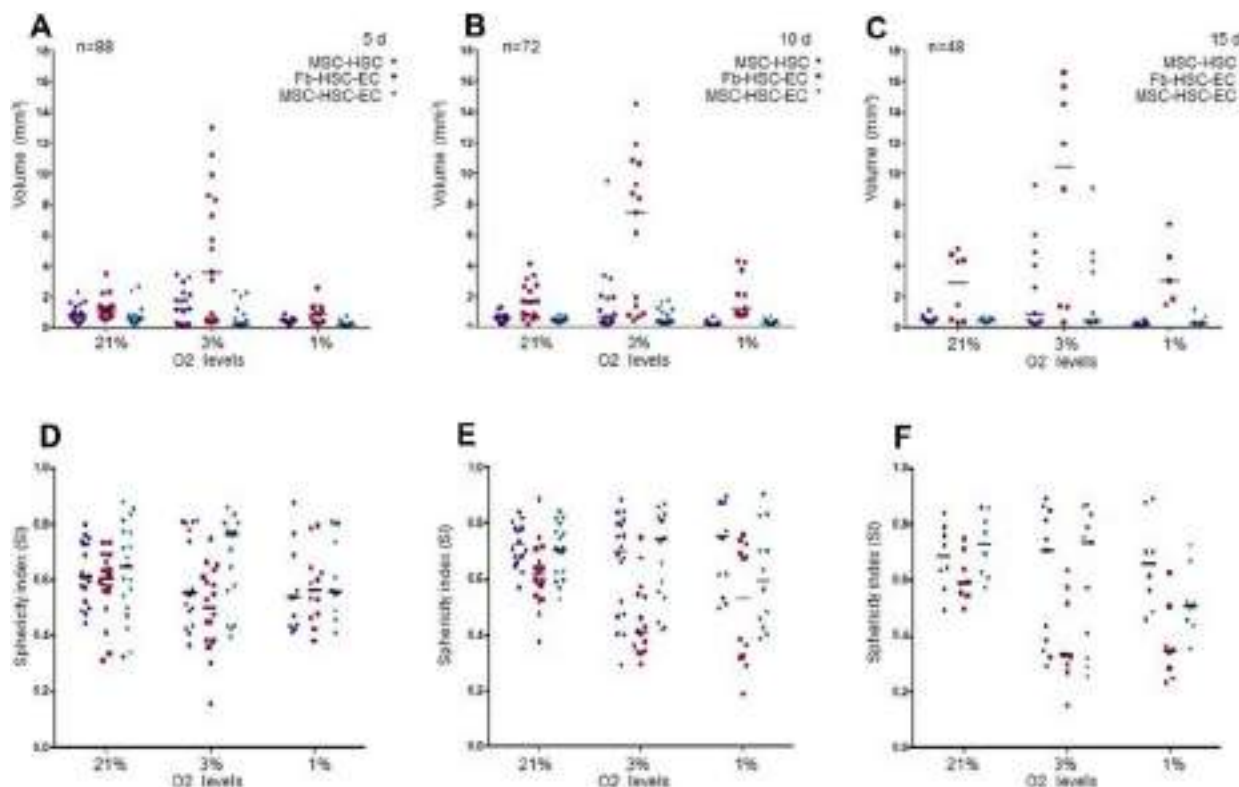
Figure 2 Volume and sphericity index (SI) of 3D cultures. (**A–C**) Volume after 5, 10 and 15 days. Friedman tests were performed to compare O$_2$ concentrations: 5, 10 and 15 days. Friedman tests were performed to compare MSC-HSC-EC structures and controls: 5 days (p=0.0278), 10 days and 15 days (p>0.,05). (**D–F**) Sphericity Index (SI) after 5, 10 and 15 days. Friedman tests were performed to compare O$_2$ concentrations and MSC-HSC-EC structures vs controls after 5, 10 and 5 days (p>0.,05).
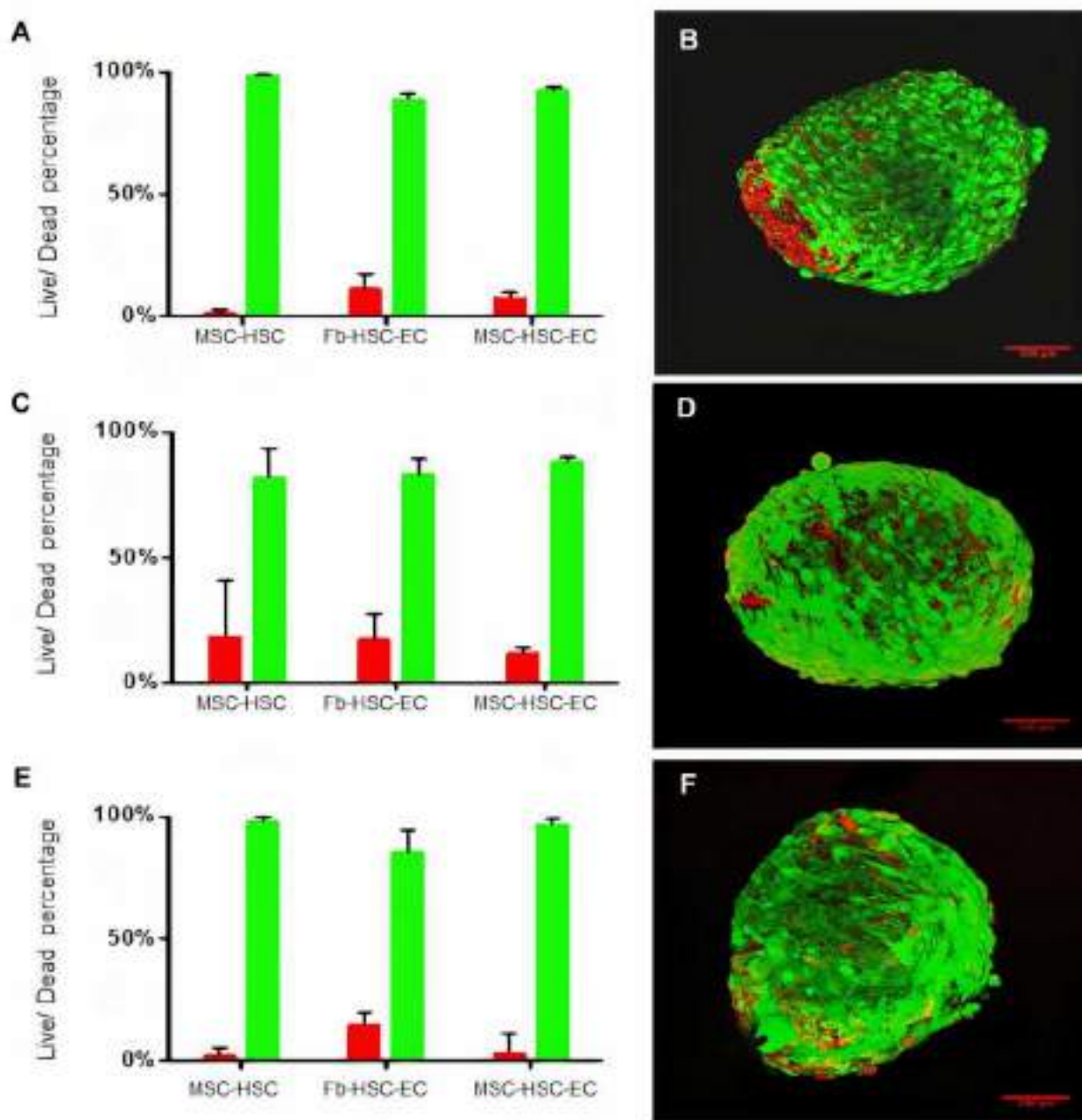
Figure 3 Viability of 3D cultures. (**A** and **B**) Cell viability in 1% O_2, (**C** and **D**) Cell viability in 3% O_2, (**E** and **F**) Cell viability 21% O_2. (**B, D, F**) LIVE/DEAD® viability kit. Red: dead cells, Green: live cells. Olympus confocal microscope FV1000, 20× (scale bar: 100μm).

F). For the CD7 antigen, there tended to be greater expression at 3% and 1% O_2, with statistically significant differences in the MSC-HSC-EC structure between 21% and 1% O_2 (Figure 6G and H).

Reactive Oxygen Species

No statistically significant differences were found in ROS production between experimental and control 3D-MS.

Nevertheless, although no statistically significant differences were found in ROS production with different oxygen conditions (p = 0.1944, Friedman test), a lower production was observed in 3D-MS with 1% O_2 (Figure 7).

Discussion and Conclusions

The niche of human HSCs in bone marrow is influenced by different cell populations; however, the perivascular niche
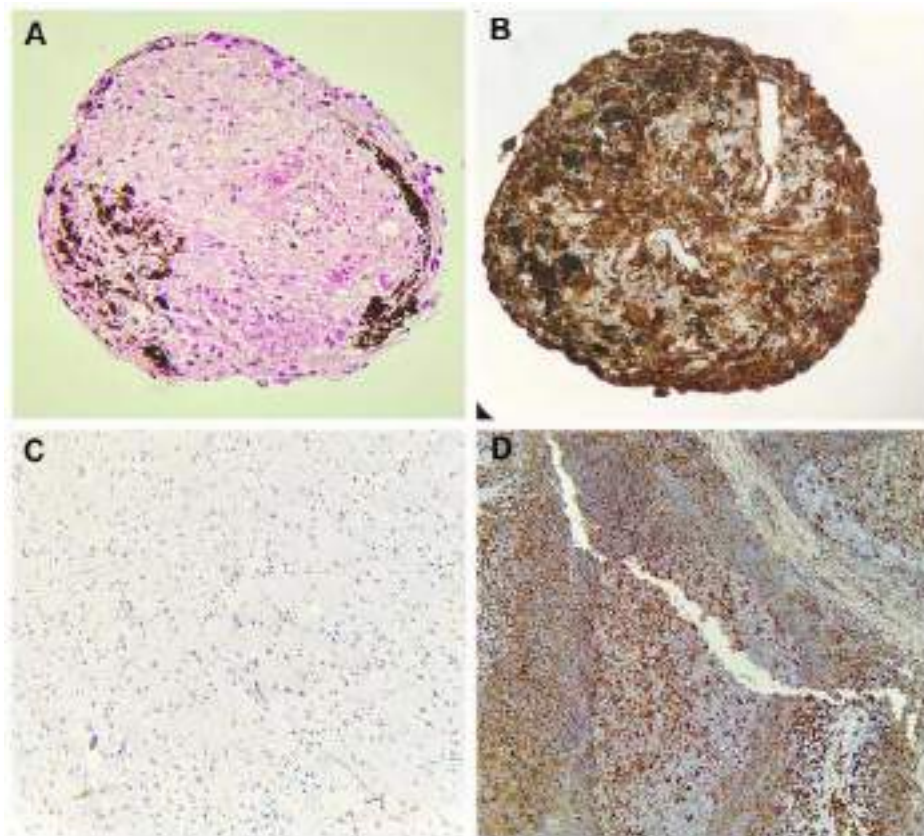
Figure 4 Histological characterization of 3D structures. (**A**) Hematoxylin and eosin stain (20×, Olympus® CX21), (**B**) Vimentin expression of MSC-HSC-EC structures after 15d with 3% O$_2$ (20×, Olympus® CX21), (**C**) Negative control for vimentin (brain tissue) (10×, Olympus® CX21), (**D**) Positive control for vimentin (lymph node tissue) (10×, Olympus® CX21).

(MSC and ECs) is increasingly important given the different phenomena that occur there and that are related to functional hematopoiesis.[36,37] In this work, we used the synergistic relationship between MSCs and ECs with different concentrations of O$_2$ on human HSCs in 3D-MS,[38] enabling the in vitro study of human HSC microenvironment.

In relation to the structure of the 3D-MS obtained, it has been previously shown that under hypoxic conditions (2–3% O$_2$), various cell populations could increase the expression of adhesion molecules.[39,40] The MSC-HSC-EC structures showed a similar sphericity index (0.6–0.8) after 5, 10 and 15 days of culture at all oxygen concentrations; however, in the controls, the sphericity index fluctuated according to the culture time and O$_2$ concentration (Figure 2). These results could possibly be related to a synergistic effect between oxygen levels and the interaction between MSCs, HSCs and ECs on the expression of adhesion molecules that is not observed in the condition with Fb or without ECs; however, addressing this possibility requires the determination of adhesion molecules that act in the 3D structure.

Interestingly, and contrary to other models of 3D cultures, we do not observe a necrotic center inside the structures. Necrotic centers in these types of structures appear in spheres with volume between 0.112 ± 0.013 mm^3[41,42]; however, the average volume of the 3D-MS generated in this work was between 0.1 and 0.5 mm^3 (MSC-HSC-CE). We propose that the absence of necrotic centers in the obtained spheres is possibly related to the secretion of soluble factors of the three cell populations and is promoted by their cell–cell interaction, which maintains the internal viability of the 3D structure and high expression of vimentin in all experimental conditions (Figures 3 and 4).

In this work, we demonstrate the effect of physiological oxygen levels of the human bone marrow (1% and 4%),[27] which some call hypoxia, on the proliferation and modulation of the human HSC phenotype. Statistically significant differences were observed in Ki67 expression levels in the different experimental conditions and controls (confocal microscopy) (Figure 5E and F) with greater expression of Ki67 at 1% and 3% O$_2$ in comparison with 21%.
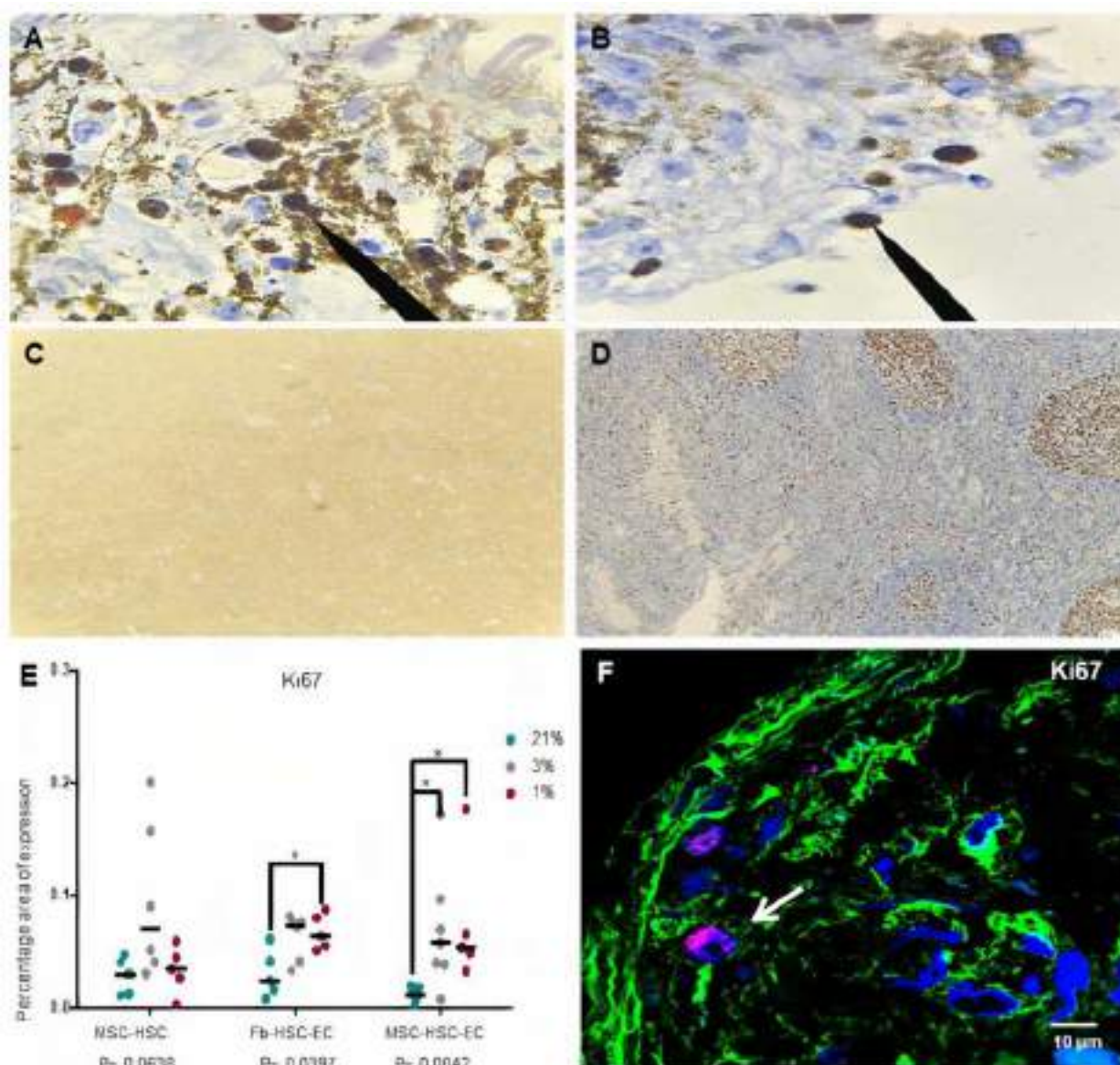
Figure 5 Ki67 expression in 3D structures. (**A** and **B**) Ki67 expression of MSC-HSC-EC structures after 15 days and 1% O_2 by immunochemistry (arrows) (100×, Olympus® CX21), (**C**) Ki67 negative control by immunochemistry (brain tissue) (10×, Olympus® CX21), (**D**) Ki67 positive control by immunochemistry (lymph node tissue) (10×, Olympus® CX21), (**E**) Ki67 expression by confocal microscopy (percentage area of expression), (**F**) Paraffin section (3 μm thick) of MSC-HSC-EC structure analyzed by confocal microscopy (Magenta: Ki67, green: α-tubulin, and blue: DAPI-nuclei) (40×, Olympus confocal microscope FV1000).

Interestingly, we show that oxygen levels between 1% and 3% increase the expression of CD34, CD133, CD33 and CD7 over the levels observed in conventional culture conditions (21% oxygen), which suggests synergy with MSCs and ECs, given that this increase was greater in the MSC-HSC-Ec condition (experimental 3D-MS) (Figure 6).

Previous studies have shown that the increase in the expression of CD34 and CD133 in human HSCs is associated with a greater capacity of repopulation of these cells in models of immunodeficient mice.[43,44] Similarly,

although the expression of CD33 and CD7 is associated with myeloid and lymphoid progenitors, respectively, it has been shown that the expression of CD33 in human HSCs from umbilical cord blood is also associated with long-term culture capacity (LTC-IC).[45,46]

We propose in this work that the 3D-MS formed by MSCs, ECs and HSCs exposed to low concentrations of oxygen (1–3% O_2) modulate human HSC expression of hematopoietic antigens associated with the engraftment and stemness capacity in an environment with low ROS
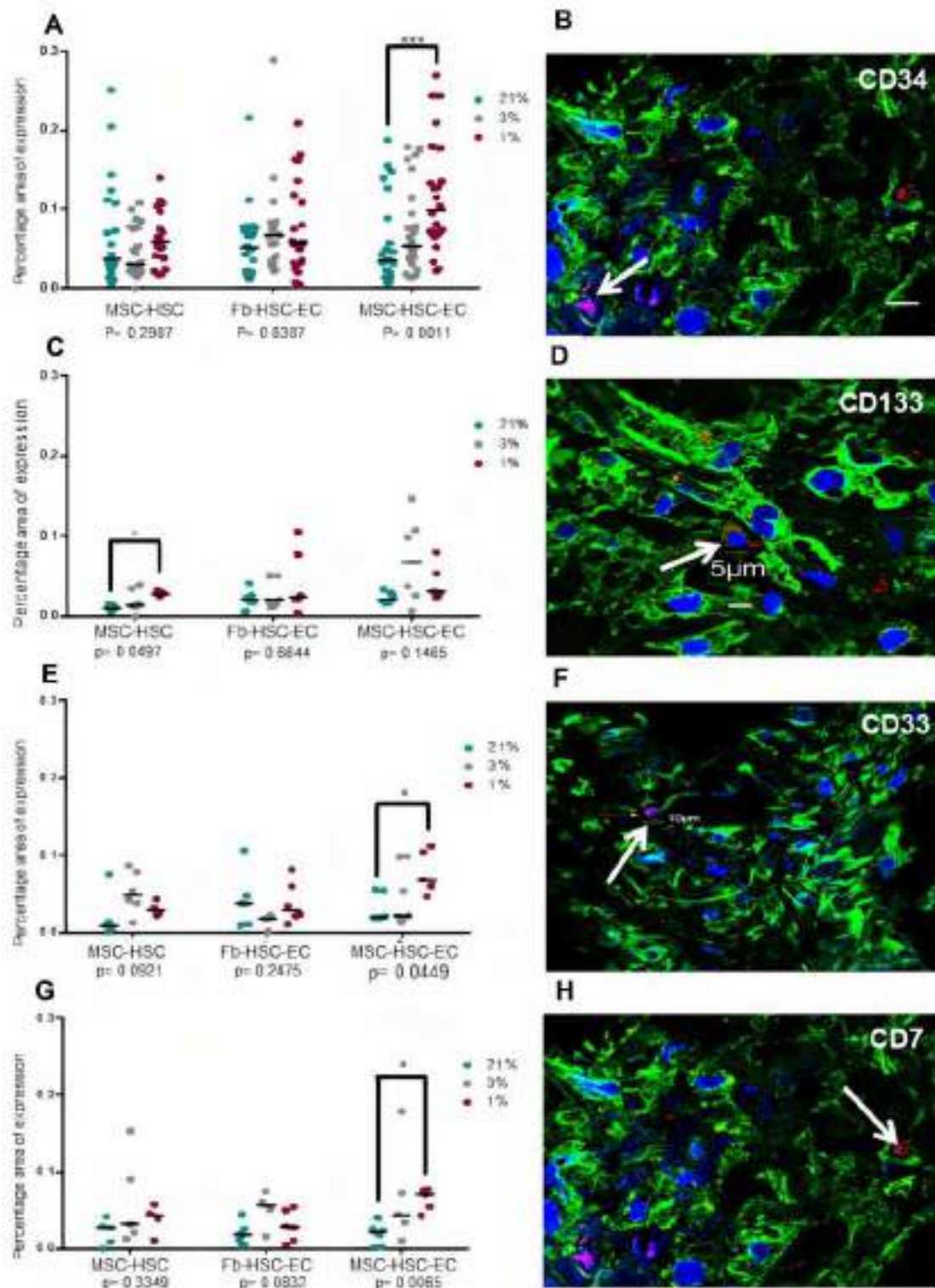
Figure 6 Hematopoietic antigens expression in 3D structures. (**A** and **B**) CD34 antigen, (**C** and **D**) CD133 antigen, (**E** and **F**) CD33 antigen, (**G** and **H**) CD7. (**B, D, F, H**) Paraffin section (3 μm thick) of MSC-HSC-EC structure analyzed by confocal microscopy after 15 days. Magenta or red: hematopoietic antigen (arrows), green: α-tubulin, and blue: DAPI-nuclei (40X, Olympus confocal microscope FV1000).
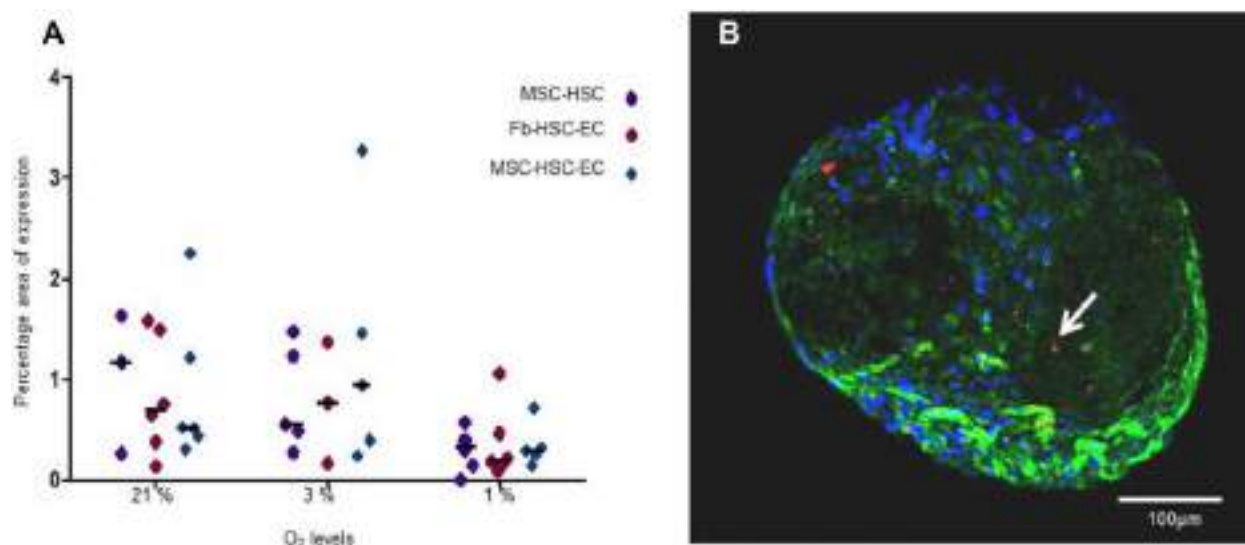
Figure 7 ROS levels in 3D structures. (**A**) ROS expression area in structures cultivated in 1%, 3% and 21% O_2. Differences between MSC-HSC-EC and controls, P> 0.9999, as determined by Friedman tests; differences between O2 concentrations, P = 0.1944, as determined by Friedman tests. (**B**) Paraffin section (3 μm thick) of MSC-HSC-EC culture stained with CellROX®. Green: α-Tubulin, blue: DAPI (nuclei), and red: ROS (20×, Olympus confocal microscope FV1000).

levels (Figure 7), and although ROS levels were detected globally and statistically significant differences were not determined, a lower production was observed in 3D-MS with 1% O_2, it has been described that this "hypoxic" environment favors the maintenance of the long-term primitive human HSC pool and mimics some features of the perivascular niche of human HSCs.

The development of human 3D-MS that mimic the microenvironment of HSCs has great potential because they may allow the testing of new drugs for the treatment of diseases such as leukemia, reduce the use of animal models, and deepen our understanding of the relationship between the microenvironment of the HSC and the evolution of different human hematological diseases.

Abbreviations

3D-MS, 3D multicellular spheroid, HSC, hematopoietic stem cells, MSCs, mesenchymal stromal cells; ECs, endothelial cells.

Acknowledgments

Disclosure

The authors declare no conflicts of interest.

References

1. Kaushansky K. Lineage-specific hematopoietic growth factors. *N Engl J Med.* 2006;354(19):2034–2045. doi:10.1056/NEJMra052706
2. Wei Q, Frenette PS. Niches for hematopoietic stem cells and their progeny. *Immunity.* 2018;48(4):632–648. doi:10.1016/j.immuni.2018.03.024
3. Lo Celso C, Fleming HE, Wu JW, et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature.* 2009;457(7225):92–96. doi:10.1038/nature07434
4. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature.* 2014;505(7483):327–334. doi:10.1038/nature12984
5. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature.* 2013;495 (7440):231–235. doi:10.1038/nature11885
6. Koechlein CS, Harris JR, Lee TK, et al. High-resolution imaging and computational analysis of haematopoietic cell dynamics in vivo. *Nat Commun.* 2016;7:12169. doi:10.1038/ncomms12169
7. Spencer JA, Ferraro F, Roussakis E, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature.* 2014;508(7495):269–273. doi:10.1038/nature13034
8. Itkin T, Gur-Cohen S, Spencer JA, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature.* 2016;532 (7599):323–328. doi:10.1038/nature17624
9. Asada N, Kunisaki Y, Pierce H, et al. Differential cytokine contributions of perivascular haematopoietic stem cell niches. *Nat Cell Biol.* 2017;19(3):214–223. doi:10.1038/ncb3475
10. Asada N, Takeishi S, Frenette PS. Complexity of bone marrow hematopoietic stem cell niche. *Int J Hematol.* 2017;106(1):45–54. doi:10.1007/s12185-017-2262-9
11. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature.* 2010;466(7308):829–834. doi:10.1038/nature09262

12. Kunisaki Y, Bruns I, Scheiermann C, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*. 2013;502 (7473):637–643. doi:10.1038/nature12612

13. Schepers K, Campbell TB, Passegue E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell*. 2015;16(3):254–267. doi:10.1016/j.stem.2015.02.014

14. Omatsu Y, Sugiyama T, Kohara H, et al. The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity*. 2010;33(3):387–399. doi:10.1016/j. immuni.2010.08.017

15. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*. 2012;481(7382):457–462. doi:10.1038/nature10783

16. Rafii S, Shapiro F, Pettengell R, et al. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood*. 1995;86(9):3353–3363. doi:10.1182/blood.V86.9.3353. bloodjournal8693353

17. Winkler IG, Barbier V, Nowlan B, et al. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med*. 2012;18(11):1651–1657. doi:10.1038/ nm.2969

18. Kobayashi H, Butler JM, O'Donnell R, et al. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol*. 2010;12 (11):1046–1056. doi:10.1038/ncb2108

19. Butler JM, Gars EJ, James DJ, Nolan DJ, Scandura JM, Rafii S. Development of a vascular niche platform for expansion of repopulating human cord blood stem and progenitor cells. *Blood*. 2012;120(6):1344–1347. doi:10.1182/blood-2011-12-398 115

20. Zhang P, Zhang C, Li J, Han J, Liu X, Yang H. The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications. *Stem Cell Res Ther*. 2019;10 (1):327. doi:10.1186/s13287-019-1422-7

21. Flores-Figueroa E, Varma S, Montgomery K, Greenberg PL, Gratzinger D. Distinctive contact between CD34+ hematopoietic progenitors and CXCL12+ CD271+ mesenchymal stromal cells in benign and myelodysplastic bone marrow. *Lab Invest*. 2012;92 (9):1330–1341. doi:10.1038/labinvest.2012.93

22. Tormin A, Li O, Brune JC, et al. CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood*. 2011;117(19):5067–5077. doi:10.1182/blood-2010-08-304287

23. Pinho S, Lacombe J, Hanoun M, et al. PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med*. 2013;210 (7):1351–1367. doi:10.1084/jem.20122252

24. Li H, Ghazanfari R, Zacharaki D, et al. Low/negative expression of PDGFR-alpha identifies the candidate primary mesenchymal stromal cells in adult human bone marrow. *Stem Cell Rep*. 2014;3 (6):965–974. doi:10.1016/j.stemcr.2014.09.018

25. van Pel M, Fibbe WE, Schepers K. The human and murine hematopoietic stem cell niches: are they comparable? *Ann N Y Acad Sci*. 2016;1370(1):55–64. doi:10.1111/nyas.12994

26. Tiwari A, Wong CS, Nekkanti LP, et al. Impact of oxygen levels on human hematopoietic stem and progenitor cell expansion. *Stem Cells Dev*. 2016;25(20):1604–1613. doi:10.1089/scd.2016.0153

27. Wielockx B, Grinenko T, Mirtschink P, Chavakis T. Hypoxia pathway proteins in normal and malignant hematopoiesis. *Cells*. 2019;8 (2):155. doi:10.3390/cells8020155

28. Nombela-Arrieta C, Pivarnik G, Winkel B, et al. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol*. 2013;15(5):533–543. doi:10.1038/ncb2730

29. Mejia-Cruz CC, Barreto-Duran E, Pardo-Perez MA, et al. Generation of organotypic multicellular spheres by magnetic levitation: model for the Study of Human Hematopoietic Stem Cells Microenvironment. *Int J Stem Cells*. 2019;12(1):51–62. doi:10. 15283/ijsc18061

30. Rodriguez-Pardo VM, Fuentes-Lacouture MF, Aristizabal-Castellanos JA, Vernot Hernandez,JP. Aislamiento y caracterización de células "stem" mesenquimales de médula ósea humana según criterios de la Sociedad Internacional de Terapia Celular. [Isolation and characterization of mesenchymal stem cells from human bone marrow according to the criteria of the International Society for Cellular Therapy]. *Univ Sci*. 2010;15 (3):224–239. doi:10.11144/javeriana.SC15-3.iaco

31. Flechas I, Mera C, Vargas L, Rodríguez-Pardo VM. Evaluación de proteínas relacionadas con la diferenciación de células madre hematopoyéticas CD34+ obtenidas de sangre de cordón umbilical. [Evaluation of proteins related to the differentiation of CD34+ hematopoietic stem cells from the umbilical cord]. *Rev Cubana Hematol Inmunol Hemoter*. 2008;24:1–17.

32. Rodríguez VM, Cuellar A, Cuspoca LM, Contreras CL, Mercado M, Gómez A. Determinacion Fenotipica de Subpoblaciones de celulas madre derivadas de sangre de cordon umbilical. [Phenotypical determinants of stem cell subpopulations derived from human umbilical cord blood]. *Biomédica*. 2006;26:51–60. doi:10.7705/biomedica. v26i1.1394

33. Barreto-Duran E, Mejia-Cruz CC, Leal-Garcia E, Perez-Nunez R, Rodriguez-Pardo VM. Impact of donor characteristics on the quality of bone marrow as a source of mesenchymal stromal cells. *Am J Stem Cells*. 2018;7(5):114–120.

34. Rodriguez-Pardo VM, Vernot JP. Mesenchymal stem cells promote a primitive phenotype CD34+c-kit+ in human cord blood-derived hematopoietic stem cells during ex vivo expansion. *Cell Mol Biol Lett*. 2013;18(1):11–33. doi:10.2478/s11658-012-0036-1

35. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng*. 2003;83 (2):173–180. doi:10.1002/bit.10655

36. Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol*. 2019;20(5):303–320.

37. Szade K, Gulati GS, Chan CKF, et al. Where hematopoietic stem cells live: the bone marrow Niche. *Antioxid Redox Signal*. 2018;29 (2):191–204. doi:10.1089/ars.2017.7419

38. Lehmann R, Lee CM, Shugart EC, et al. Human organoids: a new dimension in cell biology. *Mol Biol Cell*. 2019;30(10):1129–1137. doi:10.1091/mbc.E19-03-0135

39. Kilburn BA, Wang J, Duniec-Dmuchowski ZM, Leach RE, Romero R, Armant DR. Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. *Biol Reprod*. 2000;62(3):739–747. doi:10.1095/biolreprod62.3.739

40. Kong T, Eltzschig HK, Karhausen J, Colgan SP, Shelley CS. Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of beta2 integrin gene expression. *Proc Natl Acad Sci U S A*. 2004;101(28):10440–10445. doi:10.1073/ pnas.0401339101

41. Zanoni M, Piccinini F, Arienti C, et al. 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. *Sci Rep*. 2016;6:19103. doi:10.1038/srep19103

42. Mueller-Klieser WF, Sutherland RM. Oxygen tensions in multicell spheroids of two cell lines. *Br J Cancer*. 1982;45(2):256–264. doi:10.1038/bjc.1982.41

43. Jiang M, Chen H, Lai S, et al. Maintenance of human haematopoietic stem and progenitor cells in vitro using a chemical cocktail. *Cell Discov*. 2018;4:59. doi:10.1038/s41421-018-0059-5

44. Drake AC, Khoury M, Leskov I, et al. Human CD34+ CD133+ hematopoietic stem cells cultured with growth factors including Angptl5 efficiently engraft adult NOD-SCID Il2rgamma-/- (NSG) mice. *PLoS One*. 2011;6(4):e18382. doi:10.1371/journal.pone.00 18382

45. Pearce DJ, Taussig DC, Bonnet D. Implications of the expression of myeloid markers on normal and leukemic stem cells. *Cell Cycle*. 2006;5(3):271–273. doi:10.4161/cc.5.3.2393

46. Knapp D, Hammond CA, Hui T, et al. Single-cell analysis identifies a CD33(+) subset of human cord blood cells with high regenerative potential. *Nat Cell Biol*. 2018;20(6):710–720. doi:10.1038/s41556-018-0104-5

Profile and Management of Toxicity of Selinexor and Belantamab Mafodotin for the Treatment of Triple Class Refractory Multiple Myeloma

Karun Neupane, Ahsan Wahab, Adeel Masood, Tehniat Faraz, Saman Bahram, Hamid Ehsan, Abdul Hannan & Faiz Anwer

Published online: 01 Jul 2021.

Submit your article to this journal 🗗

View related articles 🗗

View Crossmark data 🗗

R E V I E W

Profile and Management of Toxicity of Selinexor and Belantamab Mafodotin for the Treatment of Triple Class Refractory Multiple Myeloma

Karun Neupane [1]
Ahsan Wahab[2]
Adeel Masood [3]
Tehniat Faraz [4]
Saman Bahram[5]
Hamid Ehsan [6]
Abdul Hannan[7]
Faiz Anwer[8]

[1]Department of Internal Medicine, Manipal College of Medical Sciences, Pokhara, Gandaki, Nepal; [2]Department of Internal Medicine, Baptist Medical Center South/University of Alabama at Birmingham, Montgomery, AL, USA; [3]Department of Internal Medicine, Tidal Health Peninsula Regional, Salisbury, MD, USA; [4]Department of Biochemistry, Dow University of Health Sciences, Karachi, Sindh, Pakistan; [5]Department of Internal Medicine, Dow University of Health Sciences, Karachi, Sindh, Pakistan; [6]Department of Biomedic Sciences/Biohazardous Threat Agents & Emerging Infectious Diseases, Georgetown University, Washington, DC, USA; [7]Department of Hematology/Oncology, Simmons Cancer Institute at Southern Illinois University School of Medicine, Springfield, IL, USA; [8]Department of Hematology/Oncology, Cleveland Clinic Taussig Cancer Institute, Cleveland, OH, USA

Abstract: Treatment options are limited for multiple myeloma patients who have developed four/five drug-refractory disease. Selinexor (Sel) and belantamab mafodotin (belamaf) were recently approved by the US FDA for treatment of RRMM. The toxicity profile of these drugs is a concern since these agents are used in patients who have already undergone multiple lines of treatment. In this review, we discuss the toxicity profile and strategies for the management of toxicities of Sel and belamaf for the treatment of RRMM. We conducted a comprehensive literature search on PubMed, Embase, Cochrane, and Clinicaltrials.gov using the terms "selinexor", "belantamab", "belamaf", and "multiple myeloma" without applying any limitations based on the date of the study, language, or country of origin. The most common hematological toxicity associated with these two drugs is thrombocytopenia. Cytopenias, constitutional symptoms, gastrointestinal effects, and hyponatremia are the major toxicities of Sel. Keratopathy and anemia are the major toxicities of belamaf. Treatment modifications and dose interruption are usually needed when side effects are more than grade II. As these are newer drugs with limited data, continuous surveillance and monitoring are warranted during the treatment course with early mitigation strategies.

Keywords: hematological malignancy, treatment, safety, ocular toxicity, relapsed and refractory multiple myeloma

Introduction

Multiple myeloma (MM) is the second most prevalent hematologic cancer that led to approximately 12,830 deaths in the US during 2020.[1] However, the advent of newer drugs has improved its five-year survival rate. A vast majority of MM patients require subsequent lines of therapy following relapses.[2] Treatment options are limited for those who develop the triple-class refractory disease (ie, refractory to immunomodulators (IMiDs), proteasome inhibitors (PIs), and anti-CD38 monoclonal antibodies) pressing the need for the development of newer drugs that can overcome this resistance to conventional therapy.[3]

Overview of Selinexor

Selinexor (KPT-330) is an oral, reversible inhibitor of major nuclear exporter of tumor suppressor proteins (TSPs) known as Exportin-1 (XPO1) or chromosomal maintenance 1 (CRM1) (Figure 1). XPO1 binds to the guanosine triphosphate (GTP)-binding nuclear protein called *Ran* and forms the XPO1/*Ran* GTP

Correspondence: Karun Neupane
Department of Internal Medicine, Manipal College of Medical Sciences, Phulbari, Pokhara, Gandaki, 33700, Nepal
Email krn.neupane49@gmail.com

529
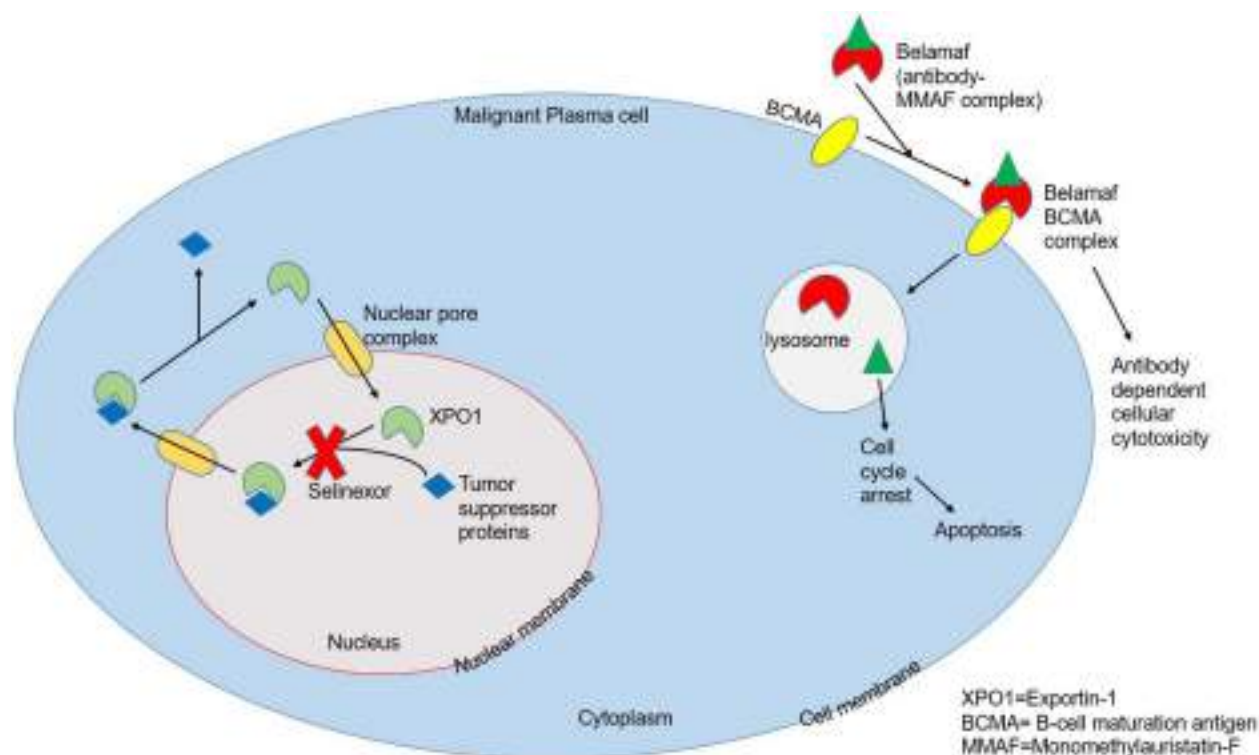
Figure 1 Mechanism of action of Selinexor and Belamaf.

nucleocytoplasmic transport complex which is responsible for the transport of many TSPs out of the nucleus such as p53, breast cancer gene 1/2 (BRCA1/2), forkhead box-O (FOXO) and growth regulatory factors (c-myc, cyclins, Mouse Double Minute 2 homolog (MDM2)). When over-expressed, XPO1 causes an aberrant distribution of these regulatory proteins localizing them within the cytoplasm, increasing the translation of oncoprotein mRNAs and functionally inactivating TSPs which gives the malignant cells a chance to evade apoptosis and thus proliferate. As a selective inhibitor of nuclear export (SINE), selinexor (Sel) causes forced nuclear retention of these compounds with subsequent cell cycle arrest and cancer cell death, largely sparing the normal cells.[3–5]

Following the encouraging results of the pivotal Phase II STORM trial,[5,6] Sel in combination with low-dose dex-amethasone (Dexa) was approved by the Food and Drug Administration (FDA) of the United States in July 2019[7] for quadrefractory (refractory to at least 2 PIs and two IMiDs) or penta-refractory MM (quadrefractory+ refrac-tory to anti-CD38 antibodies).[3,8] The adverse events (AEs) reported in the STORM,[6] STOMP[3] and BOSTON[9] trials were mostly manageable with supportive measures. Cytopenias, constitutional and gastrointestinal symptoms,

and hyponatremia were the most common AEs in these trials, with grade (G)-3 or severe thrombocytopenia occur-ring in 54% of patients.[5,10]

Overview of Belantamab Mafodotin

B-cell maturation antigen (BCMA), almost exclusively expressed on plasma cells, is an attractive drug target for the treatment of drug-resistant MM.[11,12] Belantamab mafodotin (belamaf), an anti-BCMA agent, received FDA approval in August 2020 for the treatment of adults with refractory MM who have previously received at least four therapies including PIs, IMiDs, and anti-CD38 mono-clonal antibody.[13] Belamaf is an antibody-drug conjugate (ADC) in which afucosylated humanized IgG1-antibody is conjugated to microtubule inhibitor monomethyl aurista-tin-F (MMAF) that kills myeloma cells through a multi-modal mechanism (Figure 1). This ADC complex targets BCMA and induces immunogenic cell death through anti-body-dependent cellular cytotoxicity and cellular phago-cytosis. The cytotoxic component of ADC, ie, MMAF when delivered to the target B-cells inhibits tubulin poly-merization causing cell cycle arrest at the G2/M check-point and subsequent apoptosis.[11,14,15] The use of single-agent belamaf has produced encouraging results in the

pivotal DREAMM 1 and 2 trials, with an overall response rate (ORR) of 60% in DREAMM-1, and 31% for 2.5 mg/kg vs 34% for 3.4 mg/kg cohorts of DREAMM-2.[15–17] The most common AE, keratopathy, occurring in 27% and 16% of patients receiving 2.5 mg/kg and 3.5 mg/kg doses of belamaf respectively, was manageable with supportive care along with dose adjustments and resolved after treatment completion. Thrombocytopenia and anemia were the next most common AEs.[18,19]

This review aims to discuss the toxicities associated with the use of Sel and belamaf in the treatment of heavily pretreated RRMM (relapsed/refractory MM) and to evaluate the management of these toxicities in the light of available evidence.

Materials and Methods

We conducted a comprehensive literature search on four databases including PubMed, Embase, Cochrane, and Clinicaltrials.gov. We used the search terms "selinexor", "belantamab", and "multiple myeloma". We did not apply any limitations based on the date of publication, language, or country of origin. The initial search resulted in 430 total articles. After removing duplicates and screening manually to only include articles based on human studies and those that have reported on the safety profile or management of toxicity of the two drugs belamaf and Sel, 100 articles were selected for review, including six studies on Sel and two studies on belamaf. The selection of the articles was confirmed by two authors.

Results
Dose, Combination Regimens, and Toxicity Profile of Selinexor

The dose of Sel ranged from 3–85 mg/m^2 in the first-in-human trial where Sel was used in 189 patients with advanced solid malignancies. The starting dose of 3 mg/m^2 was extrapolated from non-human studies.[20] Chen et al. investigated the safety of Sel in heavily pretreated MM patients (n=84). They administered 3–60 mg/m^2 of oral Sel either in eight doses or 10 doses per 28-day cycle in the dose-escalation phase (n=25). In the dose-expansion phase (n=59), they administered Sel 45 or 60 mg/m^2 twice-weekly along with 20 mg of Dexa in a 28-day cycle vs Sel alone with the same flat doses in the 21-day cycle.[2] Sel-Dexa combination vs Sel alone showed better overall response rates (ORR), ie, 22% vs 4%, and lower rates of serious AEs (SAEs), ie, 39% vs 61%. Given the

fewer dosage modifications, Sel 45 mg/m^2 (~80 mg) twice-weekly with 20 mg Dexa emerged as an appropriate treatment regimen for future studies.[2] The STORM phase II trial parts 1 and 2 used the same regimen of Sel-Dexa, ie, 80 mg of Sel twice-weekly along with 20 mg of Dexa in a 28-day cycle in 79 patients and 122 patients, respectively, and yielded ORR of 21% and 26%.[5,6] The STOMP phase Ib/2 study evaluated Sel-Dexa combination with bortezomib (Bort) in 42 RRMM patients. Once-weekly administration of Sel 100 mg, Dexa 40 mg, and Bort 1.3 mg/m^2 per 35-day cycle was the most tolerable regimen vs other tested regimens, with an ORR of 58%. Those without PI refractoriness had an ORR of 84% vs 43% for PI-refractory MM.[3] The Phase III BOSTON trial used the same weekly regimen of Sel-Dexa-Bort/35-day cycle and compared it with the 21-day cycle of Bort (1.3 mg/m^2)-Dexa (20 mg) twice-weekly for 8 weeks followed by Bort (1.3 mg/m^2) once-weekly and Dexa 20 mg twice-weekly.[9] Though G-3/4 hematologic (thrombocytopenia: 39% vs 17%, anemia: 16% vs 10%) and G-3/4 non-hematologic AEs except peripheral neuropathy (fatigue: 13% vs 1%, nausea: 8% vs 0%, peripheral neuropathy: 5% vs 9%) were more common with Sel-Dexa-Bort vs Bort-Dexa, once-weekly combination of Sel-Dexa-Bort showed superior median PFS (HR: 0.7, 95% CI: 0.53–0.93) and ORR (OR: 1.96, 95% CI: 1.3–3.1) compared to Bort-Dexa.[3] Recently, Jakubowiak et al evaluated the twice-weekly combination of Sel-Dexa with carfilzomib (Carf) in 21 patients with RRMM. This was a dose-escalation trial with the Sel dose ranging from 20–60 mg twice-weekly along with Carf and Dexa.[21] The recommended twice-weekly doses in this trial were Sel 60 mg, Carf 20/27 mg/m2 and Dexa 20 mg. Overall, G-3/4 thrombocytopenia (71%) and infections (24%) were the most common hematologic and non-hematologic AEs in this trial.[21]

The common G-3/4 hematological AEs as reported by these studies[2,3,5,9,20,21] were thrombocytopenia (39–71%), anemia (16–33%), leukopenia (8–33%) and neutropenia (9–33%) whereas common G-3/4 non-hematological AEs were hyponatremia (5–26%), fatigue (13–15%), diarrhea (5–10%), eye disorders (9–10%), musculoskeletal disorders (4–10%), elevated liver enzymes (10%), peripheral neuropathy (5%) and vomiting (2–4%). Gastrointestinal (GI) AEs such as nausea, vomiting, diarrhea, weight loss were mainly G-1/2 and usually reversible. The toxicities reported by these studies are summarized in Table 1. SAEs (27–63%) responsible for complications in Sel ±Dexa trials included infections (respiratory infections (n=16),

Table 1 Toxicity Profile of Selinexor

First Author, Year	Title	Subgroups	Hematological AE		Non-Hematological AE	
			Grade 1–2	Grade =/> 3	Grade 1–2	Grade =/> 3
Grosicki, 2020[9]	Once-per-week selinexor, bortezomib, and dexamethasone versus twice-per-week bortezomib and dexamethasone in patients with multiple myeloma (BOSTON): a randomized, open-label, phase 3 trial	SEL, BZM and DEX group (n=195)	Tcp: 40/195 (21%) Anemia: 40/195 (21%)	Tcp: 77/195 (39%) Anemia: 31/195 (16%)	Nausea: 83/195 (43%) Decreased appetite: 62/195 (32%) Fatigue: 56/195 (29%) Peripheral neuropathy: 54/195 (28%) Diarrhea: 51/195 (26%) Weight loss: 47/195 (24%) Cough 34/195 (17%) Constipation: 33/195 (17%) Asthenia: 32/195 (16%) Vomiting: 32/195 (16%) URTI: 30/195 (15%) Insomnia: 29/195 (15%) Back pain: 29/195 (15%) Pyrexia: 27/195 (14%) Cataract: 25/195 (13%) Peripheral edema: 22/195 (11%) Bronchitis: 20/195 (10%)	Pneumonia: 27/195 (14%) Fatigue: 26/195 (13%)

Chari, 2019[6]	Oral selinexor–dexamethasone for triple-class refractory multiple myeloma	SEL (80 mg) plus DEX (20 mg) n= 123	Anemia: 29/123 (24%) Leukopenia: 24/123 (20%) Neutropenia: 23/123 (19%) Tcp: 18/123 (15%)	Tcp: 72/123 (59%) Anemia: 54/123 (44%) Neutropenia: 26/123 (21%) Leukopenia: 17/123 (14%) Lymphopenia: 14/123 (11%)	Nausea: 76/123 (57%) Dec Appetite: 63/123 (51%) Decreased Weight: 61/123 (50%) Fatigue: 59/123 (48%) Diarrhea: 47/123 (36%) Vomiting: 43/123 (35%) URTI: 26/123 (21%) Constipation: 25/123 (20%) Dyspnea: 22/123 (18%) Cough: 21/123 (17%) Insomnia:19/123 (15%) Dizziness: 19/123 (15%) Pyrexia: 19/123 (15%) Hyponatremia: 18/123 (15%) Mental status changes: 14/123 (11%) Epistaxis:14/123 (11%) Hypokalemia: 13/123 (11%) Fall: 13/123 (11%)	Fatigue: 31/123 (25%) Hyponatremia: 27/123 (22%) Pneumonia: 13/123 (11%)
Jakubowiak, 2019[21]	Phase 1 study of selinexor plus carfilzomib and dexamethasone for the treatment of RRMM	Overall (n = 21)	Anemia: 8/21 (38%) Lymphopenia: 4/21(19%) Tcp: 2/21 (10%)	Tcp: 15/21 (71%) Anemia: 7/21 (33%) Lymphopenia: 7/21(33%) Neutropenia: 7/21(33%)	Fatigue: 14/21 (67%) Nausea: 11/21(52%) Dyspnea: 10/21 (48%) Diarrhea: 8/21 (38%) MSK disorders: 6/21(29%) Anorexia: 6/21(29%) Elevated liver and pancreatic enzymes: 6/21(29%) Eye disorders: 5/21(24%) Vomiting: 5/21(24%) Edema: 3/21(14%)	Infection: 5/21(24%) Fatigue: 3/21(14%) Diarrhea: 2/21(10%) MSK disorders: 2/21(10%) Eye disorders: 2/21(10%) Elevated liver and pancreatic enzymes: 2/21 (10%)

(Continued)

Table I (Continued).

First Author, Year	Title	Subgroups	Hematological AE		Non-Hematological AE	
			Grade 1–2	Grade =/> 3	Grade 1–2	Grade =/> 3
		Dose Level: SEL (130 mg/m²); CFZ (20/27 mg/m²); DEX (20 mg) (n = 5)	Anemia: 2/5 (40%) Lymphopenia: 1/5(20%)	Tcp: 4/5(80%) Neutropenia: 3/5(60%) Anemia: 2/5 (40%) Lymphopenia: 2/5(40%)	Nausea: 4/5(80%) Anorexia: 3/5(60%) Elevated liver and pancreatic enzymes: 3/5(60%) Dyspnea: 3/5(60%) Diarrhea: 2/5(40%) MSK disorders: 2/5(40%) Eye disorders: 2/5(40%) Vomiting: 2/5(40%) Edema: 1/5(20%) Fatigue: 1/5(20%)	Fatigue: 3/5(60%) MSK disorders: 2/5(40%) Eye disorders: 1/5(20%) Infection: 1/5(20%) Elevated liver and pancreatic enzymes: 1/5 (20%) Edema: 1/5(20%) Hyponatremia: 1/5(20%) Confusion: 1/5(20%) Psychosis: 1/5(20%)
		Dose Level: SEL (2a40 mg); CFZ (20/36 mg/m2); DEX (20 mg) (n = 3)	Anemia: 2/3 (67%)	Tcp: 3/3 (100%) Neutropenia: 2/3(67%) Lymphopenia: 1/3(33%)	Fatigue: 3/3(100%) Dyspnea: 2/3(67%) Nausea: 2/3(67%) Diarrhea: 2/3(67%) Eye disorders: 2/3(67%) MSK disorders: 1/3(33%) Elevated liver and pancreatic enzymes: 1/3(33%) Edema: 1/3(33%) Decreased appetite: 1/3(33%) Weight loss: 1/3(33%)	Dyspnea: 1/3(33%) Infection: 1/3(33%)
		Dose Level: SEL(2b60 mg); CFZ (20/27 mg/m2); DEX (20 mg) (n = 13)	Anemia: 4/13 (31%) Lymphopenia: 3/13(23%) Tcp: 2/13 (15%)	Tcp: 8/13 (62%) Anemia: 5/13 (38%) Lymphopenia: 4/13(31%) Neutropenia: 2/13(15%)	Fatigue: 10/13(77%) Dyspnea: 5/13(38%) Nausea: 5/13(38%) Diarrhea: 4/13(31%) MSK disorders: 3/13(23%) Anorexia: 3/13(23%) Vomiting: 3/13(23%) Elevated liver and pancreatic enzymes: 2/13(15%)	Infection: 3/13(23%) Diarrhea: 2/13(15%)

Chen, 2018[2]	Safety and efficacy of selinexor in RRMM and Waldenstrom Macroglobulinemia	SEL (3–23mg/m²) (n=17)	Neutropenia: 1/5(20%) Tcp: 2/17 (12%) Anemia: 2/17 (12%)	Neutropenia: 4/5(80%) Tcp: 4/17 (24%) Anemia: 2/17 (12%)	Nausea: 13/17(76%) Anorexia: 11/17(65%) Fatigue: 10/17(59%) Vomiting: 7/17(41%) Weight loss: 7/17(41%) Diarrhea: 5/17(29%) Dysgeusia: 5/17(29%) Dehydration: 3/17(18%) Blurred vision: 3/17(18%) Dyspnea: 3/17(18%)	Hyponatremia: 2/17(12%)
		SEL (30–40 mg/m2) (n=16)		Tcp: 7/16 (44%) Neutropenia: 3/16(19%) Leukopenia: 2/16(13%)	Nausea: 14/16(88%) Fatigue: 11/16(69%) Anorexia: 10/16(63%) Vomiting: 7/16(44%) Weight loss: 4/16(25%) Blurred vision: 4/16(25%) Dehydration: 3/16(19%) Diarrhea: 3/16(19%) Dysgeusia: 2/16(13%)	Hyponatremia: 6/16(38%)
		SEL (45 mg/m2) (n=11)		Tcp: 6/11 (55%) Anemia: 2/11 (18%)	Nausea: 7/11(64%) Anorexia: 5/11(45%) Vomiting: 5/11(45%) Diarrhoea: 4/11(36%) Fatigue: 4/11(36%) Dehydration: 4/11(36%) Weight loss: 2/11(18%) Blurred vision: 2/11(18%) Dysgeusia: 2/11(18%)	Fatigue: 3/11(27%) Muscle weakness: 2/11(18%)

(Continued)

Table I (Continued).

First Author, Year	Title	Subgroups	Hematological AE		Non-Hematological AE	
			Grade 1–2	Grade =/> 3	Grade 1–2	Grade =/> 3
		SEL (60 mg/m2) (n=4)	Anemia: 1/4 (25%)	Tcp: 2/4(50%) Neutropenia: 2/4(50%) Leukopenia: 1/4(25%) Anemia: 1/4 (25%)	Nausea: 3/4(75%) Anorexia: 2/4(50%) Vomiting: 2/4(50%) Weight loss: 2/4(50%) Fatigue: 1/4(25%) Dehydration: 1/4 (25%) Dysgeusia: 1/4 (25%) Muscle weakness: 1/4(25%) Diarrhea: 1/4(25%)	Hyponatremia: 1/4(25%) Confusion: 1/4(25%)
		SEL (45 mg/m2) + DEX (20mg) (n=14)		Tcp: 9/14 (64%) Anemia: 5/14 (36%) Neutropenia: 5/14 (36%) Leukopenia: 3/14 (22%)	Nausea: 12/14(86%) Anorexia: 8/14(57%) Fatigue: 7/14(50%) Blurred vision: 6/14(43%) Vomiting: 6/14(43%) Diarrhea: 4/14(29%) Dysgeusia: 4/14(29%) Dyspnea: 3/14(21.4%) Weight loss: 2/14(14%) Dehydration: 2/14(14%) Confusion: 2/14(14%)	Fatigue: 4/14(29%) Hyponatremia: 4/14(29%)
		SEL (60 mg/m2) + DEX (20mg) (n=11)	Tcp: 2/11 (18%) Anemia: 2/11 (18%)	Tcp: 4/11 (36%) Anemia: 3/11 (27%) Neutropenia: 2/11(18%)	Fatigue: 7/11 (64%) Anorexia: 7/11 (64%) Nausea: 5/11 (46%) Vomiting: 4/11(36%) Diarrhea: 3/11 (27%) Weight loss: 3/11(27%) Hyponatremia: 2/11(18%) Blurred vision: 2/11(18%) Dysgeusia: 2/11(18%) Confusion: 2/11(18%)	Hyponatremia: 6/11(55%) Nausea: 2/11 (18%) Fatigue: 2/11 (18%)

				Hyponatremia: 3/11 (27%)
	SEL (40–60mg) (n=11)	Tcp: 6/11 (55%) Anemia: 5/11 (45%) Neutropenia: 3/11 (27%)	Fatigue: 8/11 (73%) Anorexia: 8/11 (73%) Nausea: 7/11 (64%) Weight loss: 5/11 (45%) Dehydration: 4/11 (36%) Muscle weakness: 4/11 (36%) Diarrhea: 3/11 (27%) Vomiting: 2/11 (18%) Dyspnea: 2/11 (18%) Confusion: 2/11 (18%)	
Bahlis, 2018[3]	All doses (n=42)	Tcp: 19/42 (45%) Neutropenia: 10/42 (24%) Anemia: 5/42 (12%)	Nausea: 24/42 (57%) Decreased appetite: 24/42 (57%) Fatigue: 19/42 (45%) Diarrhea: 15/42 (36%) Vomiting: 12/42 (29%) Decreased weight: 8/42 (19%) Blurred vision: 8/42 (19%) Dysgeusia: 6/42 (14%) Dehydration: 5/42 (12%) Cataract: 5/42 (12%) Peripheral edema: 5/42 (12%) Confusion: 4/42 (10%) Peripheral neuropathy: 4/42 (10%)	Fatigue: 6/42 (14%)

Selinexor plus low dose bortezomib and dexamethasone for patients with RRMM

(Continued)

Table 1 (Continued).

First Author, Year	Title	Subgroups	Hematological AE		Non-Hematological AE	
			Grade 1–2	Grade =/> 3	Grade 1–2	Grade =/> 3
		60, 80 mg SEL QW/BIW + 1.3 mg/m2 BZM QW/BIW (n=16)		Tcp: 11/16 (69%) Neutropenia: 5/16 (31%) Anemia: 4/16 (25%)	Fatigue: 9/16 (56%) Decreased appetite: 8/16 (50%) Diarrhea: 7/16 (44%) Nausea: 5/16 (31%) Decreased weight: 5/16 (31%) Vomiting: 4/16 (25%) Peripheral neuropathy: 4/16 (25%) Cataract: 3/16 (19%) Peripheral edema: 3/16 (19%) Blurred vision: 2/16 (13%) Dysgeusia: 2/16 (13%) Dehydration: 2/16 (13%)	Nausea: 2/16 (13%) Diarrhea: 2/16 (13%)
		100 mg SEL QW + 1.3 mg/m2 BZM QW (n=26)		Tcp: 8/26 (31%) Neutropenia: 5/26 (19%)	Nausea: 19/26 (73%) Decreased appetite: 16/26 (62%) Fatigue: 10/26 (38%) Diarrhea: 8/26 (31%) Vomiting: 8/26 (31%) Blurred vision: 6/26 (23%) Dysgeusia: 4/26 (15%) Dehydration: 3/26 (12%) Confusion: 3/26 (12%) Decreased weight: 3/26 (12%)	Fatigue: 6/26 (23%)
Vogl, 2018[5]	Selective inhibition of nuclear export with oral selinexor for treatment of RRMM	SEL (80 mg) and DEX (20 mg) (n=79)	Leukopenia: 18/79 (23%) Anemia: 17/79 (22%) Tcp: 11/79 (14%)	Anemia: 22/79 (28%) Neutropenia: 18/79 (23%) Leukopenia: 12/79 (15%) Lymphopenia: 9/79 (11%)	Nausea: 52/79 (66%) Fatigue: 38/79 (48%) Decreased appetite: 37/79 (47%) Vomiting: 32/79 (41%) Diarrhea: 30/79 (38%) Decreased weight: 25/79 (32%) Hyponatremia: 16/79 (20%) Dysgeusia: 9/79 (11%) Dizziness: 8/79 (10%)	Hyponatremia: 17/79 (22%) Grade 3 infections 14/79 (18%) Fatigue 12/79 (15%)

Abbreviations: Tcp, thrombocytopenia; AE, adverse events; MSK, musculoskeletal; URTI, Upper respiratory tract infection; RRMM, relapsed/refractory multiple myeloma; QW, once weekly; BIW, twice weekly; SEL, selinexor; CFZ, carfilzomib; DEX, dexamethasone; BZM, bortezomib.

sepsis (n=12), bacteremia (n=4)), fever with/without neutropenia (n=14), encephalopathy/delirium/confusion (n=11), anemia (n=6), hyponatremia (n=6), dehydration (n=6), renal failure (n=4), nausea/vomiting (n=4), thrombocytopenia (n=4), elevated LFTs (n=2), intracranial hemorrhage (ICH) (n=2) and GI bleeding (n=1).[2,5,6] SAEs due to Sel-Dexa-Bort were febrile neutropenia (n=2), full-thickness macular hole in eye (n=1), and pulmonary embolism (n=1).[3] Sel-Dexa-Carf use resulted in infections more commonly (n=7) as SAEs, GI bleeding (n = 1; unrelated to treatment), syncope (n = 1), thromboembolism (n = 1), pain related to progressive disease (n = 1) and systolic heart failure (n = 1).[21]

Management of Selinexor Toxicity
Hematologic Toxicity

Thrombocytopenia, the primary hematological toxicity of Sel, occurs via inhibition of thrombopoietin (TPO)-signaling in the megakaryocyte maturation phase and can be managed with the use of TPO agonists (eltrombopag or romiplostim), platelet infusions, and drug holidays. Those with baseline thrombocytopenia are more prone to develop high-grade thrombocytopenia thereby predisposing them though rarely, to life-threatening bleeding events such as ICH or GI bleeding.[2,6] Therefore, Sel needs to be avoided unless the platelet count is ≥50,000/mm.[3,6] On a similar note, patients who received platelet infusion within one week or TPO agents within 2 weeks prior to the first dose of Sel should not be administered Sel; those patients were excluded from the STORM trial part 2. Both in-vivo and in-vitro studies affirm that Sel-mediated thrombocytopenia is reversible with TPO-agonists ensuring that Sel has been washed out and there is no more ongoing Sel use. Otherwise, TPO-agents get antagonized by the TPO-blocking mechanism of Sel causing ongoing thrombocytopenia.[20] There are no strict guidelines about the TPO-agents' use in Sel toxicity and their use is solely at the discretion of treating hematologists. In the BOSTON trial where TPO-agents were used in 18% of patients with thrombocytopenia, the events of dose reductions or interruptions were significantly reduced.[9] Sel-induced thrombocytopenia occurs in a dose-dependent fashion; one study looked at platelet drop at day 29 with different Sel doses and found evidence of less thrombocytopenia with its lower doses (50–70 mg) (n=36) vs higher doses >100 mg biweekly (n=28). The interruption of Sel dose for 8–21 days resulted in improvement in G-4 thrombocytopenia.[20] Those who are receiving FDA approved dose of Sel and

develop G-4 thrombocytopenia without bleeding should stop taking Sel until at least G-3. When at G-3, Sel dose needs to be reduced from 80 mg twice-weekly (~160 mg/week) to 100 mg once weekly until thrombocytopenia improves to G-2. Following this, Sel can be resumed in two divided doses per week (total 100 mg/week), ie, 60 mg, 40 mg.[6]

Adequate hematopoietic functions such as hemoglobin>8 gm/dl and absolute neutrophils >1000/mm^3 should be ensured before Sel treatment, preferably in the absence of recent blood transfusions, erythropoietin (EPO) analogues or colony-stimulating factors (CSF). For G-3/4 anemia and neutropenia, Sel needs to be interrupted (counts can be boosted with EPO and CSF analogues per the discretion of the hematologist) as well until toxicity improves to G-2 and then Sel can be resumed at 60 mg twice-weekly. As it is critical to modify the Sel treatment with respect to hematologic toxicity, the most important aspect of managing its toxicity is to monitor blood cell counts ideally before each dose of Sel so that the subsequent dose could be modified. However, given twice-weekly dosing of oral Sel, performing blood counts twice weekly might appear cumbersome which needs to be balanced with the clinical utility of these labs. Therefore, we recommend checking complete blood counts at least once-weekly in the first two cycles of Sel as a majority of these AEs require treatment modifications during this timeframe. Venous thromboembolism (5% of the patients in one study), requires anticoagulation.[21]

Gastrointestinal Toxicity

GI toxicity such as nausea, anorexia/decreased appetite, vomiting, diarrhea, and weight loss are usually centrally mediated and though low-grade, can limit the tolerability of Sel.[2] However, the addition of Dexa and the use of prophylactic antiemetics have improved its tolerance.[2] Most of the GI AEs, particularly vomiting are usually severe in the first 2 weeks and may decrease over time. Antiemetic use has been reported in 89–100% of the patients receiving Sel and many patients might need two (14–33%) or three antiemetics (5%).[3,5,6] For nausea/vomiting, 8 mg of ondansetron or equivalent antiemetics such as prochlorperazine, granisetron can be used before the first dose of Sel, and then ondansetron 8 mg should be given as needed twice daily or thrice daily at least for 2 days.[5,6] Those with persistent nausea despite ondansetron or equivalent can be given olanzapine and neurokinin-1 receptor blockers such as aprepitant and rolapitant.[5,22]

In STORM trial part 1, eight doses of Sel were compared with six doses of Sel in a 28-day cycle, the rate of nausea was 82% vs 69%, respectively.[5] Sel should be interrupted in case of G-3 nausea until it improves to G-2 and then Sel can be resumed at 60 mg twice-weekly dosing.[6] GI toxicity is one of the most common causes of Sel termination. In one study, the treatment was terminated due to GI or constitutional AEs in 5/8 patients.[23] Prophylactic antiemetics can successfully avoid interruptions, dose reductions, or treatment terminations. It is of note that the once-weekly maximal dose of Sel of 100 mg along with the once-weekly Bort did not result in G-3/4 nausea, vomiting, or anorexia and was well tolerated with prophylactic antiemetics.[3]

Anorexia and weight loss in cancer patients receiving Sel can be multifactorial and result from both chemotherapy and underlying malignancy itself. The use of appetite stimulants in addition to low-dose Dexa such as dronabinol, metoclopramide, and megestrol may improve appetite and cause modest weight gain.[3,24] One randomized controlled trial comparing dronabinol with megestrol reported megestrol to be superior compared to dronabinol both for improving the appetite (75% vs 49%) and causing weight gain (11% vs 3%). The study found no difference when dronabinol-megestrol combination was compared to megestrol alone.[25] In a study by Jakubowiak et al., 100% of patients (n=21) received prophylactic megestrol acetate (160–400 mg daily) and 5-HT3 antagonist but 29% (n=6) of patients remained anorexic and 5% (n=1) patients experienced ongoing weight loss. The combination of antiemetics, however, was more effective in managing treatment interruptions since none (n=0/21) of the patients required treatment discontinuations due to GI AEs.[21] Another common AE of Sel is G-1/2 diarrhea that might require antidiarrheals. G-3/4 diarrhea was reported in 10% of patients by Jakubowiak et al[21] (n=2/21) and even less commonly (5–7%) by other investigators.[5,23,26] For ≥G2 diarrhea, Sel should be interrupted and resume when resolved to G1 but at 60 mg twice-weekly dose. One unrelated meta-analysis of eight randomized controlled trials found octreotide to be effective for severe cases of chemotherapy-induced diarrhea when compared to placebo (69% vs 54%).[27,28] Dysgeusia reported in 10–17% of the patients in Sel studies is common with many other therapies but lacks evidence-based treatment strategies.[29]

Renal Toxicity and Electrolyte Derangements

Dehydration and AKI are less common G-3/4 AEs with Sel and have a favorable outcome. These can be managed with outpatient fluid resuscitation or inpatient care depending upon severity.[30] Thus, far only one case of irreversible renal failure has been reported leading to treatment discontinuation.[6] G-3/4 hyponatremia is also common with Sel and should be managed with Sel interruption and dose reductions. Sel should be resumed at 60 mg twice weekly when the hyponatremia is at least G1 or resolved. Hyponatremia, even though G-3, is mostly asymptomatic and needs sodium replacement along with frequent lab monitoring. In the STORM trial part 1, only 6% of patients required salt tablets as compared to 22% of patients diagnosed with hyponatremia.[5] Chen et al. attributed a number of cases of delirium to hyponatremia. Delirium usually requires supportive care and correction of metabolic derangements such as hyponatremia.[2]

Dose Adjustments

Early intervention with supportive care prevents the need for dose reduction and interruption. Treatment holidays and dose reductions were required in 52% and 37% of the study population in STORM part 1 and resulted in significantly less treatment termination (18%). This study had only three patients who received a higher dose of Sel 100 mg twice-weekly with all three patients requiring drug holidays or dose reduction.[5] In the STORM part 2, 80% of patients required dose modifications or holidays and the majority of those events occurred in the first 2 cycles, demanding an aggressive prophylactic treatment, and monitoring needs in the initial cycles.[6] About 17.2% of patients discontinued treatment due to treatment-related AEs. Bahlis et al. studied various dosing regimens for Bort and Sel in different combinations. There was no significant increase in G-3/4 AEs with Sel 100 mg weekly compared to 60 mg or 80 mg weekly regimen.[3] Jakubowiak et al reported 80% of patients receiving Sel needed a change in dosage or holiday from therapy but only 18% discontinued treatment due to AEs and 10% mortality (n=12/123) was attributed to major AEs. The rest recovered with conservative management.[21] Dose/treatment modifications to minimize adverse events are summarized in Table 2.

Toxicity Profile of Belantamab Mafodotin

Trudel et al. reported the first-in-human Phase I trial (DREAMM-1) of belantamab mafodotin (belamaf) which included a dose-escalation phase (part 1, n=38) and a dose-expansion phase (part 2, n=35). Based on the results of part 1, 3.4 mg/kg was the recommended dose in part 2.[16,17]

Table 2 Adverse Events Leading to Modifications in Treatment Plan in Selinexor Studies

	Treatment Holiday/Interruption	Dose Reduction	Treatment Termination
Vogl et al[5]	52% (n=41/79)	37% (n=29/79)	18% (n=14/79)
Bahlis et al[3]		50% (n=21/42)	19% (n=8/42)
Jakubowiak et al[21]	80% (n=17/21)	62% (n=13/21)	10% (n=2/21)
Chen et al[2]			31% (n=26/84)

Lonial et al conducted a phase II study (DREAMM-2) with two dosing cohorts in 196 RRMM patients. Ninety-seven patients were treated with 2.5 mg/kg and 99 patients with 3.4 mg/kg of belamaf.[15] In part 1 of the DREAMM-1, no dose-limiting AEs were reported and there was no maximum tolerated dose. The most common G-3/4 AEs were thrombocytopenia [13/38 (34%)] and anemia [6/38 (16%)]. In part 2 of DREAMM-1, G-3/4 AEs were seen in 28/35 (80%) patients, the most common being thrombocytopenia [12/35 (34%)] and anemia [5/35 (14%)]. SAEs were seen in 40% of patients, the most common of which were infusion-related reaction (IRR) (n=2) and lung infection (n=2). Five patients had drug-related SAEs including IRR (n=2), ICH (n=1), lung infection (n=1) and pericardial effusion (n=1).[17]

In the DREAMM-2, the most common G-3/4 AEs were keratopathy seen in 26/95 (27%) patients in the 2.5 mg/kg cohort and 34/99 (34%) patients in the 3.4 mg/kg cohort. Thrombocytopenia was seen in 19/95 (20%) and 33/99 (33%), respectively, for 2.5 mg/kg and 3.4 mg/kg cohorts and anemia in 19/95 (20%) and 25/99 (25%) respectively. Among those who received prophylaxis for IRR, 8/22 (2.5 mg/kg cohort) and 6/27 (3.4 mg/kg cohort) patients developed IRR. One patient in the 2.5 mg/kg cohort discontinued treatment due to G-3 IRR.[15] In part 1 of the DREAMM-1, dose reduction was required in 1/3 (33%) patients receiving 1.92 mg/kg, 1/8 (13%) patients receiving 2.50 mg/kg, 3/3 (100%) patients receiving 3.40 mg/kg and 5/6 (83%) in 4.6 mg/kg dose. Moreover, 1/4 (25%) patients receiving 1.92 mg/kg and 2/6 (33%) patients receiving 4.6 mg/kg dose in part 1 discontinued treatment due to AEs which included limbal cell defect, foreign body sensation in eyes plus thrombocytopenia, and hypercalcemia. The blurring of vision (40%) was the most common cause of interruption or delay in belamaf therapy in the DREAMM-1.[17] In part 2 of DREAMM-1, belamaf related AEs include IRR, thrombocytopenia, and corneal events. Two patients (6%) discontinued treatment and 7 (20%) required dose reduction/delays because of thrombocytopenia. AEs led to dose reduction in 23/45 (66%) and

dose interruption/delay in 25/45 (71%) patients.[17] In DREAMM-2, 93/95 (98%) patients in 2.5mg/kg cohort and 99/99 (100%) patients in 3.4 mg/kg cohort had at least one AE. AEs led to dose delays in 54% (2.5mg/kg cohort) and 62% (in 3.4mg/kg cohort) patients while dose reduction was required in 29% (2.5mg/kg cohort) and 41% (3.4 mg/kg cohort) patients. About 8% (2.5 mg/kg cohort) and 10% (3.4mg/kg cohort) patients permanently discontinued the treatment due to AEs in DREAMM-2 the most common of which being keratopathy seen in 1 (2.5mg/kg cohort) and 3 (3.4 mg/kg cohort) patients.[15] One death occurred in part 1 of DREAMM-1 study which was attributed to disease progression. Three deaths occurred in part 2 owing to disease progression. No treatment-related deaths were reported in DREAMM-1.[17] A total of two potentially treatment-related deaths were reported in DREAMM-2, one due to sepsis in the 2.5 mg/kg cohort and one due to hemophagocytic lymphohistiocytosis in the 3.4 mg/kg cohort.[15]

In part 1 of DREAMM-1, the frequency of G-3-4 corneal AEs increased with the increased dose of the drug. Corneal AEs were reported in 20/38 (53%) patients, most of which were mild G-1/2 seen in 18/38 (47%) but it resulted in treatment discontinuation in two patients. In part 2 of DREAMM-1, corneal events were seen in 22/35 (63%) patients comprising of mild-moderate (G-1/2) in 19 and G-3 in 3 (keratitis in 1, eye pain in 1, and dry eye in 1) patients. The median time to onset of corneal events was 23 days (range: 1–84 days) while the median duration of patients with a resolution date was 30 days (range: 5–224 days). Thirty-one (89%) patients had corneal findings on the ophthalmic examination which included superficial punctate keratitis 27/35 (77%), epithelial edema 22 (63%), stromal edema 5 (14%), and opacities 8 (23%). No patients discontinued treatment in part 2 due to corneal AEs.[17] In DREAMM-2, keratopathy was the most common cause of permanent treatment discontinuation with 1% (2.5mg/kg cohort) and 3% (3.4mg/kg cohort). It led to dose reduction in 23% (2.5 mg/kg cohort) and 27% (3.4mg/kg cohort) and dose delays in 27% (2.5mg/kg

cohort) and 48% (3.4 mg/kg cohort) patients. The most common reported corneal symptoms were blurred vision and dry eye in two patients without keratopathy. In the ocular sub-study (n=30; 17 patients in 2.5 mg/kg cohort and 12 patients in 3.4mg/kg cohort), G-3 AEs were reported in 29% (2.5mg/kg cohort) and 42% (3.4mg/kg cohort) in treated eye and 18% (2.5mg/kg cohort) and 50% (3.4 mg/kg cohort) in untreated eye.[15] The toxicity profile of belamaf has been summarized in Table 3.

Management of Toxicity of Belantamab Mafodotin

Cytotoxic payload and linker instability are postulated to cause the ocular toxicity associated with ADC.[31] Previously many clinical trials[31–33] of refractory hematologic malignancies have used MMAF cytotoxin and maleimidocaproyl linker and have reported similar ocular toxicity as reported in DREAMM-1 and DREAMM-2 trials due to belamaf. Ophthalmic steroid drops in DREAMM-1 were used to mitigate the ocular toxicity given the established side effects of MMAF.[16] However, ocular toxicity (blurring of vision, eye pain, and dryness, keratitis, and photophobia) still occurred, especially with the increasing doses of the drug.[17] Permanent discontinuations due to corneal events were rare in this trial and the majority of these AEs were successfully managed with dose reductions, interruptions, or delays. About 50% of individuals with corneal events showed a resolution within about 35 days.[17] On follow-up interviews of 17 patients from the second part of DREAMM-1 trial at the end of treatment, 76% of patients had reported of blurred vision while on treatment but 62% either had resolution or ongoing improvement in the complaint. The majority of those who participated in this interview never considered treatment discontinuation.[34] Ophthalmic evaluation while on belamaf plays a crucial role in the early detection of keratitis, epithelial and stromal edema. Therefore, serial ophthalmic evaluation at baseline and before subsequent doses have a critical role as a mitigation strategy and may prompt treatment adjustments. Popat et al reported a case series of 5 patients from the DREAMM-1 trial and shared experience from their center at a median follow-up of 32.6 months. When corneal AEs occurred, they increased the frequency of topical steroids (prednisolone eye drops 1–2 drops up to four times a day), used preservative-free artificial tears (such as artificial tears 1–2 drops twice a day as needed), and interrupted the next dose (median 14

days, range: 7–98 days). These patients developed increased intraocular pressure, infection, and secondary cataract formation given the excessive use of topical steroids and required topical antibiotics and cataract extractions. Therefore, Popat et al. recommended against the long-term use of topical steroids and proposed dose modifications or interruptions as the main strategy to deal with corneal side effects.[35]

DREAMM-2 trial investigators made ophthalmic evaluations a part of their protocol. The reports of changes in visual acuity were also subject to strict follow-up. Those patients with visual changes experienced ultimate resolution and none of them had permanent vision loss. An ocular substudy, part of the DREAMM-2, verified no role of topical steroids in preventing corneal side effects. Dose reductions or delays were the most effective strategies. DREAMM-2 trial, therefore, recommends 25% dose reduction if G-2 corneal events have been experienced and interruption or delay of the dose if G-3/4 corneal events have been experienced. The dose should be delayed until the corneal event is improved to G-2 and then belamaf with a 25% reduced dose should be given. In the United States, belamaf has been available since August 2020 under a Risk Evaluation and Mitigation Strategy (REMS) given ocular toxicity, called BLENREP REMS.[13] Dosage modifications of belamaf in RRMM can be made based on the Keratopathy and Visual Acuity (KVA) scale documented in the prescribing information of BLENREP (Table 4).[13,19,36] This scale was developed by GlaxoSmithKline (GSK) upon the recommendation of the FDA.

In DREAMM-1, onset of thrombocytopenia was observed for 50% at 7.5 days and 50% had resolution after 8 days of onset. Only 6% had discontinuation of therapy due to thrombocytopenia. These side effects can be managed with modification in the treatment plan.[16,17] However, in DREAMM-2, thrombocytopenia was considered self-limiting. A total of 22 patients (11%) reported bleeding of G-2 or worse.[15] Serious infections such as pneumonia or lung infection might require dose interruptions or delays.

Discussion

Despite advancements in MM treatment over decades, with many active drugs and the use of hematopoietic stem cell transplantation/autologous stem cell transplantation, it remains incurable, and invariably patients with MM relapse and require therapies for the treatment of relapse.[37] During the course of illness, a considerable

Table 3 Toxicity Profile of Belantamab

Author, Year	Title	Subgroups	Hematological AE		Non Hematological AE	
			Grade 1–2	Grade =/> 3	Grade 1–2	Grade =/> 3
Trudel, 2019[17]	Antibody–drug conjugate, GSK2857916, in RRMM: an update on safety and efficacy from dose expansion phase I study	Part 2 (n=35)	Tcp: 10/35 (29%) Anemia: 4/35 (11%)	Tcp: 12/35 (34%) Anemia: 6/35 (17%)	Blurred vision: 17/35 (49%) Cough: 14/35 (40%) Dry eye: 12/35 (34%) Inc AST: 11/35 (31%) Nausea: 11/35 (31%) Photophobia: 10/35 (29%) Pyrexia: 10/35 (29%) Chills: 9/35 (26%) Diarrhea: 8/35 (23%) Fatigue: 8/23 (23%) URTI: 8/35 (23%) Inc ALT: 7/35 (20%) Constipation: 6/35 (17%) Inc ALP: 6/35 (17%) Back pain: 5/35 (14%) Inc GGT: 5/35 (14%) Arthralgia: 5/35 (14%) Dyspnea: 5/35 (14%) Contusion: 5/35 (14%) Decreased appetite: 5/35 (14%) Headache: 5/35 (14%) Sinusitis: 5/35 (14%)	Diarrhea: 4/35 (11%)

(*Continued*)

Table 3 (Continued).

Author, Year	Title	Subgroups	Hematological AE		Non Hematological AE	
			Grade 1–2	Grade =/> 3	Grade 1–2	Grade =/> 3
Lonial, 2020[15]	Belantamab mafodotin for RRMM (DREAMM-2): a two-arm, randomised, open-label, phase 2 study	Belantamab mafodotin 2.5 mg/kg group (n=95)	Infusion related reactions[a]: 17/95 (18%) Tcp: 14/95 (15%)	Tcp: 19/95 (20%) Anemia: 19/95 (20%) Lymphopenia: 12/95 (13%)	Keratopathy or changes to corneal epithelium: 41/95 (43%). Nausea: 23/95 (24%) Fever: 18/95 (19%) Blurred vision: 17/95 (18%) Inc AST: 17/95 (18%) Fatigue: 13/95 (14%) Dry Eye: 12/95 (13%) Constipation: 12/95 (13%) Diarrhea: 11/95 (12%) Dec Appetite: 11/95 (12%) Arthralgia: 10/95 (11%)	Keratopathy or changes to corneal epithelium: 26/95 (27%).
		Belantamab mafodotin 3.4 mg/kg group (n=99)	Tcp: 24/99 (24%) Infusion related reactions[a]: 15/99 (15%) Hypercalcemia: 13/99 (13%) Anemia: 12/99 (12%) Neutropenia: 12/99 (12%)	Tcp: 34/99 (34%) Anemia: 25/99 (25%) Neutropenia: 15/99 (15%)	Keratopathy or changes to corneal epithelium: 53/99 (54%) Nausea: 31/99 (31%) Blurred vision: 28/99 (28%) Dry eye: 23/99 (23%) Fever: 21/99 (21%) Fatigue: 21/99 (21%) Vomiting: 20/99 (20%) Cough: 19/99 (19%) Inc AST: 18/99 (18%) Epistaxis: 17/99 (17%) Dec Appetite: 16/99 (16%) URTI: 16/99 (16%) Diarrhea: 14/99 (14%) Headache: 13/99 (13%) Inc ALP: 12/99 (12%) Hypokalemia: 11/99 (11%) Pain in extremity: 11/99 (11%) Inc blood creatinine: 10/99 (10%)	Keratopathy or changes to corneal epithelium: 21/99 (21%) Pneumonia: 11/99 (11%)

Notes: [a]Infusion-related reactions (considered an adverse event of special interest) includes preferred terms infusion-related reaction, pyrexia, chills, diarrhea, nausea, asthenia, hypertension, lethargy, tachycardia, vomiting, cough, and hypotension occurring within 24 hours of infusion.
Abbreviations: AE, adverse events; Tcp, Thrombocytopenia; AST, Aspartate aminotransferase; ALT, Alanine transaminase; GGT, Gamma-glutamyl transferase; URTI, Upper respiratory tract infection; Inc, increased.

Table 4 Management of Ocular Toxicity of Belamaf

Severity	Extent of Vision Loss	Treatment Recommendations
Grade 1	One line decline of best corrected visual acuity from baseline on Snellen Visual Acuity chart OR mild superficial keratopathy on corneal examination.	Treatment to be continued at current dose without any modification
Grade 2	2 or 3 lines decline of best corrected visual acuity from baseline on visual acuity chart (not worse than 20/200) OR moderate superficial keratopathy on corneal examination.	Hold belamaf until improves to grade 1 and resume same dose afterwards
Grade 3	More than 3 lines decline of best corrected visual acuity from baseline on visual acuity chart (not worse than 20/200) OR severe superficial keratopathy on corneal examination.	Hold belamaf until improves to grade 1 and then resume 25% reduced dose
Grade 4	Severely reduced vision (visual acuity worse than 20/200 on Snellen Visual Acuity chart) OR corneal epithelial defects on corneal examination.	Belamaf treatment termination with urgent ophthomology consult

number of patients develop a refractory disease to three classes of commonly used drugs (PIs, IMiDs, and monoclonal antibodies). Overall survival in three-class, quad, or penta refractory disease is short.[6] MAMMOTH study reported outcomes of MM patients who were refractory to anti-CD38-monoclonal antibody and other agents. The median overall survival after anti-CD38 antibodies refractoriness was 8.6 months, 11.2 months in patients who were not simultaneously refractory to one IMiD and one PI, and 5.6 months in patients who were refractory to anti-CD-38 antibodies, two PIs, and two IMiDs (penta-refractory).[38] After multiple lines of treatment exposures, at the time of relapses, such patients have underlying marrow suppression and cumulative toxicities. Therefore, it becomes essential that they maintain a good quality of life while we use newly approved drugs such as Sel and belamaf.

After multiple prior lines of therapy, the selection of the appropriate next line of therapy is crucial in the context of prior toxicities, including profound thrombocytopenia. Sel at recommended doses (80 mg twice weekly) may not be an appropriate treatment choice for such patients due to the risk of ICH and GI bleeding depending on the severity of thrombocytopenia and should be avoided unless the platelet count is at least 50,000. However, thrombocytopenia is reversible with drug interruption and the use of TPO agents.[39] For severe thrombocytopenia, platelet transfusions have been shown to be effective in quickly increasing the platelet levels. TPO agonists (romiplostim or eltrombopag) can be used to increase platelet counts over two to 3 weeks while continuing the treatment with selinexor. TPO agonists should be used when platelet counts fall

below 25,000/mm^3 until the count rises to ≥50,000/ mm^3.[40] Frequent monitoring of platelet counts during Sel treatment is highly recommended. Sel should be interrupted for G4 thrombocytopenia and the dose should be reduced for G3/2 thrombocytopenia. Any life-threatening bleeding event history such as ICH should be carefully weighed against the re-induction of treatment and its benefits and reintroduction should be avoided if at all possible.

When considering belamaf, it is of utmost importance that the treating hematologist is aware of its ocular toxicity and its management strategies as it may have dire visual consequences. Belamaf is currently available under REMS program that requires special certification for prescribers. About 76% of the patients in the DREAMM-1 reported some ocular complications.[16] A baseline ophthalmic evaluation and proper documentation of any visual problems using a KVA scale should be performed. Following the baseline evaluation, findings should be documented before each dose to monitor any change and tailor treatment according to the findings. It is prudent for the treating hematologist to discuss ocular toxicity with the ophthalmologist and request findings based on the KVA scale as it is a relatively newer drug, and many ophthalmologists might have limited experience. The patient on belamaf should be strictly advised to use preservative-free eye drops four times a day. The documentation of the use of contact lenses should be made and, if possible, avoided as it may worsen the keratitis. The DREAMM-2 ocular substudy data did not demonstrate a clinical benefit of prophylactic topical steroids and therefore should be avoided.[15] Preferably a strategy of dose interruption and reductions based on the KVA scale should be employed in

Table 5 Management of Toxicities of Selinexor and Belamaf

Drug	Type of Toxicity	Nature of Toxicity	Management
Selinexor	**Non-hematological**	Infection/pneumonia	Antibiotics Hospitalization for supportive care
		Fatigue[a]	Physical therapy
		Diarrhea[a]	Loperamide Diphenoxylate/atropine Octreotide (severe or refractory)
		Hyponatremia[a]	Sodium replacement (normal saline or salt tablets)
		Elevation of liver[a] and pancreatic enzymes	Watchful monitoring (transient only)
		Dehydration	Fluid resuscitation (infusion center vs inpatient)
		Nausea[a]/Vomiting	Antiemetics (Ondansetron, prochlorperazine, granisetron, aprepitant)
		Anorexia/Weight loss[a]	Combine with dexamethasone Dronabinol Megestrol acetate Metoclopramide
		Dysgeusia	Supportive
		Confusion	Correct underlying cause like hyponatremia
		Cataract	Surgical extraction
	Hematological	Thrombocytopenia	Dose reduction or interruptions Treatment holidays Platelet transfusion Thrombopoietin receptor agonists
		Anemia[a]	Packed RBC Transfusion
		Neutropenia[a]	Granulocyte Colony Stimulating Factor
Belantamab mafodotin (Belamaf)	**Non-hematological**	Keratopathy	Avoid use of ophthalmic steroid drops (risk of steroid-induced glaucoma, cataract, and infection)
		Blurred Vision	Serial ophthalmic evaluations
		Visual acuity decline	Use of keratopathy and visual acuity scale *(KVA)* to decide future treatments of belamaf.
		Photophobia	Management of Ocular toxicity: see Table 3
		Dry Eyes	Preservative free artificial tears
		Pyrexia/headache/arthralgia	Acetaminophen/Ibuprofen
		Constipation	Laxatives
		Epistaxis	Correct underlying thrombocytopenia if severe Symptomatic management
		Acute kidney injury	Fluid resuscitation can be inpatient or infusion center
		Hypokalemia	Replace potassium orally (outpatient) or intravenous (inpatient)
		Upper respiratory infection	Antibiotics

(*Continued*)

Table 5 (Continued).

Drug	Type of Toxicity	Nature of Toxicity	Management
		Cough	Dextromethorphan
		Hypercalcemia	Ca <12 mg/dl: No treatment Ca 12–14 mg/dl: Normal saline and bisphosphonates Ca >14 mg/dl: Calcitonin or zoledronic acid in addition to normal saline
		Infusion-related reactions[b]	Premedication helpful. Supportive care for symptoms
	Hematological	Thrombocytopenia	Platelet 25,000/mm^3 to <50,0000/mm^3: Withhold and/or reduce the dose Platelet <25,000/mm^3: Withhold drug

Notes: [a]Adverse Events of belantamab as well. Management same as selinexor. [b]Infusion-related reactions include a myriad of symptoms related to infusion such as pyrexia, chills, diarrhea, nausea, asthenia, hypertension, lethargy, tachycardia, vomiting, cough, and hypotension occurring within 24 hours of infusion.
Abbreviations: G, grade; RBC, red blood cell; Ca, calcium.

Table 6 Ongoing Clinical Studies for Selinexor and Belantamab (Source: Clinicaltrials.gov)

S.N	Title	Status	NCT Number
1	Selinexor Treatment for Multiple Myeloma Patients Who Are Refractory to Lenalidomide-containing Therapy.	Recruiting	NCT04519476
2	Selinexor (KPT-330) and Liposomal Doxorubicin For Relapsed and Refractory Multiple Myeloma	Active, not recruiting	NCT02186834
3	Selinexor Plus High-Dose Melphalan (HDM) Before Autologous Hematopoietic Cell Transplantation for Multiple Myeloma	Recruiting	NCT02780609
4	A Study of Selinexor Plus Low-dose Dexamethasone in Participants With Penta-refractory Multiple Myeloma or Selinexor and Bortezomib Plus Low-dose Dexamethasone in Participants With Triple-class Refractory Multiple Myeloma	Recruiting	NCT04414475
5	Selinexor, Carfilzomib, and Dexamethasone in Treating Patients With Relapsed or Refractory Multiple Myeloma	Recruiting	NCT02199665
6	Bortezomib, Selinexor, and Dexamethasone in Patients With Multiple Myeloma	Active, not recruiting	NCT03110562
7	SELIBORDARA: Selinexor, Bortezomib and Daratumumab in Multiple Myeloma	Recruiting	NCT03589222
8	Selinexor and Backbone Treatments of Multiple Myeloma Patients	Recruiting	NCT02343042
9	Selinexor, Pomalidomide, and Dexamethasone With or Without Carfilzomib for the Treatment of Patients With Relapsed Refractory Multiple Myeloma, The SCOPE Trial	Recruiting	NCT04764942
10	A Study of Evaluating the Safety and Efficacy of ATG-010 in Relapsed Refractory Multiple Myeloma	Recruiting	NCT03944057
11	Myeloma-Developing Regimens Using Genomics (MyDRUG)	Recruiting	NCT03732703
12	Study of Single Agent Belantamab Mafodotin Versus Pomalidomide Plus Low-dose Dexamethasone (Pom/Dex) in Participants With Relapsed/Refractory Multiple Myeloma (RRMM)	Recruiting	NCT04162210
13	A Study of Belantamab Mafodotin (GSK2857916) in Multiple Myeloma Participants With Normal and Impaired Hepatic Function	Recruiting	NCT04398680

(*Continued*)

Table 6 (Continued).

S.N	Title	Status	NCT Number
14	A Study of Belantamab Mafodotin (GSK2857916) in Multiple Myeloma Participants With Normal and Varying Degree of Impaired Renal Function	Recruiting	NCT04398745
15	A Study of Belantamab Mafodotin to Investigate Safety, Tolerability, Pharmacokinetics, Immunogenicity and Clinical Activity in Participants With Relapsed/Refractory Multiple Myeloma (RRMM)	Active, not recruiting	NCT04177823
16	Belantamab Mafodotin in Newly Diagnosed Transplant Eligible Multiple Myeloma Patients	Recruiting	NCT04802356
17	Study of Belantamab Mafodotin Plus Standard of Care (SoC) in Newly Diagnosed Multiple Myeloma	Recruiting	NCT04091126
18	Belantamab Mafodotin Plus Pomalidomide and Dexamethasone (Pd) Versus Bortezomib Plus Pd in Relapsed/Refractory Multiple Myeloma	Recruiting	NCT04484623
19	Evaluation of Efficacy and Safety of Belantamab Mafodotin, Bortezomib and Dexamethasone Versus Daratumumab, Bortezomib and Dexamethasone in Participants With Relapsed/Refractory Multiple Myeloma	Recruiting	NCT04246047
20	Blmf, Lenalidomide and Dexamethasone in Transplant-ineligible Patients With Newly Diagnosed Multiple Myeloma	Recruiting	NCT04808037
21	Study of Belantamab Mafodotin as Pre- and Post-autologous Stem Cell Transplant and Maintenance for Multiple Myeloma	Recruiting	NCT04680468
22	Platform Study of Belantamab Mafodotin as Monotherapy and in Combination With Anti-cancer Treatments in Participants With Relapsed/Refractory Multiple Myeloma (RRMM) (DREAMM 5)	Recruiting	NCT04126200
23	A Study to Investigate the Efficacy and Safety of Two Doses of GSK2857916 in Participants With Multiple Myeloma Who Have Failed Prior Treatment With an Anti-CD38 Antibody	Active, not recruiting	NCT03525678
24	To Evaluate Safety, Tolerability, and Clinical Activity of the Antibody-drug Conjugate, GSK2857916 Administered in Combination With Lenalidomide Plus Dexamethasone (Arm A), or in Combination With Bortezomib Plus Dexamethasone (Arm B) in Participants With Relapsed/Refractory Multiple Myeloma (RRMM)	Recruiting	NCT03544281
25	Study Evaluating Safety, Tolerability and Clinical Activity of GSK2857916 in Combination With Pembrolizumab in Subjects With Relapsed/Refractory Multiple Myeloma (RRMM)	Active, not recruiting	NCT03848845
26	An Open-label, Dose Escalation Study in Japanese Participants With Relapsed/Refractory Multiple Myeloma Who Have Failed Prior Anti Myeloma Treatments	Recruiting	NCT03828292
27	Selinexor and Backbone Treatments of Multiple Myeloma Patients	Recruiting	NCT02343042
28	Myeloma-Developing Regimens Using Genomics (MyDRUG)	Recruiting	NCT03732703

the management of ocular toxicity. Ocular toxicity also becomes important if the patient has received previous treatment with Sel, as Sel has shown to contribute to blurred vision in 10–11% of patients and cataract formation in 4%.

Another toxicity worth watchful monitoring with both Sel and belamaf treatment is thromboembolism but is not commonly reported in clinical trials.[16,21] Thromboembolism prophylaxis may be warranted but is not required. Non-hematological toxicities related to both Sel and belamaf can be managed with standard treatment

guidelines. Nausea and vomiting related to Sel may predispose patients to develop AKI. An antiemetic should be added to Sel due to its high emetogenic potential and nausea should be addressed promptly.[3] Since Dexa is added to the Sel treatment, the treating physician may find that patients do not experience nausea during the initial days of treatment. Instead, they experience delayed nausea and vomiting that will require the additional use of antiemetics.

Sel is also related to neurotoxicity and hyponatremia. The treatment for hyponatremia is usually not required

until when G3 or 4. Hyponatremia should be expected when patients report unexplained fatigue or slow thought process. Early identification of hyponatremia is crucial as it may worsen with nausea, vomiting, diarrhea, and reduced oral intake. Sodium levels should be monitored at baseline and throughout the treatment. Sel-related hyponatremia usually occurs on day eight or afterward, therefore other hyponatremia causes should be ruled out if it occurs earlier during the course. Neurotoxicity usually develops in the third or fourth week of Sel treatment and manifests as syncope, dizziness, cognitive difficulties, and mental status changes. The treatment should be interrupted and other causes of mental status changes should be ruled out.[2,6,21]

As these are newer drugs with limited data, continuous surveillance and monitoring are strictly warranted during the treatment course with early mitigation strategies. The common AEs and their management strategies have been summarized in Table 5. Various ongoing clinical studies of these two drugs have been summarized in Table 6.

Conclusion

Cytopenias, constitutional symptoms, gastrointestinal effects, hyponatremia, and anemia are the major toxicities of Sel and belamaf. Managing Sel toxicities require frequent monitoring for blood counts and basic metabolic panel along with prophylactic use of antiemetics, and appetite stimulants as needed and colony-stimulating factors/hematopoietic growth factors in addition to dose interruptions and modifications to manage neutropenia and cytopenia. We recommend following REMS program guidelines for close monitoring and evaluation of belamaf toxicities and early ophthalmic intervention. The physician should be aware of thrombocytopenia and its management as well as belamaf ocular toxicity which is best managed with dose reduction and dose delays but if missed could have serious complications.

Acknowledgments

Authors thank Ms. Marsha Halajian and Laeth George, MD for providing English language editing services and proofreading.

Disclosure

F. Anwer reports personal fees from Bristol Myers Squibb as a speaker and fee from Janssen pharmaceutical as an advisory board member, this fee was not related to the submitted work. Without receiving direct funding, served as the local principal investigator for Allogene Therapeutics, Celgene, GlaxoSmithKline, and Bristol Myers Squibb; has a consulting or advisory role for Seattle Genetics, Incyte Corporation Speakers' Bureau, Company: Incyte Corporation; receives travel and accommodations expenses from Seattle Genetics, Incyte; receives honoraria from Incyte, Company: Seattle Genetics; and received research funding from Seattle Genetics, Company: Celgene, Acetylon Pharmaceuticals, Millennium, Astellas Pharma and AbbVie; and reports no other potential conflicts of interest for this work. The other authors report no conflicts of interest for this work.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7–30. doi:10.3322/caac.21590
2. Chen C, Siegel D, Gutierrez M, et al. Safety and efficacy of selinexor in relapsed or refractory multiple myeloma and waldenstrom macroglobulinemia. *Blood*. 2018;131(8):855–863. doi:10.1182/blood-2017-08-797886
3. Bahlis NJ, Sutherland H, White D, et al. Selinexor plus low-dose bortezomib and dexamethasone for patients with relapsed or refractory multiple myeloma. *Blood*. 2018;132(24):2546–2554. doi:10.1182/blood-2018-06-858852
4. Syed YY. Selinexor: first global approval. *Drugs*. 2019;79(13):1485–1494. doi:10.1007/s40265-019-01188-9
5. Vogl DT, Dingli D, Cornell RF, et al. Selective inhibition of nuclear export with oral selinexor for treatment of relapsed or refractory multiple myeloma. *J Clin Oncol*. 2018;36(9):859–866. doi:10.1200/JCO.2017.75.5207
6. Chari A, Vogl DT, Gavriatopoulou M, et al. Oral selinexor-dexamethasone for triple-class refractory multiple myeloma. *N Engl J Med*. 2019;381(8):727–738. doi:10.1056/NEJMoa1903455
7. US-FDA. FDA grants accelerated approval to selinexor for multiple myeloma. 2019. Available from: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-grants-accelerated-approval-selinexor-multiple-myeloma. Accessed December, 2020.
8. Walker JS, Garzon R, Lapalombella R. Selinexor for advanced hematologic malignancies. *Leuk Lymphoma*. 2020;61(10):2335–2350.
9. Grosicki S, Simonova M, Spicka I, et al. Once-per-week selinexor, bortezomib, and dexamethasone versus twice-per-week bortezomib and dexamethasone in patients with multiple myeloma (BOSTON): a randomised, open-label, Phase 3 trial. *Lancet*. 2020;396 (10262):1563–1573. doi:10.1016/S0140-6736(20)32292-3
10. Malandrakis P, Ntanasis-Stathopoulos I, Gavriatopoulou M, Terpos E. Clinical utility of selinexor/dexamethasone in patients with relapsed or refractory multiple myeloma: a review of current evidence and patient selection. *Onco Targets Ther*. 2020;13:6405–6416. doi:10.2147/OTT.S227166
11. Abramson HN. B-Cell Maturation Antigen (BCMA) as a target for new drug development in relapsed and/or refractory multiple myeloma. *Int J Mol Sci*. 2020;21(15):5192. doi:10.3390/ijms21155192
12. Cho SF, Lin L, Xing L, et al. BCMA-targeting therapy: driving a new era of immunotherapy in multiple myeloma. *Cancers*. 2020;12(6):1–29. doi:10.3390/cancers12061473
13. US-FDA. FDA granted accelerated approval to belantamab mafodotin-blmf for multiple myeloma. 2020. Available from: https://www.fda.gov/drugs/drug-approvals-and-databases/fda-granted-accelerated-approval-belantamab-mafodotin-blmf-multiple-myeloma. Accessed November 25, 2020.

14. Markham AJD. Belantamab mafodotin: first approval. *Drugs.* 2020;1–7. doi:10.1007/s40265-019-01241-7

15. Lonial S, Lee HC, Badros A, et al. Belantamab mafodotin for relapsed or refractory multiple myeloma (DREAMM-2): a two-arm, randomised, open-label, Phase 2 study. *Lancet Oncol.* 2020;21 (2):207–221. doi:10.1016/S1470-2045(19)30788-0

16. Trudel S, Lendvai N, Popat R, et al. Targeting B-cell maturation antigen with GSK2857916 antibody-drug conjugate in relapsed or refractory multiple myeloma (BMA117159): a dose escalation and expansion Phase 1 trial. *Lancet Oncol.* 2018;19(12):1641–1653. doi:10.1016/S1470-2045(18)30576-X

17. Trudel S, Lendvai N, Popat R, et al. Antibody-drug conjugate, GSK2857916, in relapsed/refractory multiple myeloma: an update on safety and efficacy from dose expansion phase I study. *Blood Cancer J.* 2019;9(4):37. doi:10.1038/s41408-019-0196-6

18. Trudel S, Cohen AD, Richardson PG, et al. Safety and tolerability of single-agent belantamab mafodotin in heavily pre-treated patients with relapsed/refractory multiple myeloma: pooled data from DREAMM-1 and DREAMM-2. *HemaSphere.* 2020;4:429–430.

19. Farooq AV, Degli Esposti S, Popat R, et al. Corneal epithelial findings in patients with multiple myeloma treated with antibody-drug conjugate belantamab mafodotin in the pivotal, randomized, DREAMM-2 Study. *Ophthalmol Ther.* 2020;9(4):889–911.

20. Razak ARA, Mau-Soerensen M, Gabrail NY, et al. First-in-class, first-in-human phase I study of selinexor, a selective inhibitor of nuclear export, in patients with advanced solid tumors. *J Clin Oncol.* 2016;34(34):4142.

21. Jakubowiak AJ, Jasielec JK, Rosenbaum CA, et al. Phase 1 study of selinexor plus carfilzomib and dexamethasone for the treatment of relapsed/refractory multiple myeloma. *Br J Haematol.* 2019;186 (4):549–560. doi:10.1111/bjh.15969

22. Navari RM, Qin R, Ruddy KJ, et al. Olanzapine for the prevention of chemotherapy-induced nausea and vomiting. *N Engl J Med.* 2016;375 (2):134–142. doi:10.1056/NEJMoa1515725

23. Bahlis N, Chen C, Sebag M, et al. A phase 1B/2 study of selinexor in combination with backcone therapies for treatment of relapsed/refractory multiple myeloma. *Haematologica Conf.* 2016;101:84.

24. Ruiz Garcia V, López-Briz E, Carbonell Sanchis R, Gonzalvez Perales JL, Bort-Marti S. Megestrol acetate for treatment of anorexia-cachexia syndrome. *Cochrane Database Syst Rev.* 2013;2013(3): Cd004310.

25. Jatoi A, Windschitl HE, Loprinzi CL, et al. Dronabinol versus megestrol acetate versus combination therapy for cancer-associated anorexia: a North Central Cancer Treatment Group study. *J Clin Oncol.* 2002;20(2):567–573. doi:10.1200/JCO.2002.20.2.567

26. Chen CI, Bahlis N, Gasparetto C, et al. Selinexor, pomalidomide, and dexamethasone (SPd) in patients with relapsed or refractory multiple myeloma. *Blood.* 2019;134.

27. Andreyev J, Ross P, Donnellan C, et al. Guidance on the management of diarrhoea during cancer chemotherapy. *Lancet Oncol.* 2014;15 (10):e447–e460. doi:10.1016/S1470-2045(14)70006-3

28. Sun JX, Yang N. Role of octreotide in post chemotherapy and/or radiotherapy diarrhea: prophylaxis or therapy? *Asia Pac J Clin Oncol.* 2014;10(2):e108–e113. doi:10.1111/ajco.12055

29. Hovan AJ, Williams PM, Stevenson-Moore P, et al. A systematic review of dysgeusia induced by cancer therapies. *Support Care Cancer.* 2010;18(8):1081–1087. doi:10.1007/s00520-010-0902-1

30. Park SE, Hwang JH, Choi JH, et al. Incidence, risk factors, and clinical outcomes of acute kidney injury caused by palliative chemotherapy in lung cancer. *J Cancer.* 2019;10(22):5332–5338. doi:10.7150/jca.28399

31. Eaton JS, Miller PE, Mannis MJ, Murphy CJ. Ocular adverse events associated with antibody–drug conjugates in human clinical trials. *J Ocul Pharmacol Ther.* 2015;31(10):589–604. doi:10.1089/jop.2015. 0064

32. Fathi AT, Chen R, Trippett TM, et al. *Interim Analysis of a Phase 1 Study of the Antibody-Drug Conjugate SGN-CD19A in Relapsed or Refractory B-Lineage Acute Leukemia and Highly Aggressive Lymphoma.* Washington, DC: American Society of Hematology; 2014.

33. Moskowitz CH, Forero-Torres A, Shah BD, et al. *Interim Analysis of a Phase 1 Study of the Antibody-Drug Conjugate SGN-CD19A in Relapsed or Refractory B-Lineage Non-Hodgkin Lymphoma.* Washington, DC: American Society of Hematology; 2014.

34. Eliason L, Opalinska J, Martin ML, et al. Dreamm-1: patient perspectives from the first-in-human study of single-agent belantamab mafodotin for relapsed and refractory multiple myeloma (RRMM). *HemaSphere.* 2020;4:936.

35. Popat R, Warcel D, O'Nions J, et al. Characterisation of response and corneal events with extended follow-up after belantamab mafodotin (GSK2857916) monotherapy for patients with relapsed multiple myeloma: a case series from the first-time-in-human clinical trial. *Haematologica.* 2020;105(5):e261. doi:10.3324/haematol.2019.235 937

36. Accessdata.fda.gov. BLENREP (belantamab mafodotin-blmf) for injection, for intravenous use. 2020. Available from: https://www. accessdata.fda.gov/drugsatfda_docs/label/2020/761158s000lbl.pdf. Accessed February 1, 2021.

37. Goldschmidt H, Ashcroft J, Szabo Z, Garderet L. Navigating the treatment landscape in multiple myeloma: which combinations to use and when? *Ann Hematol.* 2019;98(1):1–18. doi:10.1007/s00277-018-3546-8

38. Gandhi UH, Cornell RF, Lakshman A, et al. Outcomes of patients with multiple myeloma refractory to CD38-targeted monoclonal antibody therapy. *Leukemia.* 2019;33(9):2266–2275. doi:10.1038/s413 75-019-0435-7

39. Machlus KR, Wu SK, Vijey P, et al. Selinexor-induced thrombocytopenia results from inhibition of thrombopoietin signaling in early megakaryopoiesis. *Blood.* 2017;130(9):1132–1143. doi:10.1182/ blood-2016-11-752840

40. Gavriatopoulou M, Chari A, Chen C, et al. Integrated safety profile of selinexor in multiple myeloma: experience from 437 patients enrolled in clinical trials. *Leukemia.* 2020;34(9):2430–2440. doi:10.1038/ s41375-020-0756-6

Taylor & Francis

ABO and Rhesus Blood Group Distribution and Blood Donation Willingness Among First-Year Health Students in a Saudi University

Nouf A AlShamlan, Malak A Al Shammari, Reem S AlOmar, Danya Gari, Assim M AlAbdulKader, Sameerah Motabgani, Abdulaziz Farea & Magdy A Darwish

Published online: 05 Jul 2021.

Submit your article to this journal 🗗

Article views: 512

View related articles 🗗

View Crossmark data 🗗

Citing articles: 5 View citing articles 🗗

Open Access Full Text Article

ORIGINAL RESEARCH

ABO and Rhesus Blood Group Distribution and Blood Donation Willingness Among First-Year Health Students in a Saudi University

Nouf A AlShamlan [ID]
Malak A Al Shammari [ID]
Reem S AlOmar [ID]
Danya Gari
Assim M AlAbdulKader [ID]
Sameerah Motabgani
Abdulaziz Farea
Magdy A Darwish

Department of Family and Community Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

Background: Understanding the distribution of blood groups and blood donation willingness in a population is crucial in managing blood banks and transfusion services. Moreover, awareness of one's own blood group is essential especially in emergencies that mandate blood donation. This study aimed to determine the distribution of ABO and Rhesus (Rh) blood groups among health students, the students' knowledge about their blood group, and their willingness to donate blood.

Methods: This cross-sectional study included all newly accepted health students in a large university in the Eastern Province of Saudi Arabia (N=1145) during August 2020. The data included a self-administered questionnaire and the serology results of ABO and Rh factors. Chi-squared and Fisher's exact tests were performed followed by a multivariable binary logistic regression analysis which identified the predictors of willingness of blood donation.

Results: Blood group O was the most frequent type among students (51.1%), followed by group A (24.5%) and B (20.4%). The majority (93.3%) of students had Rh-positive factor. When we compared students' answers with their sample results, most students (75.5%) correctly reported their ABO and Rh blood groups. Male students and those with a previous history of blood donation correctly reported their blood group more than others. Of the total sample, 47.3% were willing to donate blood within the next year. Positive predictors of the willingness of student to donate blood included being male, and those with a history of blood donation. Interestingly, students with a family member in the healthcare field were significantly less likely to donate blood.

Conclusion: Blood group O and Rh positive were the most frequent blood groups. Most students had a good knowledge about their blood groups, and about half of students were willing to donate blood. Efforts to encourage the young population to participate in blood donation are crucial.

Keywords: ABO blood group, Rh factor, blood donation, Saudi, students

Introduction

ABO and Rhesus (Rh) blood groups, the most recognized blood group systems, are important for transfusion and transplantation safety, and have been linked with susceptibility to certain diseases.[1] Frequencies of blood groups vary in different ethnic groups. In the United States, among Caucasians, the distribution of blood groups O, A, B, and AB was 45.0%, 40.0%, 11.0%, and 4.0%, respectively. In Hispanics, the frequencies were reported to be 57.0%, 31.0%, 10.0% and 3.0%, respectively. In Blacks, the distributions were 50.0%, 26.0%, 20.0% and 4.0%,

Correspondence: Nouf A AlShamlan
Department of Family and Community Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia
Tel +966504901406
Email nashamlan@iau.edu.sa

respectively.[2] In China, the most common blood group was A (30.5%), followed by O (30.4%) and B (29.4%), and the least common was AB (9.7%). Moreover, only 1.02% of the population had Rh-negative factor.[1] In Saudi Arabia, a previous report among 9939 stem cell donors from different regions in the Kingdom showed that Blood group O was the most frequent type (50.4%), followed by group A (28.6%), and B (17.1%), and the least common was group AB (4.0%), Moreover, about 90.0% of donors were Rh-positive.[2] Blood-derived products are frequently used as a life-saving procedure in both routine and emergency situations that require a replacement of blood.[3] Awareness of one's own blood group is essential, primarily because it is important in case an urgent transfusion is required. The awareness rate varies between different countries, ranging between 50.0% in the UK and 97.0% in Japan.[4]

The blood donation system in Saudi Arabia allows only healthy adults, with an age range from 18 to 65 years, to donate.[5] The contribution of the young population in blood donation is essential, since they are less likely to suffer from certain conditions that would disqualify from donating.[4] Moreover, the presence of health students in the teaching hospitals, with a variety of blood groups, can serve as an essential pool of potential blood donors for many reasons. These students have the benefit of easy accessibility to blood banks, are usually young and healthy, and more aware of the local needs of blood products.[4,6] The attitude of health students towards blood donation is different among countries. In Portugal, only 12.7% of health science students had ever donated blood, however, 88.0% of non-donors will donate blood if necessary.[7] In Northwest Ethiopia, 16.8% of students in a university donated blood voluntarily.[8] In India, 22.9% of students in a medical college had a history of blood donation, and the majority (91.0%) were willing to donate in the future.[6] In Poland, 30.2% of students were blood donors.[4] In Tanzania, around 30.0% of university students donated blood, and 89.3% reported willingness to donate blood to anyone upon request.[9] In Hong Kong, 49.45% of students from one university were blood donors.[10] In Saudi Arabia, a study conducted between 2014 and 2015 revealed that 30.1% of healthcare students had a history of blood donation and the majority (98.0%) reported willingness to donate blood to their relatives and non-relatives (90%).[11]

Up-to-date knowledge on the pattern of blood types and the rates of willingness of young population to donate blood is essential for national health services. Moreover, there is limited data concerning the knowledge of one's own blood group in Saudi Arabia. Hence, the objective of this study was to estimate the frequency of different ABO and Rh blood groups among health track students in a large university in the Eastern Province of Saudi Arabia, as well as to determine students' knowledge about their blood group and their willingness to donate blood.

Materials and Methods
Ethical Approval
The Institutional Review Board committee at Imam Abdulrahman Bin Faisal University approved the study. The researcher obtained the written consent from all participants, and from the parents/guardians if less than 18 years old after explaining the study purpose and to reassure them that there were no negative consequences for them. Confidentiality of the data was assured. This study complied with the principles of the Declaration of Helsinki.

Study Design, Setting, and Population
This cross-sectional study included all first-year, newly accepted, health students at Imam Abdulrahman Bin Faisal University in the Eastern Province of Saudi Arabia (N=1145) and was conducted in the Family and Community Medicine center of the Imam Abdulrahman Bin Faisal University during August 2020 after ethical approval and informed consent was obtained.

Data Collection
The data had two components. The first part was a self-administered questionnaire (Supplementary File). It was designed by the researchers after a review of recent literature and similar studies based on the objectives of the study.[3,4] This part included questions on sociodemographic factors, self-reported ABO and Rh blood groups, participant's willingness to donate blood in the next year, and other questions that could affects the participant's knowledge on self-blood type or willingness to donate such as having chronic disease or the presence of health care workers in the family. This part was developed initially in English then double translated from English to Arabic then to English. It was tested by a pilot study among 30 students and was revised for content validity by three professional experts.

The second part of the data was the serology results and included ABO and Rh factors tests. Blood samples were collected from participants and tested for the ABO and Rh blood groups and both were performed simultaneously with reagents (anti-A, anti-B, and anti-D). The red blood cell agglutination method was used for blood type analysis. Prior to blood extraction, the investigators approached the students and distributed the online-based self-reported questionnaires.

Data Management and Analysis

After checking for completeness and consistency, data were analyzed using IBM SPSS for Windows, version 26 (IBM Corp., Armonk, NY, USA). Good knowledge about self-blood group included participants who correctly identified their ABO blood group and/or Rh factors by comparing their answers in the survey with their serology results. Participants who incorrectly reported their ABO and/or Rh blood groups along with those who answered (Not sure) were included as poor knowledge. Categorical variables, were presented as percentages and frequency distribution, and the 95% confidence intervals for proportions were determined using the formula for standard error of measurement. Variables were compared using the chi-squared or Fisher's exact tests. Bonferroni-corrected post-hoc comparisons were conducted as appropriate. Multivariable binary logistic regression analysis was conducted to identify the independent predictors of the willingness to donate blood next year. Candidate variables were selected based on medical literature and bivariate analyses. Odds ratio (OR) with 95% confidence intervals (CI) were estimated using the full model fit and were reported in comparison with the designated reference group. The goodness-of-fit of the model was evaluated using the Omnibus and Hosmer-Lemeshow tests. The significance level was defined as $\alpha = 0.05$.

Results
Participants Characteristics

The study included 1145 participants, and comprised of 54.9% female and 45.1% male students. The majority (80.4%) of students was aged 18 years old, and 72.1% students were originally from the Eastern Province of Saudi Arabia. Only 10.5% students had a history of chronic diseases. The hematological disorders, including glucose-6-phosphate dehydrogenase deficiency (n=62) and sickle cell diseases (n=14) constituted a major part of these

conditions. Other reported diseases included asthma (n=17), type 1 diabetes mellitus (n=7), psoriasis (n=4), and eczema (n=4). About 40.5% of students had healthcare workers in the family (Table 1).

Blood Groups of Participants

Overall, 51.1% of students had a blood group O making it the most frequent ABO blood group while the least frequent blood type was blood group AB (4.0%). Furthermore, 280 (24.5%) and 234 (20.4%) students had blood group A and B, respectively. The majority (93.3%) of students had Rh positive blood group.

There was no statistically significant difference in the ABO and Rh blood group types according to age and gender. However, there was a significant association between the ABO and Rh blood group types and the origin in Saudi Arabia ($P < 0.05$). While the pattern of frequency of the blood groups was similar across all the regions, it was notable that the students who were from the Southern Province had the lowest prevalence (7.5%) of blood group B ($P = 0.004$) (Table 2).

Awareness of Blood Group Types

Most students had a good knowledge about their ABO (81.9%; 95% *CI*: 79.7–84.1) and Rh (81.0%; 95% *CI*: 78.7–83.3) blood group types. Overall, three-fourths (75.5%; 95% *CI*: 73.0–78.0) of students reported their ABO–Rh blood group correctly. Table 3 demonstrates the self-reported blood groups of the participants and their serology results. The majority of students with Rh positive correctly reported their Rh group. On the other hand, 59.8% of students who reported their Rh group as negative had a good knowledge about their Rh type. Table 4 summarizes the associations with the good knowledge about the ABO and Rh groups. For instance, male students had a higher proportion (89.1%) of good knowledge about their ABO blood groups than their female counterparts (76.0%) ($P < 0.001$). Additionally, students who had a previous history of blood donation had a higher proportion of good knowledge about their ABO and Rh blood groups compared with those who had not (90.9% and 93.9% vs 81.4% and 80.2%, respectively) ($P < 0.05$). Moreover, other demographic and socioeconomic factors, including age, marital status, parental education, and having a chronic disease were not significantly associated with a good knowledge about the blood group of the student ($P > 0.05$).

Table 1 Sociodemographic Characteristics of Participants

Variable		N (%)	[95% CI]
Age	17 years	145 (12.7)	[10.8–14.6]
	18 years	921 (80.4)	[78.1–82.7]
	19 years	79 (6.9)	[5.4–8.4]
Gender	Female	629 (54.9)	[52.0–57.8]
	Male	516 (45.1)	[42.2–48.0]
Marital Status	Single	1135 (99.1)	[98.6–99.6)
	Married	10 (0.9)	[0.4–1.4]
Origin in Saudi Arabia	Eastern Province	825 (72.1)	[69.5–74.7]
	Central Province	84 (7.3)	[5.8–8.8]
	Northern Province	55 (4.8)	[3.6–6.0]
	Western Province	61 (5.3)	[4.0–6.6]
	Southern Province	120 (10.5)	[8.7–12.3]
Have Chronic Diseases	Yes	120 (10.5)	[8.7–12.3]
	No	1025 (89.5)	[87.7–91.3]
Paternal Education	Less Than Secondary School	124 (10.8)	[9.0–12.6]
	Secondary School	319 (27.9)	[25.3–30.5]
	Diploma or Bachelor	569 (49.7)	[46.8–52.6]
	Master or Doctorate	133 (11.6)	[9.7–13.5]
Maternal Education	Less Than Secondary School	167 (14.6)	[12.6–16.6]
	Secondary School	306 (26.7)	[24.1–29.3]
	Diploma or Bachelor	629 (54.9)	[52.0–57.8]
	Master or Doctorate	43 (3.8)	[2.7–4.9]
Have HCW in the Family	Yes	464 (40.5)	[37.7–43.3]
	No	681 (59.5)	[56.7–62.3]

Abbreviations: N, Number of participants; HCW, Healthcare workers; CI, confidence interval.

Attitudes Towards Blood Donation

Only 66 (5.8%) students reported previous experience of blood donation and most of them (83.1%) donated only once. Moreover, only 17 (1.5%) students had a history of blood transfusion. Additionally, around half (47.3%; 95% *CI*: 44.4–50.2) of participants reported a willingness to donate blood in the next 12 months.

Male students reported a higher willingness to donate blood in the next 12 months than their female counterparts (57.8% vs 38.8%) (*P* < 0.001). Students with a history of chronic diseases (37.5%) were less willing to donate blood

than those without (48.5%) (*P* = 0.023). Students who had a family member in the healthcare field were slightly less willing to donate blood next year (43.5% vs 49.9%) (*P* = 0.033). Moreover, a good knowledge about the blood groups was not found to be significantly associated with the willingness to donate blood (*P* > 0.05) (Table 5).

Multivariable Analysis of Factors Associated with Blood Donation

Multivariable binary logistic regression analysis was performed to identify the independent predictors of the

Table 2 ABO and Rh Blood Groups According to Demographic Factors

Variable		ABO Group				P value	Rh Positive N (%)	P value
		A N (%)	**B N (%)**	**AB N (%)**	**O N (%)**			
Age	17 years	42 (29.0)	27 (18.6)	7 (4.8)	69 (47.6)	0.142	136 (93.8)	0.951
	18 years	225 (24.4)	191 (20.7)	32 (3.5)	473 (51.4)		858 (93.2)	
	19 years	13 (16.5)	16 (20.3)	7 (8.9)	43 (54.4)		74 (93.7)	
Gender	Female	137 (21.8)	134 (21.3)	24 (3.8)	334 (53.1)	0.120	591 (94.0)	0.308
	Male	143 (27.7)	100 (19.4)	22 (4.3)	251 (48.6)		477 (92.4)	
Origin in Saudi Arabia (Province)	Eastern	195 (23.6)	184 (22.3)	32 (3.9)	414 (50.2)	**0.032**	779 (94.4)	**0.003**
	Central	21 (25.0)	22 (26.2)	4 (4.8)	37 (44.0)		71 (84.5)	
	Northern	10 (18.2)	11 (20.0)	3 (5.5)	31 (56.4)		54 (98.2)	
	Western	17 (27.9)	8 (13.1)	4 (6.6)	32 (52.5)		55 (90.2)	
	Southern	37 (30.8)	9 (7.5)	3 (2.5)	71 (59.2)		109 (90.8)	

Note: P values are in bold if statistically significant.
Abbreviation: N, Number of participants.

Table 3 Self-Reported and Serology Results of Participants' Blood Groups

Blood groups		Serology Result			
ABO Groups		A N (%)	B N (%)	AB N (%)	O N (%)
Self-Reported	A	**223 (89.9)**	7 (2.8)	4 (1.6)	14 (5.7)
	B	3 (1.6)	**177 (91.7)**	2 (1.0)	11 (5.7)
	AB	1 (2.6)	2 (5.1)	**35 (89.7)**	1 (2.6)
	O	13 (2.5)	14 (2.6)	0 (0)	**503 (94.9)**
	Not Sure	40 (29.6)	34 (25.2)	5 (3.7)	56 (41.5)
Rh Groups		Positive N (%)		Negative N (%)	
Self-Reported	Positive	**875 (98.1)**		17 (1.9)	
	Negative	35 (40.2)		**52 (59.8)**	
	Not Sure	158 (95.2)		8 (4.8)	

Note: Correctly identified blood groups are in bold.
Abbreviation: N, Number of participants.

willingness for blood donation in the next 12 months. The model revealed that male students were 1.9-times (OR = 1.09; 95% CI: 1.50–2.44) more likely to donate blood than their female counterparts. Additionally, the previous history of blood donation is an independent predictor (OR = 4.57; 95% CI: 2.33–8.94) of the willingness to donate blood next year. In contrast, having a chronic disease (OR = 0.64; 95% CI: 0.43–0.96) or having a family member in

the healthcare field (OR = 0.76; 95% CI: 0.59–0.97) were independent predictors of the unwillingness to donate blood (Table 6).

Discussion

Awareness of one's own blood group is of great importance especially in an emergency situation that mandates blood donation. The current study determined the

Table 4 Good Knowledge About the Self-Blood Groups in Participants

Variable		Good Knowledge About Self-Blood Group			
		ABO Group N (%)	P value	Rh Factor N (%)	P value
Age	17 years	118 (81.4)	0.687	115 (79.3)	0.160
	18 years	758 (82.3)		754 (81.9)	
	19 years	62 (78.5)		58 (73.4)	
Gender	Female	478 (76.0)	**<0.001**	478 (76.0)	**<0.001**
	Male	460 (89.1)		449 (87.0)	
Marital Status	Single	931 (82.0)	0.400	919 (81.0)	1.000
	Married	7 (70.0)		8 (80.0)	
Paternal Education	Less Than Secondary School	100 (80.6)	0.531	95 (76.6)	0.591
	Secondary School	254 (79.6)		258 (80.9)	
	Diploma or Bachelor	475 (83.5)		464 (81.5)	
	Master or Doctorate	109 (82.0)		110 (82.7)	
Maternal Education	Less Than Secondary School	138 (82.6)	0.712	136 (81.4)	0.850
	Secondary School	244 (79.7)		251 (82.0)	
	Diploma or Bachelor	520 (82.7)		507 (80.6)	
	Master or Doctorate	36 (83.7)		33 (76.7)	
Chronic Disease	Yes	100 (83.3)	0.671	97 (80.8)	0.970
	No	838 (81.8)		830 (81.0)	
HCW in Family	Yes	385 (83.0)	0.445	393 (84.7)	**0.008**
	No	553 (81.2)		534 (78.4)	
Donated Blood	Yes	60 (90.9)	**0.049**	62 (93.9)	**0.006**
	No	878 (81.4)		865 (80.2)	
Received Blood	Yes	13 (76.5)	0.528	12 (70.6)	0.344
	No	925 (82.0)		915 (81.1)	
Had Surgery	Yes	187 (83.1)	0.605	187 (83.1)	0.359
	No	751 (81.6)		740 (80.4)	

Note: P values are in bold if statistically significant.
Abbreviations: N, Number of participants; HCW, Healthcare workers.

frequencies of ABO and Rh blood groups among first-year health students in a large university in the largest Province of Saudi Arabia, where there is a lack of data on this subject. Moreover, to the best of our knowledge, there have been no previous studies in Saudi Arabia concerning knowledge about one's own blood group.

This study showed that the most frequent blood type is O (51.1%), followed by blood group A (24.5%) and B (20.4%), and the least frequent is type AB (4.0%). Additionally, the majority (93.3%) of students had Rh-positive blood group. This is consistent with studies conducted in different countries, and is especially similar to the distribution of ABO and Rh blood groups in Tanzania.[12] Moreover, a similar pattern was reported in studies from Ethiopia, Kenya, Mauritania, Nigeria, Uganda, and among blood and stem cell donors in Saudi

Table 5 Willingness to Donate Blood Next Year in Participants

Variable			Willingness to Donate Blood		
			N	**(%)**	**P value**
Age	17 years		72	(49.7)	0.152
	18 years		425	(46.1)	
	19 years		45	(57.0)	
Gender	Female		244	(38.8)	**<0.001**
	Male		298	(57.8)	
Marital Status	Single		536	(47.2)	0.531
	Married		6	(60.0)	
Paternal Education	Less Than Secondary School		63	(50.8)	0.341
	Secondary School		146	(45.8)	
	Diploma or Bachelor		278	(48.9)	
	Master or Doctorate		55	(47.3)	
Maternal Education	Less Than Secondary School		89	(53.3)	0.121
	Secondary School		135	(44.1)	
	Diploma or Bachelor		293	(46.6)	
	Master or Doctorate		25	(58.1)	
Chronic Diseases	Yes		45	(37.5)	**0.023**
	No		497	(48.5)	
HCW in Family	Yes		202	(43.5)	**0.033**
	No		340	(49.9)	
Donated Blood	Yes		55	(83.3)	**<0.001**
	No		487	(45.1)	
Received Blood	Yes		9	(52.9)	0.641
	No		533	(47.3)	
Good Knowledge about Self-Blood Groups	ABO	Yes	441	(47.0)	0.643
		No	101	(48.8)	
	Rh	Yes	446	(48.1)	0.278
		No	96	(44.0)	
	Both	Yes	414	(47.9)	0.490
		No	128	(45.6)	

Note: *P* values are in bold if statistically significant.
Abbreviations: N, Number of participants; HCW, Healthcare workers.

Table 6 Multivariable Regression of Factors Associated with Willingness to Donate Next Year

Variables		Univariable Logistic Regression			Multivariable Logistic Regression		
		OR	[95% CI]	P value	OR	[95% CI]	P value
Age	17 years	1.0	Reference Group		1.0	Reference Group	
	18 years	0.87	[0.61–1.23]	0.431	0.82	[0.57–1.17]	0.268
	19 years	1.34	[0.77–2.33]	0.296	1.09	[0.62–1.94]	0.758
Male Gender		2.16	[1.70–2.73]	<0.001	1.91	[1.50–2.44]	<0.001
Have Chronic Diseases		0.64	[0.43–0.94]	0.023	0.64	[0.43–0.96]	0.031
Donated Blood Previously		6.08	[3.15–11.74]	<0.001	4.57	[2.33–8.94]	<0.001
Have HCW in the Family		0.77	[0.61–0.98]	0.034	0.76	[0.59–0.97]	0.026

Note: P values are in bold if statistically significant.
Abbreviations: OR, odds ratio; CI, confidence interval; HCW, healthcare workers.

Arabia in which blood group O was the predominant group and AB was the least prevalent.[2,12,13] Demand for blood products is high, and blood banks need up-to-date information regarding the frequency of blood groups in the region to ensure sufficient supply, especially for the most required blood types.[12] Our study found a significant association between the ABO and Rh blood group types and participant's origin in Saudi Arabia. While the pattern of frequency of the blood groups was similar across all the regions, students from the Southern Province had the lowest prevalence (7.5%) of blood group B. It has been previously described that societies practicing endogamy tend to be genetically isolated. For instance, genetic studies among several endogamous populations in Bihar, India, revealed that these populations have less gene diversity and thus have less variability in ABO and Rh blood groups.[14] Thus, the cultural tradition of endogamy may contribute to the observed pattern of blood group distribution in the Southern region.

When we compared students' answers to questions about their blood group with their sample results, most students correctly reported their ABO (81.9%) and Rh (81.0%) blood group types. Moreover, three-fourths (75.5%) of students reported their ABO and Rh blood groups correctly. In line with these findings, a study among 1121 students with different health specialties in Poland found that 86.8% of students were aware of their blood group.[4] Slightly higher than our findings, a study on 235 medical students in North India found that 95.7% of students were aware of their blood types. However, the data was collected through a self-reported survey rather than comparing students' answers with a more objective

tool.[6] Another study in Nigeria among 155 undergraduate medical and dental students showed that less than half of them (43.9%) knew their blood groups.[15] Although the small sample size was a major limitation in that study, their finding of a significant association between a history of blood donation and student's awareness of their blood groups was in line with our study.[15] This study showed that male students had a higher proportion of knowledge about their ABO and Rh blood groups than their female counterparts. This observation could be attributed to some regulations in the Kingdom where until very recently, females were not allowed to drive. Information on ABO and Rh factors are reported in the driver license. Therefore, this discrepancy in knowledge between males and females may be eliminated in the next few years with the growing number of females drivers in the country.

The relatively small number of students with a history of donation may be attributed to the policy implemented by Saudi Arabia's Ministry of Health where donation is not permitted for individuals below 18 years of age, and our population's age ranged between 17 and 19 years old.[5]

While most of blood donors in this study (83.1%) reported donating blood only once in their lifetime, those who donated were more likely to do it again over the next year. These results corroborate the findings of Huis In 'T Veld et al in which blood shortages were more likely to be alleviated by previous donors.[16]

Willingness to donate blood has been a question of interest in similar studies with a remarkably wide range of results. A survey conducted in Kilimanjaro, Tanzania, reported that 89.3% of their university students' sample (n=422) were willing to donate blood to anyone

voluntarily, and 94.5% were willing to donate blood to their relatives.[9] In our study, we found that about half (47.3%) of participants reported a willingness to donate blood in the next 12 months.

Consistent with previous research, we found that male students report more willingness to donate blood than their female counterparts.[9,12] Additionally, this finding was documented in a Saudi study in which females constituted less than 5.0% of blood donors.[17] Bani and Giussani have described in a literature review how gender appeared to play a critical role in the motivation to donate blood. They reported that females seemed more guarded about repeated blood donations and were more fearful of adverse reactions making them less frequent blood donors.[18] Another study that surveyed 469 female students revealed that 89.3% of them had never donated blood. When asked about reasons, they cited fear, accessibility issues to donation sites, and not having time to donate.[19] These observations highlight the need to raise awareness among females about the safety of giving blood and breaking the barriers to blood donation.

In this study, we found that students with a reported history of chronic diseases were less willing to donate blood in the next 12 months than those without. It is not precisely clear why, however, most of the reported chronic diseases were hematologic conditions such as sickle cell disease, which may explain why these students seem to be less willing to donate blood. Another possible explanation for this might be linked to some blood donors' safety concerns reported in a large cross-sectional study in the European Union.[16] However, we did not assess the students' knowledge about the contraindications of a blood donation. Nonetheless, misconceptions and myths around blood donation and blood transfusion are not uncommon.[3,11,20]

Contrary to expectations, students with a family member in the healthcare field were slightly less willing to donate blood. Our analysis showed that this was an independent predictor for the unwillingness for blood donation. It is known that blood donation is remarkably safe, and the perceived risk for a transfusion has not been associated with objective knowledge.[21,22] However, experimental studies to examine the impact of message cueing and framing on young adults indicated that people's perception might be altered by how the information is presented. For instance, Farrell et al suggested that presenting blood donation/transfusion risk information as a positive frame, compared with either a mixed or negative frame, resulted in people voicing more confidence on the safety of blood donation/transfusion.[23] Likewise, it could be argued that learning more about the potential risks, or in a negative frame, from a relative healthcare worker may adversely impact the person's willingness to donate blood. There is limited data available to explain this observation. Hence, this question remains unanswered at present.

This study has some limitations; it was cross-sectional, and we cannot establish the temporal relationship between the associated factors we observed with a willingness to blood donation among the population in this study. Data on the history of previous blood donation and its frequency depend on self-reported information by the students and was not verified with any registry or medical reports. Therefore, recall bias could not be excluded. Moreover, involving a limited age range is another limitation in this study.

Conclusion

This study provides epidemiological information about the blood groups' distribution, knowledge about self-blood groups, blood donation willingness and the associated characteristics among the first-year health students in a large university in the Eastern Province of Saudi Arabia. ABO blood group O and positive Rh type were the most frequent blood groups, and most students knew their blood groups. Additionally, about half of students were willing to donate blood during the next year. Encouraging the young population, especially females, to participate in blood donation is important. Moreover, similar studies across the Kingdom are recommended.

Acknowledgments

Disclosure

References

1. Liu J, Zhang S, Wang Q, Shen H, Zhang Y, Liu M. Frequencies and ethnic distribution of ABO and RhD blood groups in China: a Population-Based Cross-Sectional Study. *BMJ Open*. 2017;7(12): e018476. doi:10.1136/bmjopen-2017-018476

2. Alzahrani M, Jawdat D, Alaskar A, Cereb N, Hajeer AH. ABO and Rh blood group genotypes in a cohort of Saudi stem cell donors. *Int J Immunogenet*. 2018;45(2):63–64. doi:10.1111/iji.12354

3. Urgesa K, Hassen N, Seyoum A. Knowledge, attitude, and practice regarding voluntary blood donation among adult residents of Harar Town, Eastern Ethiopia: a Community-Based Study. *J Blood Med*. 2017;8:13–20. doi:10.2147/JBM.S121460

4. Ciepiela O, Jaworska A, Łacheta D, Falkowska N, Popko K, Demkow U. Awareness of blood group and blood donation among medical students. *Transfus Apher Sci*. 2017;56(6):858–864. doi:10.1016/j.transci.2017.10.002

5. Ministry of Health, Saudi Arabia. Blood donation. Facts and guidelines [Internet]; 2014. Available from: https://www.moh.gov.sa/en/HealthAwareness/EducationalContent/Blog/1435/Pages/Artical-2014-06-08.aspx. Accessed June 30, 2021.

6. Chauhan R, Kumar R, Thakur S. A study to assess the knowledge, attitude, and practices about blood donation among medical students of a medical college in North India. *J Fam Med Prim Care*. 2018;7 (4):693–697. doi:10.4103/jfmpc.jfmpc_54_17.

7. Gomes MJ, Nogueira AJ, Antão C, Teixeira C. Motivations and attitudes towards the act of blood donation among undergraduate health science students. *Transfus Apher Sci*. 2019;58(2):147–151. doi:10.1016/j.transci.2018.12.018

8. Talie E, Wondiye H, Kassie N, Gutema H. Voluntary blood donation among bahir dar university students: application of integrated behavioral model, Bahir Dar, Northwest Ethiopia, 2020. *J Blood Med*. 2020;11:429–437. doi:10.2147/JBM.S277411

9. Elias E, Mauka W, Philemon RN, Damian DJ, Mahande MJ, Msuya SE. Knowledge, attitudes, practices, and factors associated with voluntary blood donation among university students in Kilimanjaro, Tanzania. *J Blood Transfus*. 2016;2016:8546803. doi:10.1155/2016/8546803

10. Suen LKP, Siu JY, Lee YM, Chan EA. Knowledge level and motivation of Hong Kong young adults towards blood donation: a cross-sectional survey. *BMJ Open*. 2020;10(1):e031865. doi:10.1136/bmjopen-2019-031865.

11. Alsalmi MA, Almalki HM, Alghamdi AA, Aljasir BA. Knowledge, attitude and practice of blood donation among health professions students in Saudi Arabia; a Cross-Sectional Study. *J Fam Med Prim Care*. 2019;8(7):2322–2327. doi:10.4103/jfmpc.jfmpc_415_19

12. Jahanpour O, Pyuza JJ, Ntiyakunze EO, Mremi A, Shao ER. ABO and Rhesus blood group distribution and frequency among blood donors at Kilimanjaro Christian Medical Center, Moshi, Tanzania. *BMC Res Notes*. 2017;10(1):738. doi:10.1186/s13104-017-3037-3

13. Bashwari LA, Al-Mulhim AA, Ahmad MS, Ahmed MA. Frequency of ABO blood groups in the eastern region of Saudi Arabia. *Saudi Med J*. 2001;22(11):1008–1012.

14. Pandey BN, Das PK, Husain S, Anwer MR, Jha AK. Genetic studies among seven endogamous populations of the Koshi Zone, Bihar (India). *Anthropol Anz*. 2003;61(3):269–274. doi:10.1127/anthranz/61/2003/269

15. Akingbola TS, Yuguda S, Akinyemi OO, Olomu S. Awareness and distribution of ABO, Rhesus blood groups and haemoglobin phenotypes among medical undergraduates in a Nigerian university. *Afr J Med Med Sci*. 2016;45(3):275–280.

16. Huis In 'T Veld EMJ, de Kort W, Merz EM. Determinants of blood donation willingness in the European Union: a cross-country perspective on perceived transfusion safety, concerns, and incentives. *Transfusion*. 2019;59(4):1273–1282. doi:10.1111/trf.15209

17. Al-Johar AW, Al-Saud A, Abalkhail Y, et al. Why do-Saudi women refrain donating their blood?--a study on the attitude, belief and motivation of Saudi female university students towards blood donation. *Clin Lab*. 2016;62(5):771–779. doi:10.7754/Clin.Lab.2015.150718

18. Bani M, Giussani B. Gender differences in giving blood: a review of the literature. *Blood Transfus*. 2010;8(4):278–287. doi:10.2450/2010.0156-09

19. Tariq S, Tariq S, Jawed S, Tariq S. Knowledge and attitude of blood donation among female medical students in Faisalabad. *J Pak Med Assoc*. 2018;68(1):65–70.

20. Alfouzan N. Knowledge, attitudes, and motivations towards blood donation among King Abdulaziz Medical City population. *Int J Family Med*. 2014;2014:539670. doi:10.1155/2014/539670

21. Lowe KC, Ferguson E. Benefit and risk perceptions in transfusion medicine: blood and blood substitutes. *J Intern Med*. 2003;253 (5):498–507. doi:10.1046/j.1365-2796.2003.01149.x

22. Ferguson E, Farrell K, Lowe KC, James V. Perception of risk of blood transfusion: knowledge, group membership and perceived control. *Transfus Med*. 2001;11(2):129–135. doi:10.1046/j.1365-3148.2001.00295-4.x

23. Farrell K, Ferguson E, James V, Lowe KC. Confidence in the safety of blood for transfusion: the effect of message framing. *Transfusion*. 2001;41(11):1335–1340. doi:10.1046/j.1537-2995.2001.41111335.x

Hepatitis B and Hepatitis C Viral Infections and Associated Factors Among Prisoners in Northeast Ethiopia

Yeshimebet Kassa, Yihenew Million, Sirak Biset & Feleke Moges

Published online: 05 Jul 2021.

Submit your article to this journal ☑

View related articles ☑

View Crossmark data ☑

ORIGINAL RESEARCH

Hepatitis B and Hepatitis C Viral Infections and Associated Factors Among Prisoners in Northeast Ethiopia

Yeshimebet Kassa[1]
Yihenew Million [ID][2]
Sirak Biset [ID][2]
Feleke Moges [ID][2]

[1]Department of Medical Laboratory Sciences, College of Medicine and Health Sciences, Wollo University, Dessie, Ethiopia; [2]Department of Medical Microbiology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia

Background: Hepatitis is an inflammation of the liver and often caused by viruses. Hepatitis viruses are the leading causes of liver-related morbidity and mortality worldwide, with Hepatitis B and C viruses share the great majority. Studies have shown that prison settings are one of the high-risk environments for the transmission of these viruses. However, there is limited information on the seroprevalence and associated factors of hepatitis B and C viral infection among Ethiopian prisoners.

Methods: A facility-based cross-sectional study was conducted among 339 prisoners in Dessie town, Ethiopia from February to April 2020. Hepatitis B surface antigen and antibody against hepatitis C virus in serum were determined using Enzyme-Linked Immunosorbent Assay. We imputed the data using "EpiData 3.1" software and exported it to Statistical Package for Social Sciences version 20.0 for analysis, and a p-value of <0.05 was considered statistically significant.

Results: The overall seroprevalence of hepatitis B surface antigen and anti-hepatitis C virus among prisoners was 22/339 (6.5%) (95% CI = 3.8–9.4), and 4/339 (1.2%) (95% CI = 0.0–2.4), respectively. Multiple sexual partners, previous imprisonment, body tattooing, and contact with the jaundiced patient were independently associated with hepatitis B virus infection. Prisoners who had a history of blood transfusion, and dental extraction were independently associated with hepatitis C virus infection.

Conclusion: The seroprevalence of hepatitis B and hepatitis C viral infection among Dessie town prisoners was intermediate and low, respectively. The finding of a significant association between the presence of Hepatitis B surface antigen and hepatitis C virus antibodies among prisoners and factors calls for the need of serological testing for both Hepatitis B and C viruses to high-risk individuals. Strengthening screening strategies and prevention programs in prison settings is advisable to prevent disease transmission.

Keywords: hepatitis B virus, hepatitis C virus, prison, associated factors, Ethiopia

Background

Viral hepatitis is considered a significant public health problem worldwide;[1] it is responsible for the deaths of approximately 1.4 million people per year. Of those deaths, ~96% are attributable to the Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection.[1,2] Hepatitis B virus, along with HCV, accounts for 60% of cirrhosis and 80% of hepatocellular carcinoma (HCC) and causes one million deaths each year around the globe. Worldwide, an estimated 71 million people (African region accounts for 11 million) are living with chronic HCV infection,

Correspondence: Sirak Biset
University of Gondar, PO Box: 196, Ethiopia
Email serbis33@gmail.com

with a seroprevalence of between 2% - 3%.[1–3] Hepatitis C virus can cause both acute and chronic infection and is a major cause of liver cancer.[4] Globally, HCV accounts for an estimated 28% and 26% of cases of cirrhosis and HCC, respectively.[5,6]

Even though there are effective vaccine and antiviral therapies for HBV infection that makes the elimination of HBV possible, there is a lot to do, especially in low-income countries.[7] In the case of HCV, in addition to the absence of an effective vaccine, the presence of diversified genotypes, drug-resistant variants, occult HCV infection, and other cost and awareness-related factors make its elimination difficult.[8,9] For the elimination of HBV and HCV to be possible, working hard on awareness creation, testing, and vaccinating people in high-risk groups, including people in prisons and those who are intravenous drug users is mandatory.[8–11]

Insufficient infection control, poor medical diagnosis, inaccessible to treatment, and the absence of harm reduction practice in prisons make prisoners exposed to various infectious diseases.[12–14] As a result, prisoners are considered as one of the high-risk group populations and are the focus of many researchers across the world.[12,15–21] Since sharing needles and sharp materials is common in the prison setting, the incidence of HCV infection is expected to be high in these populations.[22] Furthermore, different studies documented that the prevalence of HCV and HBV infection among prisoners is much higher than that of the general population.[13,23–25]

Worldwide, more than 10.74 million people are incarcerated in penal institutions. In Africa, Ethiopia has the second-highest number of prisoners. According to the 12th edition of the world prison population list, in 2018, there were 113,727 prisons distributed across Ethiopia.[26] In Ethiopia, studies conducted on HBV[27,28] and HCV[27] infection among prisoners are limited to a few reports. We believed that investigating the seroprevalence of HBV and HCV among prisoners is relevant to public health for maintaining the chain of infection transmission. Therefore, this study aimed to determine the prevalence and associated factors of HBV and HCV infections among prisoners in Dessie town.

Methods
Study Design and Setting
A facility-based cross-sectional study was conducted among prisoners in Dessie town from February to April 2020. Dessie town is located at South Wollo Zone of Amhara Regional State, 401 km north of the capital city, Addis Ababa. Based on the information from the Dessie Prison Administration Office, during the data collection period, there were about 1350 detainees at the prison center. The prison has a clinic with six health professionals delivering healthcare service to the prisons. A total of 339 prisoners were tested for HBsAg and HCV antibody.

Sample Size and Sampling Technique
We calculated the sample size for this study using a single population proportion formula[29] ($n = (Z_{a/2})^2 XP(1 - P)d^2$) for estimation of prevalence based on a 10.4% prevalence of HBV infection among prisoners.[28] The 10.4% prevalence provided us with the larger sample size (n = 398) at 95% CI, 3% margin of error (d) and 10% non-response rate. Since the number of prisoners in the Dessie prison center (N = 1350) was less than 10,000, we used a correction formula ($nf = \frac{n}{1+\frac{n}{N}}$) and got a final sample size of 339. Study participants were selected using the systematic random sampling technique, where after the first participant was selected using the lottery method, we selected other participants at a regular interval.[30]

Study Variables
The presence of HBsAg and anti-HCV was the outcome variable while socio-demographic characteristics, type of crime, current duration of stay in prison, number of sexual partners, history of blood transfusion, history of operation, dental extraction, sharing of sharp materials in prison, injectable drug use (IDU), tattooing practice or ear-piercing, and history of sexually transmitted diseases (STDs) were the predictor variables.

Definitions
Hepatitis B infection is diagnosed when HBsAg in the serum sample is detected using a serological test.[10]

Hepatitis C infection is diagnosed when anti-HCV antibodies in the serum sample are detected using serological tests.[11]

Data Collection and Laboratory methods
Data Collection
Data including socio-demographic characteristics and associated factors or history of high-risk behaviors were collected using a structured questionnaire. The

questionnaire was completed by trained data collectors who can speak and write the local language and under the supervision of the principal investigator.

Specimen Collection and Processing

Trained laboratory technologist collected five milliliters of venous blood with a plain tube from each study participant. The blood sample was allowed to clot at room temperature and centrifuged at 5000 rpm for 15 minutes, and then the serum separated. We transported the serum sample to the Ethiopian Red Cross Society Blood Bank of Dessie branch by using a cold box and stored it at −20°C until tested.[27]

Laboratory Methods

We tested the serum specimen for HBsAg and anti-HCV using the Beijing Wantai's enzyme-linked immune sorbent assay (ELISA) test kits, developed by Wantai Biological Pharmacy Enterprise Co., Ltd. Wantai AiD[TM] HBsAg ELISA test kit with a sensitivity of 100% and specificity of 99.92% and Wantai AiD[TM] anti-HCV ELISA test kit with a sensitivity of 100% and specificity of 99.55% were used to test the presence of HBsAg and HCV antibodies in the serum, respectively.

Quality Control

A pre-tested and structured questionnaire was used to collect the data. Furthermore, the quality of data was also maintained by providing training for data collectors and conducting regular supervision. Standard operating procedures of sample collection and laboratory work were strictly followed.

Data Analysis

The collected data was entered into EpiData 3.1 software then exported to SPSS version 20.0 (SPSS, Chicago, IL, USA) for analysis. A descriptive and inferential statistics is used to present findings. Variables that show a p-value of <0.2 during univariate analysis were selected for multi-variable analysis. Adjusted odds ratios (AOR) and their 95% confidence intervals (CIs) were used as indicators of the strength of association. A p-value <0.05 was used to indicate statistical significance. We have used previously defined endemicity levels to report the prevalence of HBV and HCV infection. The prevalence of HBsAg has been categorized as low (<2%), intermediate (2–8%), and high (>8%),[31] and the prevalence of anti-HCV antibodies as high (>3.5%), moderate (1.5–3.5%), and low (<1.5%).[32]

Results

A total of 339 prisoners have participated in our study, with the mean age of 34 (± 13.2) years, ranged from 18 to 83 years. Above eighty-nine percent (89.4%) of the prisoners were male, 54.6% were from urban areas, and 49.3% were single (Table 1).

Prevalence

The seroprevalence of HBV infection was 22/339 (6.5%). More than half of the participants tested positive were age greater than 44. A relatively higher prevalence of HBsAg 18/ 154 (11.7%) was observed among rural dweller prisoners. The difference in seroprevalence of HBV among age groups, marital statuses, residences, and educational statuses of the prisoners was significant (p-value <0.05). The overall sero-prevalence of HCV infections was 1.2% (4/339). The Fisher's exact test showed that none of the socio-demographic characteristics of the prisoners were associated with the presence of HCV antibody (p-value >0.05) (Table 1).

History of the Prisoners

The mean duration of stay in prison was 3.5 ± 3.2 years, with a range of 1 month to 13 years. One hundred twenty-four (36.6%) participants had a history of the previous imprisonment and are more likely to be positive for HBsAg (p-value <0.001). One hundred forty-three 143 (42.2%) and 191 (56.3%) of the prisoners had a history of multiple sexual partner and tattooing, respectively. The presence of antibody to HCV was significantly higher among prisoners with a history of blood transfusion (p-value = 0.010), history of operation (p-value = 0.023), and history of dental extraction (p-value = 0.004) (Table 2).

Associated Factors of HBV and HCV Infections

In multivariate analysis, individuals with age >44 years, rural residence, history of multiple sexual partners, previous imprisonment, body tattooing, and contact with jaundiced patients were significantly associated with the presence of HBsAg in the prisoners (p-value <0.05) (Table 3). Seroprevalence of HCV was significantly higher among prisoners with a history of blood transfusion (p-value = 0.008) and dental extraction (p-value = 0.003) (Table 4).

Discussion

People detained in prisons are at a higher risk of being infected with different infectious agents, including

Table 1 Socio-Demographic Characteristics of Prisoners in Dessie, Northeast Ethiopia (N=339)

Variables	Category	Frequency: N° (%)	HBsAg (22/339)		Fisher's Exact Test (p-value)	Anti-HCV (4/339)		Fisher's Exact Test (p-value)
			Positive: N° (%)	Negative: N° (%)		Positive: N° (%)	Negative: N° (%)	
Gender	Male	303 (89.4)	21 (6.9)	282 (93.1)	0.490	4 (1.3)	299 (98.7)	1.000
	Female	36 (10.6)	1 (2.7)	35 (97.3)		0	36 (100)	
Age (years)	18–24	82 (24.2)	1 (1.2)	81 (98.8)	0.003*	2 (2.4)	80 (97.6)	0.548
	25–34	126 (37.2)	7 (5.5)	119 (94.5)		2 (1.8)	124 (98.4)	
	35–44	64 (18.8)	3 (4.7)	61 (95.3)		0	64 (100)	
	≥45	67 (19.8)	11 (16.4)	56 (83.5)		0	67 (100)	
Marital status	Married	134 (39.5)	2 (1.5)	132 (98.5)	0.002*	1 (0.7)	133 (99.3)	0.583
	Single	167 (49.3)	18 (10.8)	149 (89.2)		2 (1.2)	165 (98.8)	
	Divorced/ Widow	38 (11.2)	2 (5.26)	36 (94.74)		1 (2.63)	37 (97.37)	
Residence	Rural	154 (45.4)	18 (11.7)	136 (88.3)	0.001*	2 (1.2)	152 (98.7)	1.000
	Urban	185 (54.6)	4 (2.1)	181 (97.8)		2 (1.1)	183 (98.9)	
Education status	Unable to read and write	78 (23.0)	12 (15.4)	66 (84.6)	0.009*	1 (1.3)	77 (98.7)	0.644
	Primary school	129 (38.1)	6 (4.7)	123 (95.3)		1 (0.8)	128 (99.2)	
	Secondary school	77 (22.7)	2 (2.6)	75 (97.4)		2 (2.6)	75 (97.4)	
	College/University	55 (16.2)	2 (3.6)	53 (96.4)		0	55 (100)	
Occupational status	Farmer	101 (29.8)	7 (6.9)	94 (93.1)	0.311	0	101 (100)	0.090
	Daily laborer	58 (17.1)	6 (10.3)	52 (89.7)		2 (3.4)	56 (96.6)	
	Governmental employee	76 (22.4)	3 (3.9)	73 (96.1)		0	76 (100)	
	Unemployed	40 (11.8)	4 (10.0)	36 (90.0)		0	40 (100)	
	Student	53 (15.6)	1 (1.9)	52 (98.1)		2 (3.8)	51 (96.2)	
	Housewife	11 (3.2)	1 (9.1)	10 (90.9)		0	11 (100)	

Notes: *The observed difference is statistically significant (p < 0.05).
Abbreviations: HBsAg, hepatitis B surface antigen; Anti-HCV, anti- hepatitis C virus antibodies.

hepatitis-causing viruses; they can be a reservoir of these agents and a source of infection within the community.[14,33] In the prison setting, HBV and HCV can be easily transmitted within inmates because of overcrowded conditions, injecting drug use practices, or sharing blood contaminated supplies (syringes, needles, scissors, or razors).

Prevalence of HBsAg

In the present study, the prevalence of HBsAg among prisoners was 6.5% (95% CI = 3.8–9.4); can be categorized as intermediate.[31] This result is in line with studies in Jimma, Ethiopia (5.8%),[27] India (3.89%),[34] Switzerland (5.9%),[35] and Iran (6.9%).[36] The prevalence in the present study is lower than studies conducted in Woldia prison, Ethiopia 10.4%,[28] West Africa 12.5%,[37] Nigeria 13.7%,[38] and Iran 18%.[39] However, it was higher than the result reported from Mexico (0.4%)[40] and Iran (3.3%).[41] The

discrepancy might be due to variation in geographical regions, time of study period, diagnostic methodology, types of risk exposure, the immunization status of the population, behavioral differences for the potential risk factors of HBV infection, and sample size. It may also be due to variation in circulating genotypes, which is responsible for disease severity as well as treatment responses.[42]

Prevalence of anti-HCV

This study has found a 1.2% (95% CI = 0.0–2.4) prevalence of anti-HCV antibodies, and can be reported as very low, considering the prison population.[32] Our finding is in agreement with results reported in India (1.27%),[34] West Africa (0.5%),[37] and Turkey (0.5%)[17] but lower than results in Jimma (2.6%), Ethiopia.[27] Higher prevalence of HCV infection was reported in United States

Table 2 Prison History and Risk Behaviors of Prisoners in Dessie, Northeast Ethiopia (N=339)

Variables	Category	Frequency: N^{O} (%)	HBsAg (22/339)		Fisher's Exact Test p-value	Anti-HCV (4/339)		Fisher's Exact Test p-value
			Positive: N^{O} (%)	Negative: N^{O} (%)		Positive: N^{O} (%)	Negative: N^{O} (%)	
Duration spent	<1year 1–3 years >3 years	112 (33) 90 (26.5) 137 (40.5)	9 (8.0) 2 (2.2) 11 (8.0)	103 (92.0) 88 (97.8) 126 (92.0)	0.138	2 (1.8) 1 (1.1) 1 (0.7)	110 (98.2) 89 (98.9) 136 (99.3)	0.826
History of previous imprisonment	Yes No	124 (36.6) 215 (63.4)	17 (13.7) 5 (2.3)	107 (86.3) 210 (97.7)	<0.001*	2 (1.6) 2 (0.9)	122 (98.4) 213 (99.1)	0.625
Multiple sexual partner	Yes No	143 (42.2) 196 (57.8)	18 (12.6) 4 (2.0)	125 (87.4) 192 (98.0)	<0.001*	1 (0.7) 3 (1.53)	142 (99.3) 193 (98.47)	0.641
History of sexually transmitted disease	Yes No	124 (36.6) 215 (63.4)	7 (5.64) 15 (6.97)	117 (94.36) 200 (93.03)	0.820	0 4 (1.86)	124 (100) 211 (98.14)	0.301
History of blood transfusion	Yes No	48 (14.2) 291 (85.8)	4 (8.33) 18 (6.18)	44 (91.67) 273 (93.82)	0.532	3 (6.25) 1 (0.34)	45 (93.75) 290 (99.66)	0.010*
History of surgical procedures	Yes No	65 (19.2) 274 (80.8)	6 (9.23) 16 (5.84)	59 (90.77) 258 (94.16)	0.398	3 (4.62) 1 (0.36)	62 (95.38) 273 (99.64)	0.023*
History of sharing sharp materials	Yes No	74 (21.8) 265 (78.2)	8 (10.8) 14 (5.28)	66 (89.2) 251 (94.72)	0.108	2 (2.7) 2(0.75)	72 (97.3) 263 (99.25)	0.209
Injectable drug use	Yes No	13 (3.8) 326 (96.2)	1 (7.7) 21 (6.44)	12 (92.3) 305 (93.56)	0.589	0 4 (1.23)	13 (100) 322 (98.77)	1.000
Tattooing	Yes No	191 (56.3) 148 (43.7)	20 (10.47) 2 (1.35)	171 (89.53) 146 (98.65)	0.001*	3 (1.57) 1 (0.67)	188 (98.43) 147 (99.33)	0.635
Ear/ nose piercing	Yes No	90 (26.5) 249 (73.5)	8 (8.9) 14 (5.62)	82 (91.1) 235 (94.38)	0.319	0 4 (1.6)	90 (100) 245 (98.4)	0.577
Homosexual	Yes No	7 (2.1) 332 (97.9)	1 (14.3) 21 (6.33)	6 (85.7) 311 (93.67)	0.378	0 4 (1.2)	7 (100) 328 (98.8)	1.000
History of dental extraction	Yes No	35 (10.3) 304 (89.7)	4 (11.43) 18 (5.9)	31 (88.57) 286 (94.1)	0.264	3 (8.57) 1 (0.33)	32 (91.43) 303 (99.67)	0.004*
Contact with jaundiced patient	Yes No	179 (52.8) 160 (47.2)	16 (8.9) 6 (3.75)	163 (91.1) 154 (96.25)	0.076	3 (1.68) 1 (0.63)	176 (98.32) 159 (99.37)	0.625

Note: *The observed difference is statistically significant (p < 0.05).
Abbreviations: HBsAg, hepatitis B surface antigen; Anti-HCV, anti- hepatitis C virus antibodies.

(10.1%),[43] Iranian (national data, 2015) (9.48%)[44] and Brazilian (5.3%)[19] prisoners. This noticeable difference could be due to the higher number of subjects who use injectable drugs (eg, injectable drug users among Iranian prisoners were reported as 17%, while in our case, they accounted 3.8%) and homosexuals reported in the above studies. In Ethiopia, IDU and homosexuality may be under-reported or practiced at a very minimal level since they are not acceptable by the community and by law.[45–47] The difference in the number of participants (sample size) can also impact the prevalence estimation.

Associated Factors for HBV Infection

In the current study, being over 44 years of age was significantly associated with the presence of HBsAg, a finding that was in line with a report from another study.[16] The reason for this association may be a proxy for lifetime exposure, indicating that, over time, there is a high risk of HBV infection linked to sexual activity and percutaneous exposures. There was a higher seroprevalence of HBsAg among rural dweller prisoners. This finding was in agreement with a previous study conducted in Nigeria.[48] The reasons for high seroprevalence in rural

Table 3 Bivariate and Multivariate Analysis of Risk Factors for HBV Infection Among Prisoners in Dessie, Northeast Ethiopia (N=339)

Variables	Categories	HBsAg		COR (95% CI)	p-value	AOR (95% CI)	p-value
		Positive: N⁻° (%)	Negative: N⁻° (%)				
Age	18–24	1 (1.2)	81 (98.8)	1		1	
	25–34	7 (5.5)	119 (94.5)	4.76 (0.57–39.46)	0.15	2.26 (0.19–26.06)	0.51
	35–44	3 (4.7)	61 (95.3)	3.98 (0.40–39.2)	0.23	5.19 (0.38–70.997)	0.21
	≥45	11 (16.4)	56 (83.5)	15.9 (1.99–26.75)	0.009	18.7 (1.73–20.37)	0.016*
Educational level	Not read & write	12 (15.4)	66 (84.6)	4.08 (1.03–22.47)	0.045	3.29 (0.35–30.88)	0.29
	Primary school	6 (4.7)	123 (95.3)	1.29 (0.257–6.61)	0.758	1.2 (0.122–12.27)	0.86
	Secondary school	2 (2.6)	75 (97.4)	0.7 (0.096–5.176)	0.73	0.4 (0.02–5.67)	0.50
	College/University	2 (3.6)	53 (96.4)	1		1	
Residence	Rural	18 (11.7)	136 (88.3)	4.47 (1.60–12.4)	0.004	8.51 (2.00–36.12)	0.004*
	Urban	4 (2.1)	181 (97.8)	1		1	
Number of sexual partners	1	3 (2.7)	110 (97.3)	2.23 (0.22–21.8)	0.48	0.81 (0.06–11.09)	0.879
	≥2	18 (12.6)	125 (87.4)	11.8 (1.54–90.16)	0.02	8.65 (1.79–93.8)	0.026*
	0	1 (1.2)	82 (98.8)	1		1	
History of previous imprisonment	Yes	17 (13.7)	107 (86.3)	6.67 (2.39–18.5)	0.00	7.15 (1.91–26.7)	0.003*
	No	5 (2.3)	210 (97.7)	1		1	
Tattooing	Yes	20 (10.5)	171 (89.5)	8.53 (1.96–37.14)	0.004	15.03 (1.68–134.3)	0.015*
	No	2 (1.4)	146 (98.6)	1		1	
History of sharing sharp materials	Yes	8 (11.0)	66 (89.0)	2.20 (0.88–5.4)	0.08	2.93 (0.77–11.1)	0.11
	No	14 (5.3)	251 (94.7)	1		1	
History of dental extraction	Yes	6 (4.8)	118 (95.2)	2.05 (0.65–6.44)	0.01	1.09 (0.20–5.85)	0.91
	No	16 (7.4)	199 (92.6)	1		1	
Contact with jaundiced patient	Yes	16 (8.9)	163 (91.1)	3.02 (1.08–8.45)	0.03	4.35 (1.14–16.5)	0.03*
	No	6 (3.1)	154 (96.9)	1		1	

Note: *The observed difference is statistically significant (p < 0.05).
Abbreviations: HBsAg, hepatitis B surface antigen; COR, crude odds ratio; AOR, adjusted odds ratio.

Table 4 Bivariate and Multivariate Analysis of Risk Factors for HCV Infection Among Prisoners in Dessie, Northeast Ethiopia (N=339)

Variables		Anti-HCV		COR (95% CI)	AOR (95% CI)	p-value
		Positive: N° (%)	Negative: N° (%)			
History of blood transfusion	Yes	3(6.3)	45(93.8)	19.33(1.96–189.93)	27.63(2.34–325.59)	0.008*
	No	1(0.3)	290(99.7)	1	1	
History of operation	Yes	3(4.6)	62(95.4)	13.21(1.35–129.135)	5.68(0.41–78.59)	0.19
	No	1(0.4)	273(99.6)	1	1	
Sharing of sharp material	Yes	2(2.7)	72(97.3)	3.70(0.51–26.75)	7.07(0.05–10.05)	0.843
	No	2(0.8)	263(99.2)	1	1	
History of dental extraction	Yes	3(2.4)	121(97.6)	28.4(2.87–281.15)	39.94(3.40–68.58)	0.003*
	No	1(0.5)	214(99.5)	1	1	

Notes: *The observed difference is statistically significant (p < 0.05).
Abbreviations: Anti-HCV, anti- hepatitis C virus antibodies; COR, crude odds ratio; AOR, adjusted odds ratio

dwellers might be due to low economic status, low educational level, inability to obtain healthcare information from the media, or limited access to medical care than urban dwellers.[49,50]

In this study, the history of imprisonment had a significant association with the presence of HBsAg (AOR = 7.15, 95% CI: 1.91–26.7) and this result is supported by studies among prisoners in Iran.[39,44] Several risk factors act together with the crowded prison condition may facilitate the transmission of this virus. Prisoners having multiple sexual partners had about 8.6 times higher HBsAg in their serum compared to their counterparts. It is consistent with the reports of the study conducted among prisoners in Jimma, Ethiopia[27] and Indonesia.[51] The high seroprevalence rate among promiscuous prisoners may be the fact that HBV is sexually transmitted, and the transmission rises with the duration of sexual activity and the number of sexual partners. Tattooing is also significantly associated with the presence of HBsAg among prisoners. A high proportion of the inmates (56.3%) are practicing tattooing while incarcerated. Similar findings were reported from Mexico[40] and Indonesia.[51] The high prevalence rate among tattooed prisoners may be due to the sharing and reuse of instruments among inmates.

Associated Factors for HCV Infection

In our study, all the socio-demographic characteristics of the participants were not significantly associated with the presence of HCV antibody. In contrast, studies by Kinner et al., 2017,[52] Soholm et al., 2019,[53] and Miller et al.[20] reported that the prevalence was higher among older prisoners. Increased exposure to the risk of infection at the

prison and the high probability of the presence of irreversible HCV seroconversion may explain higher prevalence in older age groups. In terms of sex, some studies also reported a higher prevalence among female prisoners than males. Intravenous drug use (IDU) is reported as the main risk factor for HCV infection in prison settings, where widespread sharing of contaminated equipment is prevalent. According to a WHO report, most HCV infections (67%) are related to IDU practices.[1] Studies such as Zampino et al.,[33] Soholm et al.,[53] Puga et al.,[54] Guimaraes et al.,[55] and Bahzadifar et al.[15] reported that a history of IDU was a risk factor for HCV infection. In our study, we reported that prisoners use injecting drugs for medical purposes only, which was administered by health professionals, but not for other purposes. Since illegal use of intravenous drugs is not widely and openly practiced in Ethiopia, especially in the study area, the seroprevalence of HCV infection is expected to be lower than results from other countries where intravenous drug use is prevalent.

The finding of greater HCV antibody prevalence in prisoners with blood transfusion is consistent with the study from Jimma.[27] The possible explanation for a significant association between HCV infection and blood transfusion might be the lack of improved laboratory screening methods of HCV infection from blood donors before transfusion. The seroprevalence of HCV was higher in participants with a history of dental extraction is supported by a study conducted among prisoners in Egypt.[16] The possible reason may be due to traditional tooth extraction practices,[56] shortage of electricity, dental equipment

spare parts, or trained healthcare workers in health institutions of the developing world, which hinders the proper decontamination or sterilization techniques.[57]

Limitation

This research article is limited in assessing anti-HBcAg. Since individuals having infection prior to six months may not be positive for HBsAg (resolution of infection), the prevalence of HBV infection might be underestimated. Furthermore, due to availability and financial reasons, HCV-RNA could not be measured, which is a more specific test for infectious individuals.

Conclusion

In Ethiopia, it was reported that awareness of hepatitis virus disease, complications, transmission, control, prevention, and treatment options were poor. Furthermore, screening for viral hepatitis was not widely practiced, and diagnosed patients were not receiving available treatments.[58] We reported an intermediate seroprevalence rate of HBV infection among prisoners and is associated with high-risk behavior, including history of multiple sexual partners, previous imprisonment, body tattooing, and contact with the jaundiced patient. Although many studies classified prison settings as a high-risk environment for HCV infection, we reported a lower prevalence of HCV antibodies among them. However, higher prevalence of HCV antibodies among prisoners with a history of blood transfusion and dental extraction calls for the need for serological testing. The regional health bureau, the prison authorities, and healthcare workers are expected to deliver health education on the reduction of high-risk behaviors, mode of HBV and HCV transmission, and control and prevention mechanisms to prisoners.

Abbreviations

ELISA, Enzyme Linked Immunosorbent Assay; HBsAg, Hepatitis B Surface Antigen; HBV, Hepatitis B Virus; HCV, Hepatitis C virus; HCC, Hepatocellular Carcinoma; IDU, Injection drug use; STD, Sexually Transmitted Disease; WHO, World Health Organization.

Data Sharing Statement

The datasets used and/or analyzed during the study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the research and Ethical Review Committee of the School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar and a formal letter was obtained. Official permission also obtained from Dessie town prison Administration Office after explaining the aim of the study. In addition to that, following an explanation of the purpose of the study, written informed consent was obtained from study participant and/or their guardian officers before data collection. Furthermore, the study was conducted in accordance with the declaration of Helsinki (59). Personal identifiers were not used, and data were retrieved only for the study purpose to ensure confidentiality. Finally, prisoners who are positive for HBsAg and anti-HCV was linked to prison physicians for further investigation and treatment.

Consent for Publication

Each study participant was informed and signed for publication.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests in this work.

References

1. World Health Organization [WHO]. Global health sector strategy on viral hepatitis. Towards ending viral hepatitis. In: *Global Hepatitis Programme Department of HIV/AIDS*; 2016–2021.

2. World Health Organization. Global Hepatitis Report 2017; 2017 Licence: CC BY-NC-SA 3.0 IGO. Geneva: World Health Organization. 2017.

3. Blach S, Zeuzem S, Manns M, et al. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol*. 2017;2:3.

4. World Health Organization. *Hepatitis C [Internet]*. WHO; 2020. Accessed https://www.who.int/news-room/fact-sheets/detail/hepatitis-c. 2020 Nov 14.

5. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380:9859.

6. Perz JF, Armstrong GL, Farrington LA, Hutin YJF, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol*. 2006;45:4.

7. Cox AL, El-Sayed MH, Kao JH, et al. Progress towards elimination goals for viral hepatitis. *Nat Rev Gastroenterol Hepatol*. 2020;17:9.

8. Taherkhani R, Farshadpour F. Global elimination of hepatitis C virus infection: progresses and the remaining challenges. *World J Hepatol*. 2017;9:1239–1252.

9. Taherkhani R, Farshadpour F. Lurking epidemic of hepatitis C virus infection in Iran: a call to action. *World J Hepatol*. 2017;9 (24):1040–1042.

10. WHO. Hepatitis B [Internet]. Fact Sheets. 2020. Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-b. Accessed May 31, 2021.

11. WHO. Hepatitis C [Internet]. Fact Sheets. 2020. Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-c. Accessed May 25, 2021.

12. De La Hoya PS, Marco A, García-Guerrero J, Rivera A. Hepatitis c and b prevalence in Spanish prisons. *Eur J Clin Microbiol Infect Dis*. 2011;30:7.

13. Kamarulzaman A, Reid SE, Schwitters A, et al. Prevention of transmission of HIV, hepatitis B virus, hepatitis C virus, and tuberculosis in prisoners. *The Lancet*. 2016;388.

14. Dolan K, Wirtz AL, Moazen B, et al. Global burden of HIV, viral hepatitis, and tuberculosis in prisoners and detainees. *The Lancet*. 2016;388.

15. Behzadifar M, Gorji HA, Rezapour A, Bragazzi NL. Prevalence of hepatitis C virus infection among prisoners in Iran: a systematic review and meta-analysis. *Harm Reduct J*. 2018;15.

16. Mohamed HI, Saad ZM, Abd-Elreheem EM, et al. Hepatitis C, hepatitis B and HIV infection among Egyptian prisoners: seroprevalence, risk factors and related chronic liver diseases. *J Infect Public Health*. 2013;6:3.

17. Kose S, Adar P, Gozaydin A, Kuzucu L, Akkoclu G. Hepatitis B and Hepatitis C in prisons: a prevalence study. *Int J Prison Health*. 2019;15:2.

18. Larney S, Kopinski H, Beckwith CG, et al. Incidence and prevalence of hepatitis C in prisons and other closed settings: results of a systematic review and meta-analysis. *Hepatology*. 2013;58:4.

19. El Maerrawi I, Carvalho HB. Prevalence and risk factors associated with HIV infection, hepatitis and syphilis in a state prison of São Paulo. *Int J STD AIDS*. 2015;26:2.

20. Miller ER, Bi P, Ryan P. The prevalence of HCV antibody in South Australian prisoners. *J Infect*. 2006;53:2.

21. Masarone M, Caruso R, Aglitti A, et al. Hepatitis C virus infection in jail: difficult-to-reach, not to-treat. Results of a point-of-care screening and treatment program. *Dig Liver Dis*. 2020;52:5.

22. Belaunzarán-Zamudio PF, Mosqueda-Gomez JL, Macias-Hernandez A, Sierra-Madero JG, Ahmed S, Beyrer C. Risk factors for prevalent hepatitis C virus-infection among inmates in a state prison system in Mexico. *PLoS One*. 2017;12(6):e0179931–e0179931.

23. Bautista-Arredondo S, González A, Servan-Mori E, et al. A cross-sectional study of prisoners in Mexico city comparing prevalence of transmissible infections and chronic diseases with that in the general population. *PLoS One*. 2015;10:7.

24. Gidding HF, Mahajan D, Reekie J, Lloyd AR, Dwyer DE, Butler T. Hepatitis B immunity in Australia: a comparison of national and prisoner population serosurveys. *Epidemiol Infect*. 2015;143:13.

25. Moradi G, Goodarzi E, Khazaei Z. Prevalence of Hepatitis B and C in prisons worldwide: a meta-analysis during the years 2005-2015. *Biomed Res Ther*. 2018;5:4.

26. Walmsley R. World Prison Population List (twelfth edition). *Inst Crim Policy Res*. 2018;12.

27. Kebede W, Abdissa A, Seid Y, Mekonnen Z. Seroprevalence and risk factors of hepatitis B, hepatitis C and HIV infections among prisoners in Jimma Town, Southwest Ethiopia. *Asian Pacific J Trop Dis*. 2017;7:5.

28. Berhe H, Yismaw G, Addis Z, Anagaw H, Uc B. Seroprevalence and associated risk factors of human immunodeficiency virus and Hepatitis B Virus infections among prison inmates. *J Pharm Biomed Sci*. 2013;30(30):1035–1045.

29. Charan J, Biswas T. How to calculate sample size for different study designs in medical research? *Indian J Psychol Med*. 2013;35.

30. Elfil M, Negida A. Sampling methods in clinical research; an educational review. *Arch Acad Emerg Med*. 2019;7:1.

31. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet. 2015;386(10003).

32. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*. 2013;57:4.

33. Zampino R, Coppola N, Di Caprio G, Sagnelli C, Sagnelli E. Hepatitis C virus infection and prisoners: epidemiology, outcome and treatment. *World J Hepatol*. 2015;7.

34. Ramamoorthy M, Venketeswaran A, Seenivasan P, et al. Risk factors and prevalence, hepatitis B virus and hepatitis C virus among prison inmates, Chennai, India, 2015. *Int J Infect Dis*. 2016;53.

35. Gétaz L, Casillas A, Siegrist CA, et al. Hepatitis B prevalence, risk factors, infection awareness and disease knowledge among inmates: a cross-sectional study in Switzerland's largest pre-trial prison. *J Glob Health*. 2018;8:2.

36. Crowley D, Lambert JS, Betts-Symonds G, et al. The seroprevalence of untreated chronic hepatitis c virus (HCV) infection and associated risk factors in male Irish prisoners: a cross-sectional study, 2017. *Eurosurveillance*. 2019;24:14.

37. Jaquet A, Wandeler G, Tine J, et al. HIV infection, viral hepatitis and liver fibrosis among prison inmates in West Africa. *BMC Infect Dis*. 2016;16:1.

38. Dan-Nwafor C, Adeoye I, Aderemi K, et al. Knowledge, serological markers and risk factors associated with Hepatitis B and C virus infection among Kuje Prison Inmates, Federal Capital Territory, Nigeria. *Int J Infect Dis*. 2018;73.

39. Taleban R, Moafi M, Ataei B, et al. Seroprevalence of Hepatitis B infection and associated risk factors among drug users in drop-in centers of Isfahan, Iran. *Int J Prev Med*. 2018;9.

40. Belaunzaran-Zamudio PF, Mosqueda-Gomez JL, MacIas-Hernandez A, Rodríguez-Ramírez S, Sierra-Madero J, Beyrer C. Burden of HIV, Syphilis, and Hepatitis B and C among Inmates in a Prison State System in Mexico. *AIDS Res Hum Retroviruses*. 2017;33.

41. Ghafari S, Sharifzadeh G, Jamali S, Taji B, Javadmoosavi SY, Ziaee M. Prevalence of Hepatitis B and C among drug-abusing male prisoners in Birjand, South Khorasan, Iran. *Arch Iran Med*. 2019;22:9.

569

42. Sunbul M. Hepatitis B virus genotypes: global distribution and clinical importance. *World J Gastroenterol*. 2014;20:18.

43. Alvarez KJ, Befus M, Herzig CTA, Larson E. Prevalence and correlates of hepatitis C virus infection among inmates at two New York State correctional facilities. *J Infect Public Health*. 2014;7:6.

44. Moradi G, Gouya MM, Azimizan Zavareh F, et al. Prevalence and risk factors for HBV and HCV in prisoners in Iran: a national bio-behavioural surveillance survey in 2015. *Trop Med Int Heal*. 2018;23:6.

45. Overs C. booshtee! Survival and resilience in Ethiopia. *Inst Dev Stud*. 2015.

46. FDRE. *The Criminal Code of the Federal Democratic Republic of Ethiopia*. Negarita Gazeta. 414. 2004.

47. Tadele G, Amde WK. Health needs, health care seeking behaviour, and utilization of health services among lesbians, gays and bisexuals in Addis Ababa, Ethiopia. *Int J Equity Health*. 2019;18:1.

48. Meka IA, Onodugo OD, Obienu O, Okite J. Hepatitis B surface antigenemia in two rural communities in Enugu, Nigeria. *Niger J Clin Pract*. 2019;22:7.

49. Lingani M, Akita T, Ouoba S, et al. High prevalence of hepatitis B infections in Burkina Faso (1996-2017): a systematic review with meta-analysis of epidemiological studies. *BMC Public Health*. 2018;18.

50. Andriamandimby SF, Olive MM, Shimakawa Y, et al. Prevalence of chronic hepatitis B virus infection and infrastructure for its diagnosis in Madagascar: implication for the WHO's elimination strategy. *BMC Public Health*. 2017;17:1.

51. Rey I, Saragih RH, Effendi-Ys R, Sembiring J, Siregar GA, Zain LH Profile of hepatitis B and C virus infection in prisoners in Lubuk Pakam correctional facilities. In: IOP Conference Series: Earth and Environmental Science. 2018.

52. Kinner SA, Snow K, Wirtz AL, Altice FL, Beyrer C, Dolan K. Age-specific global prevalence of Hepatitis B, Hepatitis C, HIV, and tuberculosis among incarcerated people: a systematic review. *J Adolescent Health*. 2018;62.

53. Søholm J, Holm DK, Mössner B, et al. Incidence, prevalence and risk factors for hepatitis C in Danish prisons. *PLoS One*. 2019;14:7.

54. Puga MAM, Bandeira LM, Pompilio MA, et al. Prevalence and incidence of HCV infection among prisoners in Central Brazil. *PLoS One*. 2017;12:1.

55. Guimarães T, Granato CF, Varella D, Ferraz ML, Castelo A, Kallás EG. High prevalence of hepatitis C infection in a Brazilian prison: identification of risk factors for infection. *Braz J Infect Dis*. 2001;5:3.

56. Taye BW. A path to ending hepatitis C in Ethiopia: a phased public health approach to achieve micro-elimination. *Am J Trop Med Hygiene*. 2019;101.

57. Al-Kubaisy W, Al-Naggar RA, Shamsidah NIN, Bobryshev YV, Al-Kubaisy MW. Is dental extraction a risk factor for contracting HCV infection: abs, RNA and genotype detection. *Oral Biol Dent*. 2014;2:1.

58. Shiferaw F, Letebo M, Bane A. Chronic viral hepatitis: policy, regulation, and strategies for its control and elimination in Ethiopia. *BMC Public Health*. 2016;16:1.

59. World Medical Association declaration of Helsinki.:Ethical principles for medical research involving human subjects. *JAMA*. 2013;310.

Identification and Validation of Hemophilia-Related Outcomes on Japanese Electronic Medical Record Database (Hemophilia-REAL V Study)

Takashi Fujiwara, Chisato Miyakoshi, Takashi Kanemitsu, Yasuyuki Okumura & Hironobu Tokumasu

Published online: 06 Jul 2021.

Submit your article to this journal

Article views: 183

View related articles

View Crossmark data

Citing articles: 5 View citing articles

ORIGINAL RESEARCH

Identification and Validation of Hemophilia-Related Outcomes on Japanese Electronic Medical Record Database (Hemophilia-REAL V Study)

Takashi Fujiwara [1,2]
Chisato Miyakoshi[3]
Takashi Kanemitsu[4]
Yasuyuki Okumura [5]
Hironobu Tokumasu[2,6]

[1]Department of Management, Clinical Research Center, Kurashiki Central Hospital, Okayama, Japan; [2]Department of Public Health Research, Kurashiki Clinical Research Institute, Okayama, Japan; [3]Department of Pediatrics, Kobe City Medical Center General Hospital, Hyogo, Japan; [4]Medical Affairs Division, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan; [5]Department of Psychiatry and Behavioral Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; [6]Real World Data Co., Kyoto, Japan

Correspondence: Takashi Fujiwara
Department of Management, Clinical Research Center, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki City, Okayama, Japan
Tel +81-86-422-0210
Fax +81-86-421-3424
Email tf14817@kchnet.or.jp

Purpose: Routinely collected data are useful for epidemiological study in hemophilia, but few studies validated the algorithm accuracy. We aimed to develop and validate algorithms to identify patients with hemophilia A and hemophilia A-related events.

Patients and Methods: This validation study compared data from medical chart reviews to a database of routinely collected health data, including claims data and discharge abstracts, and especially electronic medical records (EMR), at a single Japanese hospital (Kurashiki Central Hospital) using a stratified sampling method. Two physicians reviewed the charts for all patients at high risk for hemophilia A, and randomly sampled patients with moderate risk. Diagnostic accuracy was determined based on sensitivity, specificity, positive predictive value (PPV), and negative predictive value.

Results: There were 1,033,845 eligible patients, of whom 31 had a diagnosis of hemophilia A. ICD-10 diagnosis code D66 in the EMR identified hemophilia A with a sensitivity of 93.5% (95% confidence interval: 78.6–99) and PPV of 61.7% (95% confidence interval: 46.4–75.5). The administration of ≥10,000 units/month of factor VIII products, as documented in the EMR, identified 81.3% of patients with prophylactic factor replacement therapy. The ICD-10 diagnosis code for intracranial bleeding in the EMR identified 75.0% of patients with intracranial bleeding, but those of gastrointestinal bleeding and major joint bleeding identified only 11.1% and 1.7%, respectively.

Conclusion: We developed and validated algorithms to identify congenital hemophilia A and hemophilia A-related events. Hemophilia A could be identified with high sensitivity and PPV, but it was still challenging to identify hemophilia A-related events.

Keywords: congenital hemophilia, electronic health record, positive predictive value, sensitivity, validation study

Introduction

Congenital hemophilia A is a rare, chronic, heritable bleeding disorder caused by deficiency of clotting factor VIII.[1] Hemophilia A is the most common type of congenital hemophilia, occurring in approximately 1 in 5000 live-born males.[2] Severe hemophilia A is defined as factor VIII activity of <1%, as seen in two-thirds of hemophilia A patients. Patients with hemophilia A suffer from lifelong bleeding, but the availability of factor replacement products has markedly improved the care for patients with these conditions over the past decade.[3,4]

The low prevalence of hemophilia A complicates large-scale epidemiological studies. Therefore, routinely collected health data, such as electronic medical

records (EMR) and claims data, are vital to understand the clinical course of hemophilia A.[5,6] Accurate identification of patients with hemophilia A is crucial, but misclassification can occur. Algorithms for identifying patients with hemophilia A in EMR and claims databases have been developed and validated in the United States.[7,8] However, there have been no reports of validation studies of hemophilia in Japan. Health care systems, including EMR and claims data, vary among countries; therefore, the accuracy of algorithms for identifying disease may also vary. It is important to develop algorithms for identifying patients with hemophilia A to conduct epidemiological studies using EMR and claims data. In addition, no study has developed a validated algorithm to identify hemophilia A-related outcomes.[7,8] The present study was performed to develop and validate algorithms for identifying patients with hemophilia A and hemophilia A-related outcomes using the EMR and claims database in Japan.

Patients and Methods
Study Design
This validation study compared data from medical chart reviews to a database of routinely collected health data, including EMR, claims data, and discharge abstracts for a single Japanese hospital (Kurashiki Central Hospital) using a stratified sampling method.[9] Kurashiki Central Hospital is an urban hospital with 1172 beds that serves 800,000 people in the western area of Okayama Prefecture, Japan.[10]

This study was conducted and reported in accordance with the statement of the Japanese Society for Pharmacoepidemiology and the Standards for Reporting of Diagnostic Accuracy Studies criteria.[11–13] The study was approved by the institutional review board of Kurashiki Central Hospital and the Research Institute of Healthcare Data Science. This study was registered in the UMIN Clinical Trials Registry (Trial Number: UMIN000038212; https://www.umin.ac.jp/ctr/index-j.htm).

Data Sources
This study used anonymized, routinely collected health data stored in a database.[10] The Health, Clinic, and Education Information Evaluation Institute (HCEI) has contracts with more than 190 healthcare institutions, including Kurashiki Central Hospital, to collect EMR and claims data from those institutions and develop a large-scale database, known as the RWD database. The

anonymized data for this research study were collected by the HCEI on August 28, 2019. The RWD database included approximately 20.5 million inpatients and outpatients. In the database, disease data are extracted from EMRs and are recorded based on the International Classification of Diseases, 10th revision (ICD-10) codes. Drugs are labeled based on the Japanese receipt code and YJ code. Laboratory test results are standardized and labeled according to the Japanese Laboratory Code version 10.

Study Population
Due to the low prevalence of hemophilia A, it would be difficult to ensure precise diagnostic value with a random sample of patients from the overall patients at Kurashiki Central Hospital. Therefore, we used a stratified sampling method to identify all possible cases of hemophilia A.[11,14,15]

Patients were classified as a having high, moderate, or low risk for hemophilia A based on diagnostic codes (ICD-10), drug codes, procedural codes, and notes in the medical records. Patients who received an ICD-10 diagnosis or suspected code D66 (congenital factor VIII disorder, hemophilia A) were classified as being at high risk for hemophilia A. We defined moderate risk of hemophilia A as follows: diagnosed or suspected congenital factor IX disorder or hemophilia B (ICD-10 D67), von Willebrand disease (D680), or acquired factor VIII disorder or hemophilia A (D684); prescription for hemophilia treatment (factor VIII products); blood test related to hemophilia (factor VIII activity); and "hemophilia A" in chart notes (see Appendix 1). We defined all other patients as being at low risk for hemophilia A. Chart review was conducted for all high-risk patients and randomly sampled moderate-risk patients.

Ascertaining Hemophilia A and Disease-Related Outcomes
Detailed medical chart reviews of all high-risk patients and a subset of moderate-risk patients were conducted. Hemophilia A-related outcomes including disease, treatment and disease-related events were analyzed. Two physicians (an adult hematologist and a pediatric hematologist) independently conducted the paper and electrical chart reviews for all available periods. Kurashiki Central Hospital introduced an EMR system in 2003, before which paper charts were used. The reviewers also

identified hemophilia A-related outcomes, including disease characteristics (severity and history of factor VIII inhibitor), treatment issue (prophylactic factor VIII replacement therapy), and disease-related events (intracranial bleeding, gastrointestinal bleeding, and major joint bleeding). Disagreements were resolved by discussion.

Data Linkage and Data Extraction from Database

The RWD database contained healthcare EMR data from individual medical institutions. When extracting EMR data from medical institutions, patients' EMR numbers were removed, and each patient was hash-encoded to anonymize the records. We also used chart review data, which contained personal identification information; these data were anonymized, and a hash was provided encoding the RWD for each patient. The chart review data were subsequently linked with the RWD database.

Statistical Analysis

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each algorithm were calculated, accepting the chart review results as the reference standard. When evaluating the diagnostic values of disease (hemophilia A), we calculated these diagnostic values adjusted with weighted sampling according to a task force report on the validation of diagnosis codes in Japan.[11] When calculating the diagnostic values of disease characteristics (severity and history of factor VIII inhibitor) and treatment issue (prophylactic factor VIII replacement therapy), we examined data for patients with ICD-10 diagnosis or suspected code D66. When assessing disease-related events (intracranial bleeding, gastrointestinal bleeding, and major joint bleeding), we determined the sensitivity and PPV. If a disease-related event occurred within 3 days of the recorded outcome, it was recorded as a true positive.

For evaluating the diagnostic value of severity of hemophilia A, the algorithm used laboratory test results of factor VIII activity. Laboratory test results of factor VIII activity are often stored as numeric data in the RWD database, but the results are sometimes stored as characteristic data, which we defined as follows: severe; ≤1, <2, and <3, moderate; ≤5; mild; <5.0, <5.1, and ≥5.0, normal; (+).

In accordance with the task force report on the validation of diagnosis codes in Japan,[11] we evaluated how representative the population in this validation study was of the entire RWD population. We compared patients who received an

ICD-10 diagnosis code D66 in Kurashiki Central Hospital and those in the RWD database. We assessed patient characteristics (age at data extraction, sex), comorbidities (hypertension, diabetes mellitus, hyperlipidemia, arteriosclerosis, and cardiovascular disease), and hemophilia-related data (age at receiving ICD-10 diagnosis code D66, factor VIII activity, factor VIII inhibitor, prescription of factor VIII products, and prophylactic factor replacement therapy). The presence of comorbidities was defined using the ICD-10 diagnosis codes (Appendix 2).

Statistical analyses were performed using R software (version 3.4.1; R Foundation for Statistical Computing, Vienna, Austria).

Study Ethics

This study was approved by the institutional ethics committee of the Research Institute of Healthcare Data Science (https://rihds.org/ethic/) and the institutional ethics committee of Kurashiki Central Hospital. Optout consent was used.

Results
Patient Selection

We identified 128 patients with a high risk of hemophilia A from the medical records of Kurashiki Central Hospital. We also identified 895 patients with moderate risk of hemophilia A and randomly selected 120 of these patients (Figure 1). We conducted a chart review of the 248 patients, and the data were linked with the RWD database. The chart review revealed no hemophilia patients among those with a moderate risk of the disease. After data linkage of the chart review and the RWD database, 12 patients were excluded due to a lack of data in the RWD database (Appendix 3).

Of the 236 patients included in the study, 31 were identified as hemophilia A and were in the high-risk group. There were no cases of hemophilia A in the moderate-risk group (Figure 1). The characteristics of the 31 patients with hemophilia A are shown in Table 1.

Diagnostic Value

The definitions of each algorithm and the diagnostic values are shown in Tables 2–4. In the outcome condition of disease (hemophilia A), ICD-10 diagnosis or suspected code D66 showed 100% sensitivity, with low PPV (24.4%). Compared with ICD-10 diagnosis or suspected code D66, ICD-10 diagnosis code D66, and male sex had similar sensitivity (93.5%) but higher PPV (73.3%; Table 2). Among 47 patients with
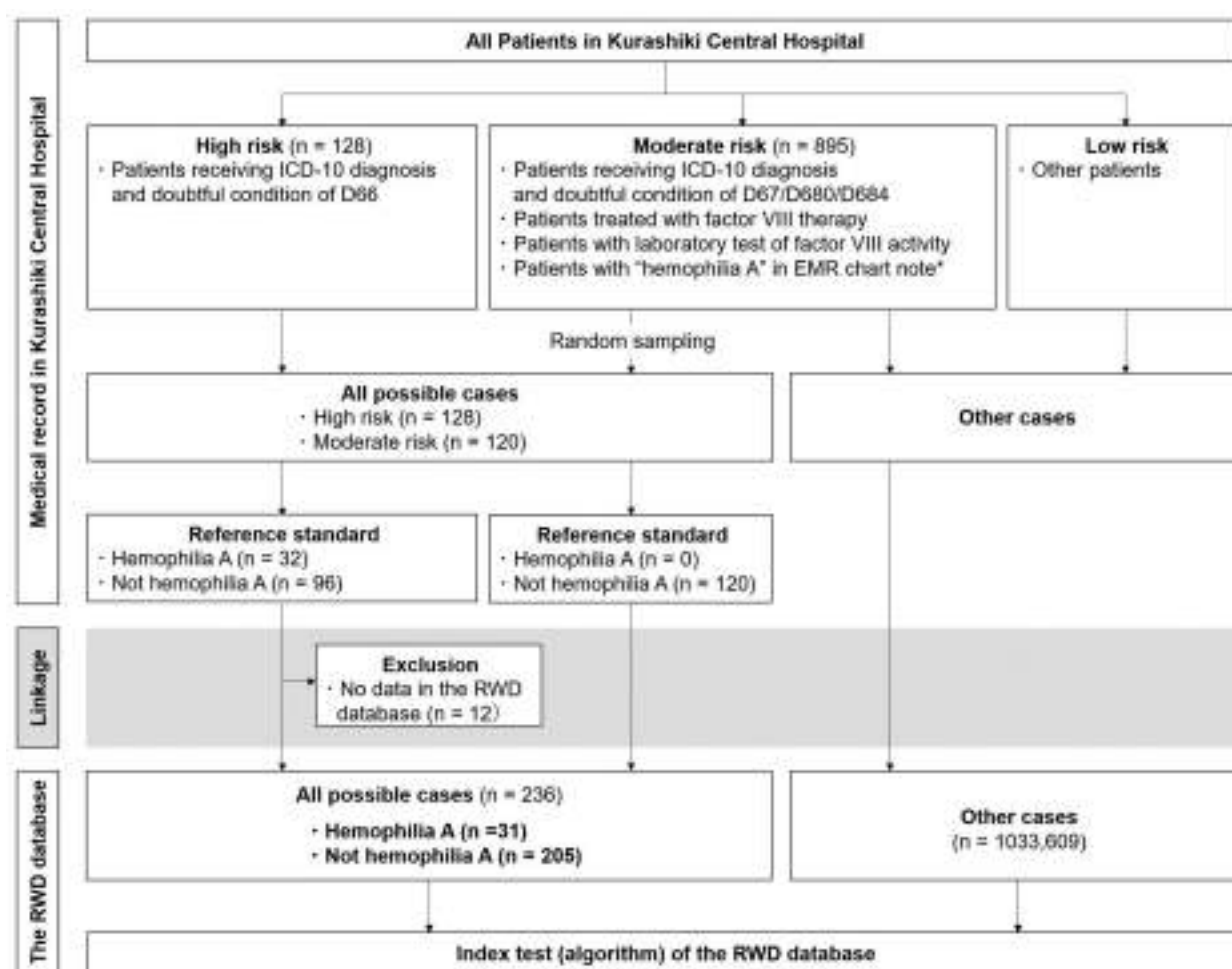
Figure 1 Flow diagram of patients' selection and validation.
Note: *We used Japanese written language (Kanji) when searching of "hemophilia A" in electronic medical record (EMR).

a definitive diagnosis of hemophilia (ICD-10 D66) according to the Kurashiki Central Hospital EMR database, the sensitivity and specificity of disease characteristics and treatment issues were about 70–90% (Table 3). However, the sensitivity and PPV of disease-related events were very low except for intracranial bleeding (Table 4). The data for other diagnoses are listed in Appendix 4. The definition of treatment products are listed in Appendix 5, Appendix 6, and Appendix 7.

Comparison of Kurashiki Central Hospital Data and the RWD Database

In the outcome definition of hemophilia A, the ICD10 diagnosis code D66 algorithm using EMR data had useful diagnostic value. Therefore, we used this algorithm to assess how representative the population in this study was of the entire RWD database. Table 5 shows the patients receiving

ICD-10 diagnosis code D66 in Kurashiki Central Hospital and the RWD database. The definition of co-morbidity disease for the comparison are listed in Appendix 8.

Discussion

Japan has recently promoted the use of RWD, including EMR and claims databases,[16] but there have been no comprehensive validation studies. A recent systematic review suggested that only six validation studies had been performed in Japan as of 2017.[17] Several studies identified and validated algorithms of hemophilia A.[7,8] However, these studies only validated the algorithms of disease condition and not algorithms of hemophilia A-related events (eg, joint bleeding, and intracranial bleeding). By contrast, the present study revealed the diagnostic value of disease condition, treatment condition, and hemophilia A-related events. The results of this study will

Table I Characteristics of Patients with Congenital Hemophilia A of Kurashiki Central Hospital

Variable		Congenital Hemophilia A (n = 31)	
Mean age, standard deviation (range)		37.6 ± 21.1	(1–90)
Male, n, (%)		31	(100.0%)
Severity, n, (%)	Severe	13	(41.9%)
	Moderate	10	(32.3%)
	Mild	8	(25.8%)
History of prophylactic factor replacement therapy, n, (%)		16	(51.6%)
History of factor VIII inhibitor, n, (%)		3	(9.7%)
History of immune tolerance induction, n, (%)		2	(6.5%)

promote epidemiological studies of hemophilia A using the EMR and claims database.

Algorithm for Hemophilia A

The sensitivity of the ICD-10 diagnosis or suspected code D66 algorithm was 100.0%, but the PPV was 24.4%. The sensitivity of the ICD-10 diagnosis code D66 algorithm had a higher PPV (61.7%), but the sensitivity was 93.5%. Of the 31 hemophilia patients identified in this study, the treatment period in Kurashiki Central Hospital varied. Two patients were treated 30 years ago, and the medical records were mainly stored in paper charts; these patients appeared as false negatives in the ICD-10 diagnosis code D66 algorithm. The sensitivity and PPV would be higher when evaluating diagnostic values using populations currently undergoing treatment.

Algorithm for Disease-Related Events

This study identified and validated an algorithm for hemophilia A-related events. In hemophilia A, clinical practice guidelines define intracranial, neck/throat, and gastrointestinal bleeding as life-threatening.[3] Therefore, we attempted to identify and validate an algorithm for these types of bleeding conditions, although there were no cases of neck/throat bleeding in this study. We also evaluated the diagnostic value of the algorithm for major joint bleeding

because that is one of the most frequent hemophilia A-related events.

Intracranial bleeding is the most critical hemophilia A-related event, and the algorithm based on its ICD-10 diagnosis code had 75.0% sensitivity and 33.3% PPV. During the study period, intracranial bleeding occurred in four patients. No disease-related ICD-10 code was provided for one event; the patient had asymptomatic intracranial bleeding after brain tumor resection. Disease-related ICD-10 was provided in the three symptomatic cases. The algorithm based on ICD-10 code for intracranial bleeding identified symptomatic intracranial bleeding with 100% sensitivity.

Strengths of the EMR Database

In the healthcare field, there are two primary types of databases for observational research; claims databases and EMR databases, and previous validation studies of hemophilia A used claims data.[8,9] This study used the RWD database, which is classified as an EMR database. Compared with claims databases, EMR databases can provide laboratory test results. In this study, we attempted to identify severe hemophilia A using the test results of factor VIII activity. The minimum test results of factor VIII activity <1% algorithm had a sensitivity of 69.2% and specificity of 76.5%. The algorithm yielded false-negative results in four patients: one of these patients had visited our hospital 30 years ago, and the laboratory test results were not stored; two patients had already been treated when Kurashiki Central Hospital introduced EMR, and factor VIII activity was >1%, and the remaining patient had been treated at another hospital before visiting Kurashiki Central Hospital, and factor VIII activity was >1%. Therefore, the limitations of EMR databases must be considered when conducting epidemiological studies stratified by disease severity. Researchers should exclude patients initially treated ≥10 years ago, and consider the influence of transfer from another hospital.

This study revealed that algorithms based on ICD-10 codes for hemophilia A had at least 90% PPV and NPV. However, the accuracy of algorithms based on the ICD-10 codes for disease-related events was very low. Free-text notes in the EMR were not used in any algorithms because of their unstructured nature. However, use of unstructured free-text notes in algorithms for disease-related events could be a target for future research.

Table 2 Diagnostic Value of Algorithm of Outcome Conditions: Disease (Hemophilia A)

Outcome Definition	Algorithm	True Positive (n)	False Positive (n)	False Negative (n)	True Negative (n)	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)
Hemophilia A	ICD-10 diagnosis or Suspected code D66 (EMR)	31	96	0	1033718	100.0 (88.8–100.0)	100.0 (100.0–100.0)	24.4 (17.2–32.8)	100.0 (100.0–100.0)
	ICD-10 diagnosis code D66 (EMR)	29	18	2	1033796	93.5 (78.6–99.2)	100.0 (100.0–100.0)	61.7 (46.4–75.5)	100.0 (100.0–100.0)
	ICD-10 diagnosis code D66 (Claim data)	22	8	9	1033806	71.0 (52.0–85.8)	100.0 (100.0–100.0)	73.3 (54.1–87.7)	100.0 (100.0–100.0)
	ICD-10 code D66 in DPC data (diagnosis that triggered hospitalization or diagnosis with highest medical cost)	10	1	21	1033813	32.3 (16.7–51.4)	100.0 (100.0–100.0)	90.9 (58.7–99.8)	100.0 (100.0–100.0)
	Administration of factor VIII products	20	1	11	1033813	64.5 (45.4–80.8)	100.0 (100.0–100.0)	95.2 (76.2–99.9)	100.0 (100.0–100.0)
	ICD-10 diagnosis code D66 in EMR and administration of factor VIII products without administration of factor IX products (See Appendix 6)	20	1	11	1033813	64.5 (45.4–80.8)	100.0 (100.0–100.0)	95.2 (76.2–99.9)	100.0 (100.0–100.0)
	Administration of factor VIII products excepting those with universal insurance coverage for vWD	20	1	11	1033813	64.5 (45.4–80.8)	100.0 (100.0–100.0)	95.2 (76.2–99.9)	100.0 (100.0–100.0)
	ICD-10 diagnosis code D66 in EMR and administration of factor VIII products excepting those with universal insurance coverage for vWD	20	1	11	1033813	64.5 (45.4–80.8)	100 (100–100)	95.2 (76.2–99.9)	100 (100–100)
	ICD-10 diagnosis code D66 in EMR without acquired hemophilia disease name in EMR	29	18	2	1033796	93.5 (78.6–99.2)	100 (100–100)	61.7 (46.4–75.5)	100 (100–100)
	ICD-10 diagnosis code D66 in EMR without Acquired hemophilia disease name in EMR (male patients)	29	12	2	1033800	93.5 (78.6–99.2)	100 (100–100)	73.3 (54.1–87.7)	100 (100–100)

Abbreviations: DPC, diagnosis procedure combination; EMR, electronic medical record; PPV, positive predictive value; NPV, negative predictive value; vWD, von Willebrand Disease.

Table 3 Diagnostic Value of Algorithm of Outcome Conditions: Disease Characteristics and Treatment

Outcome Definition	Algorithm	True Positive (n)	False Positive (n)	False Negative (n)	True Negative (n)	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)
Disease severity (Severe hemophilia A)	Initial test results of factor VIII activity < 1%	8	3	5	31	61.5 (31.6–86.1)	91.2 (76.3–98.1)	72.7 (39.0–94.0)	86.1 (70.5–95.3)
	Minimum test results of factor VIII activity < 1%	9	8 *	4	26	69.2 (38.6–90.9)	76.5 (58.8–89.3)	52.9 (27.8–77.0)	86.7 (69.3–96.2)
History of factor VIII inhibitor	Laboratory test results of ≥ 0.6 BU/mL factor VIII inhibitor	2	7 *	1	37	66.7 (9.4–99.2)	84.1 (69.9–93.4)	22.2 (2.8–60.0)	97.4 (86.2–99.9)
	Administration of bypassing products **	2	0	1	44	66.7 (9.4–99.2)	100.0 (92.0–100.0)	100.0 (15.8–100.0)	97.8 (88.2–99.9)
History of prophylactic factor replacement therapy	Medical claim with a Japanese procedural code of C101 or C153 ***	12	3	4	28	75.0 (47.6–92.7)	90.3 (74.2–98.0)	80.0 (51.9–95.7)	87.5 (71.0–96.5)
	Administration of factor VIII (≥ 10 bottles per month)	12	3	4	28	75.0 (47.6–92.7)	90.3 (74.2–98.0)	80.0 (51.9–95.7)	87.5 (71.0–96.5)
	For 3 months	8	0	8	31	50 (24.7–75.3)	100 (88.8–100)	100 (63.1–100)	79.5 (63.5–90.7)
	Administration of ≥ 10,000 units/month of factor VIII products	13	3	3	28	81.3 (54.4–96.0)	90.3 (74.2–98.0)	81.3 (54.4–96.0)	90.3 (74.2–98.0)
	For 3 months	8	1	8	30	50.0 (24.7–75.3)	96.8 (83.3–99.9)	88.9 (51.8–99.7)	78.9 (62.7–90.4)

Notes: Diagnostic values were calculated based on patients receiving a diagnosis or suspected code D66. *The Minimum test results of factor VIII activity < 1% and Laboratory test results ≥ 0.6 BU/mL factor VIII inhibitor algorithms yielded false positives in some patients because patients receiving a diagnosis or suspected code D66 (ICD-10) included severe acquired hemophilia with factor VIII inhibitor. We defined these patients as false positives. **See Appendix 7. ***Medical claim of C101 is the management fee for home drug injection, and that of C153 is the fee for needle prescription.
Abbreviations: DPC, diagnosis procedure combination; PPV, positive predictive value; NPV, negative predictive value.

Table 4 Diagnostic Value of Algorithms of Outcome Conditions: Hemophilia A-Related Events

Outcome Definition	Algorithm	Total Event (n)	True Positive (n)	False Positive (n)	Sensitivity (%, 95% CI)	PPV (%, 95% CI)
Intracranial bleeding	ICD-10 diagnosis code of related disease * (EMR)	4	3	6	75.0 (19.4–99.4)	33.3 (7.5–70.1)
	With hospitalization on the same day	4	2	0	50.0 (6.8–93.2)	100.0 (15.8–100)
	With factor VIII # or bypassing products	4	1	4	25.0 (0.6–80.6)	20.0 (0.5–71.6)
Gastrointestinal bleeding	ICD-10 diagnosis code of related disease ** (EMR)	9	1	3	11.1 (0.3–48.2)	25.0 (0.6–80.6)
	ICD-10 diagnosis code of related disease ** (EMR) and administration of factor VIII products/bypass products	9	1	2	11.1 (0.3–48.2)	33.3 (0.8–90.6)
Major joint bleeding	ICD-10 diagnosis code of related disease *** (EMR)	302	5	13	1.7 (0.5–3.8)	27.8 (9.7–53.5)
	With administration of factor VIII products/bypass products	302	3	4	1.0 (0.2–2.9)	42.9 (9.9–81.6)

Notes: *Head trauma or intracranial bleeding-related ICD-10 = I600/I601/I602/I603/I604/I605/I606/I607/I608/I609/I610/I611/I612/I613/I614/I615/I616/I618/I619/I620/I621/I629/P520/P521/P522/P523/P524/P525/P526/P528/P529; **Gastrointestinal bleeding-related ICD-10 = K228/K250/K260/K270/K272/K274/K276/K280/K282/K284/K286/K573/K768/K838/K922/S361/S368/S369; ***Major joint bleeding-related ICD-10 = M2506/M2509/S500/S800/T140. # See Appendix 5.
Abbreviations: EMR, electronic medical record; PPV, positive predictive value.

Table 5 Characteristics of Patients with a Diagnosis of ICD-10 D66

	Kurashiki Central Hospital Data from the RWD Database		RWD Database	
Patients' baseline characteristics				
Patient number	47		646	
Sex (male n, %)	41	(87.2%)	550	(85.1%)
Age at data extraction (mean ± standard deviation)	43.9 ± 23.3		40.2 ± 24.1	
Hemophilia-related characteristics				
Age when the patients received ICD-10 diagnosis code D66 (mean ± standard deviation)	31.7 ± 25.2		28.1 ± 25.1	
Factor VIII activity level *				
< 1%	17	(36.2%)	195	(30.2%)
≥ 1%, < 5%	7	(14.9%)	50	(7.7%)
≥ 5%, < 40%	7	(14.9%)	104	(16.1%)
History of factor VIII inhibitor (≥ 0.6 BU/mL) **	9	(19.1%)	39	(6.0%)
Administration of factor VIII	21	(44.7%)	199	(30.8%)
Co-morbid disease ***				
Hypertension	9	(19.1%)	101	(15.6%)
Diabetes mellitus	17	(36.2%)	110	(17.0%)
Hyperlipidemia	8	(17.0%)	61	(9.4%)
Arteriosclerosis	2	(4.3%)	21	(3.3%)
Cardiovascular disease	24	(51.1%)	143	(22.1%)
Ischemic heart disease	14	(29.8%)	72	(11.1%)
Arrhythmia	11	(23.4%)	65	(10.0%)
Cerebrovascular disease	13	(27.7%)	59	(9.1%)

Notes: *Factor VIII activity level was estimated using the lowest laboratory test results of factor VIII activity in each patient. **History of factor VIII inhibitor was defined as laboratory test results of factor VIII inhibitor level ≥ 0.6 BU/mL even once. ***See Appendix 2.

Representativeness of the Validation Dataset

To evaluate how representative the population in this validation study was of the entire population in the RWD database, we compared the characteristics of patients

receiving ICD-10 diagnosis code D66 in Kurashiki Central Hospital and the RWD database. The ratio of patients with factor VIII activity level <1% in Kurashiki Central Hospital was high, and patients in Kurashiki Central Hospital tended to have comorbidities. Kurashiki Central Hospital is one of the largest hospitals in Japan and treats cases with greater disease severity and higher rates of complications. It would be useful for physicians to understand these differences in patient characteristics between Kurashiki Central Hospital and the RWD database, which could be explained by the role of Kurashiki Central Hospital.

The population in this study is representative of the entire population (ie, of all patients in the RWD database). Therefore, the sensitivity, specificity, PPV, and NPV data could be applied in other epidemiological studies using the RWD database. However, this validation study was conducted at a single large hospital, so the results may not apply to other hospital settings. Future epidemiological studies using the RWD database should consider performing sensitivity analyses based on the hospital volume data contained therein.

Conclusion

In conclusion, we developed and validated EMR- and claims-based definitions of hemophilia A-related outcomes, including disease, treatment, and disease-related events. These results support outcomes research studies using RWD for hemophilia A.

Acknowledgments

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

References

1. Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. *Lancet*. 2003;361 (9371):1801–1809. doi:10.1016/S0140-6736(03)13405-8
2. Carcao MD. The diagnosis and management of congenital hemophilia. *Semin Thromb Hemost*. 2012;38(7):727–734. doi:10.1055/s-0032-1326786
3. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1–47.
4. Berntorp E, Shapiro AD. Modern haemophilia care. *Lancet*. 2012;379 (9824):1447–1456. doi:10.1016/S0140-6736(11)61139-2
5. National Research Council. The Learning Healthcare System: workshop Summary (IOM Roundtable on Evidence-Based Medicine). Washington, DC: The National Academies Press, 2007.
6. Berger ML, Mamdani M, Atkins D, Johnson ML. Good research practices for comparative effectiveness research: defining, reporting and interpreting nonrandomized studies of treatment effects using secondary data sources: the ISPOR Good Research Practices for Retrospective Database Analysis Task Force Report--Part I. *Value Health*. 2009;12(8):1044–1052. doi:10.1111/j.1524-4733.2009 .00600.x
7. Wang M, Cyhaniuk A, Cooper DL, Iyer NN. Identification of patients with congenital hemophilia in a large electronic health record database. *J Blood Med*. 2017;8:131–139. doi:10.2147/JBM.S133616
8. Lyons J, Desai V, Xu Y, et al. Development and validation of an algorithm for identifying patients with hemophilia A in an administrative claims database. *Value Health*. 2018;21(9):1098–1103. doi:10.1016/j.jval.2018.03.008
9. Health, Clinic, and Education Information Evaluation Institute. Secondary use of the RWD database. Available from: https://www. hcei.or.jp/page/application. Accessed February 25, 2021.
10. Fujiwara T, Okamoto H, Ohnishi Y, et al. Diagnostic accuracy of lateral neck radiography in ruling out supraglottitis: a prospective observational study. *Emerg Med J*. 2015;32(5):348–352. doi:10.1136/ emermed-2013-203340
11. Iwagami M, Aoki K, Akazawa M, et al. Validation study using administrative data in Japan Task Force Report. Available from: http://www.jspe.jp/committee/pdf/validationtrr120180523.pdf. Accessed July 18, 2019.

12. Benchimol EI, Manuel DG, To T, Griffiths AM, Rabeneck L, Guttmann A. Development and use of reporting guidelines for assessing the quality of validation studies of health administrative data. *J Clin Epidemiol*. 2011;64(8):821–829. doi:10.1016/j.jclinepi.2010.10.006

13. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ*. 2015;351:h5527. doi:10.1136/bmj.h5527

14. Widdifield J, Ivers NM, Young J, et al. Development and validation of an administrative data algorithm to estimate the disease burden and epidemiology of multiple sclerosis in Ontario, Canada. *Mult Scler*. 2015;21(8):1045–1054. doi:10.1177/1352458514556303

15. Kumamaru H, Judd SE, Curtis JR, et al. Validity of claims-based stroke algorithms in contemporary Medicare data: reasons for geographic and racial differences in stroke (REGARDS) study linked with medicare claims. *Circ Cardiovasc Qual Outcomes*. 2014;7 (4):611–619. doi:10.1161/CIRCOUTCOMES.113.000743

16. Yamada K, Itoh M, Fujimura Y, et al. The utilization and challenges of Japan's MID-NET ® medical information database network in postmarketing drug safety assessments: a summary of pilot pharmacoepidemiological studies. *Pharmacoepidemiol Drug Saf*. 2019;28 (5):601–608. doi:10.1002/pds.4777

17. Koram N, Delgado M, Stark JH, et al. Validation studies of claims data in the Asia-Pacific region: a comprehensive review. *Pharmacoepidemiol Drug Saf*. 2019;28(2):156–170. doi:10.1002/pds.4616

Profiling the Genetic and Molecular Characteristics of Glanzmann Thrombasthenia: Can It Guide Current and Future Therapies?

Alan Nurden

Published online: 08 Jul 2021.

Submit your article to this journal 🗗

View related articles 🗗

View Crossmark data 🗗

REVIEW

Profiling the Genetic and Molecular Characteristics of Glanzmann Thrombasthenia: Can It Guide Current and Future Therapies?

Alan Nurden [ID]

Institut Hospitalo-Universitaire LIRYC, Pessac, France

Abstract: Glanzmann thrombasthenia (GT) is the most widely studied inherited disease of platelet function. Platelets fail to aggregate due to a defect in platelet-to-platelet attachment. The hemostatic plug fails to form and a moderate to severe bleeding diathesis results. Classically of autosomal recessive inheritance, GT is caused by defects within the *ITGA2B* and *ITGB3* genes that encode the αIIbβ3 integrin expressed at high density on the platelet surface and also in intracellular pools. Activated αIIbβ3 acts as a receptor for fibrinogen and other adhesive proteins that hold platelets together in a thrombus. Over 50 years of careful clinical and biological investigation have provided important advances that have improved not only the quality of life of the patients but which have also contributed to an understanding of how αIIbβ3 functions. Despite major improvements in our knowledge of GT and its genetic causes, extensive biological and clinical variability with respect to the severity and intensity of bleeding remains poorly understood. I now scan the repertoire of *ITGA2B* and *ITGB3* gene defects and highlight the wide genetic and biological heterogeneity within the type II and variant subgroups especially with regard to bleeding, clot retraction, the internal platelet Fg storage pool and the nature of the mutations causing the disease. I underline the continued importance of gene profiling and biological studies and emphasize the multifactorial etiology of the clinical expression of the disease. This is done in a manner to provide guidelines for future studies and future treatments of a disease that has not only aided research on rare diseases but also contributed to advances in antithrombotic therapy.

Keywords: Glanzmann thrombasthenia, inherited platelet disorder, bleeding syndrome, integrin, gene profiling, mutation analysis

Introduction

In Glanzmann thrombasthenia (GT) (OMIM#273,800) platelets fail to aggregate when stimulated by physiologic agonists while clot retraction is often defective.[1] Platelets interact with exposed subendothelium but a platelet-rich hemostatic plug fails to form. Spontaneous or trauma-dependent mucocutaneous bleeding is usually observed from birth. Epistaxis, gum bleeding, easy bruising, ecchymoses and petechiae are frequent and gastro-intestinal (GI) bleeding is a major problem particularly in older patients.[2,3] Menorrhagia is critical for women while pregnancy and childbirth present severe hemorrhagic risks. Surgery including tooth extraction requires preventative measures. The molecular basis of GT was clarified when, in Paris, I located a deficit of two major platelet membrane glycoproteins (GPs) in this disease.[4] As the complexity of the platelet surface topography became apparent

Correspondence: Alan Nurden
Institut Hospitalo-Universitaire LIRYC, Pessac, 33600, France
Email nurdenat@gmail.com

these were named GPIIb and GPIIIa; it was quickly rea-lized that they form a Ca²⁺-dependent complex in platelets.[5–7]

The inability of GPIIb-IIIa to bind fibrinogen (Fg) or other adhesive proteins accounts for the lack of aggrega-tion in GT. Clinical and biological heterogeneity led to the definition of three subgroups (type I, type II and variant forms) largely depending on the ability of platelets to retract a clot, store Fg and the level of GPIIb-IIIa expres-sion (Table 1). A major advance came when GPIIb-IIIa was revealed as αIIbβ3, a member of the integrin super-family of cellular receptors (reviewed by Coller and Shattil[8]). Electron microscopy initially showed GPIIb-IIIa to have a head and two legs; later crystallography strik-ingly revealed αIIbβ3 in a bent conformation that on activation straightened as it took on its ligand-binding conformation.[8–10] Precision crystallography and modeling showed αIIb and β3 to have precisely defined subdomains (shown in Figure 1).

ITGA2B (OMIM # 607,759) with 26 exons and *ITGB3* (OMIM # 173,470) with 15 exons encode αIIb and β3; both genes localizing to a 260-kb segment on the long arm of chromosome 17.[11,12] In pioneering work, Newman et al showed that GT was caused by defects in either gene with in Israeli-Arabs a founder *ITGA2B* mutation (c.IVS3(−3)-418del + frameshift (Fs)) while in Iraqi-Jewish patients there was a c.2031–2041del/premature termination in *ITGB3*.[13] Over the years, several hundred mutations with AR inheritance have been identified in GT including small deletions and insertions and splice site variants causing a Fs as well as abundant nonsense and missense mutations sometimes also associated with mRNA instability.[14–16] While repeated mutations may identify gene hotspots, others within ethnic groups are clearly founder mutations; yet for most families they remain private. Large deletions are rare.[17] In addition to αIIbβ3, platelets contain trace amounts of αvβ3 mostly lacking when the genetic lesion affects *ITGB3* but persisting and even in increased density

Table 1 Glanzmann Thrombasthenia in All Its Forms

Disease Description	Comments
Type I Subgroup	
- Absence of platelet aggregation and little or no clot retraction. Levels of αIIbβ3 <5% or absent. Platelet Fg storage pool lacking or negligible. AR inheritance.	- The most common type of GT, given by defects in *ITGA2B* and *ITGB3 genes*. With *ITGA2B* defects αvβ3 may still be present and functional. Patients susceptible to form isoantibodies reactive with αIIbβ3 and/or αvβ3 after blood transfusion or pregnancy.
Type II Subgroup	
- Absence of platelet aggregation but clot retraction can be partial or normal. Residual αIIbβ3 historically defined as 5–15% of normal levels. Platelet Fg pool can be substantial. AR inheritance.	- Frequency variable within populations but usually less than 20% of the patients. Given by defects in *ITGA2B* and *ITGB3*. Clot retraction defects and the platelet Fg storage capacity are mutation dependent.
Variant Forms	
- Absence of platelet aggregation but clot retraction and Fg storage highly variable. Residual αIIbβ3 mainly >50% or even normal but non-functional with little or no activation-dependent Fg binding as also shown by a lack of PAC-1 binding. AR inheritance.	- Rare. Can be given by defects in *ITGA2B* but mostly by *ITGB3* variants. Extracellular mutations directly or indirectly abrogate Fg-binding sites. Intracellular mutations stop signals for αIIbβ3 activation. Clot retraction and Fg storage are mutation dependent. Can be confused with defects in *FERMT3* and *RASGRP2* that prevent kindlin-3 (LAD-III disease) and CalDAG-GEFI signaling.
Upregulated αIIbβ3 and Macrothrombocytopenia (MTP)	
- Much reduced platelet aggregation with clot retraction and Fg storage again variable. Residual αIIbβ3 normally >30% but with spontaneous binding of PAC-1 (but rarely Fg). MTP mostly moderate with subpopulations of enlarged even giant platelets. AD inheritance.	- Rare. Patients with up-regulated αIIbβ3 interfering with megakaryocyte maturation and platelet biogenesis with enlarged platelets in variable numbers. Bleeding mostly due to defective αIIbβ3 function. Single allele mutations on *ITGA2B* but mostly *ITGB3*. Often these affect cytoplasmic domains.

Notes: The above criteria are basic for each subtype, but there is much overlap between them and clear boundaries do not exist. PAC-1 is an activation dependent IgM monoclonal antibody to αIIbβ3.
Abbreviations: AR, autosomal recessive; AD, autosomal dominant; LAD-III, leukocyte adhesion deficiency syndrome type III.

582

Journal of Blood Medicine 2021:12
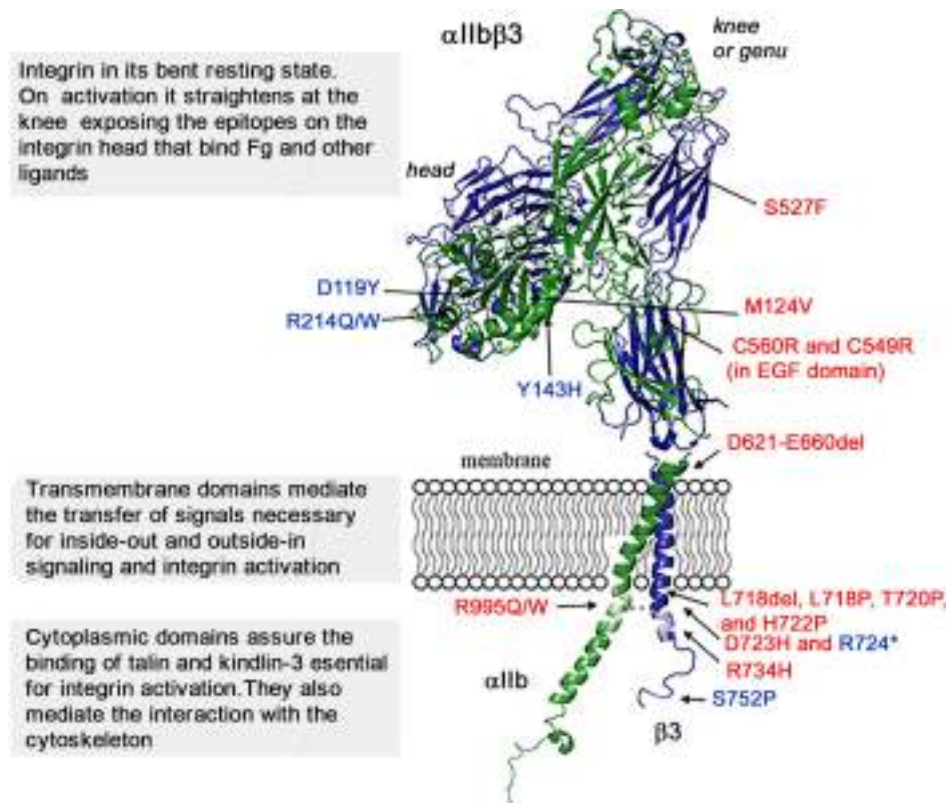
Figure 1 Structural representation of αIIbβ3 in its bent conformation showing the mutations that give rise to selected variant forms of GT or to related phenotypes. This model is based on the crystal structure of αIIbβ3; it was constructed using the PyMol Molecular Graphics System, version 1.3 Schrödinger, LLC and 3fcs and 2knc pdb files as described.[16] The αIIb subunit is in green and β3 is in blue. Precision crystallography and modeling showed that αIIb has 4 major extracellular domains (β-propeller, thigh, calf-1 and calf-2) whereas β3 has more (β-I or β-A, hybrid, plexin-semaphorin-integrin (PSI), 4 epidermal growth factor (EGF) and the β-tail domain).[8,10] Loss-of-function mutations (in blue) in the β3 extracellular head prevent binding of Fg or other adhesive proteins to the opened integrin headpiece following platelet activation, while those in the β3 cytoplasmic tail prevent binding of kindlin-3 and/or talin, and block steps essential for integrin activation. Gain-of-function mutations (in red) lead to at least partial activation of αIIbβ3 and often associated with MTP accompanied by a variable loss of αIIbβ3 function. All mutations are detailed and referenced in the text.

when *ITGA2B* mutations are the cause.[18] While αIIbβ3 is more or less specific for MKs and platelets, αvβ3 through the more promiscuous translation of *ITGB3* is widespread but without influencing the bleeding phenotype in GT.[19]

An objective of this review is to question whether in 2021 it is valid to classify GT into distinct subgroups. In so doing, I ask 1) what are the genetic causes allowing the expression of residual αIIbβ3, 2) whether this residual αIIbβ3 is functional, and 3) whether type II and variant GT give rise to a milder form of GT. Likewise, I ask how the advent of high-throughput sequencing procedures can be used to better advantage in determining patient care.

Biological Testing Within the Type I and Type II Subgroups

Diagnosis of GT is straightforward given the clinical characteristics and phenotype as revealed by platelet function and biological testing. Platelets fail to form large aggregates in response to physiologic agonists in a platelet aggregometer although small clusters were noted by microscopy.[1] Using flow cytometry (FC), others showed how GT platelets formed small aggregates when interacting with collagen through the α2β1 receptor.[20] Ristocetin-induced platelet agglutination (RIPA) mediated through the binding of VWF to GPIb is mostly normal in GT but can be reversible or occur in cycles. Clot retraction provides useful information on the type II and variant forms while measuring the closure time in the point-of-care Platelet Function Analyzer-100 (PFA-100) has largely replaced the bleeding time. Highly recommended for patient management is the International Society of Thrombosis and Haemostasis-Bleeding Assessment Tool (ISTH-BAT) that uses a standardized questionnaire to enable the clinician to establish a comprehensive clinical file.[21]

An inherent difficulty in characterizing type II and variant GT is accurately measuring αIIbβ3 expression on platelets. Currently, the binding of monoclonal antibodies (MoAbs) is assessed directly or indirectly by FC. However, copy number can be influenced by bivalent IgG MoAbs cross-linking adjacent αIIbβ3 complexes; a less likely occurrence at a low surface density of αIIbβ3 and although the use of Fab fragments is advisable their use is rare. The genetic variants causative of GT can also influence MoAb binding, either directly or through long-range allosteric effects. Thus, measures are approximate and many groups report results for a combination of MoAbs to different epitopes as well as performing Western blotting (WB) but as this review will underline the results are frequently disparate. Overlap of αIIbβ3 density between heterozygotes and normal donors confuses family studies. Key to understanding the heterogeneity within the type II and variant subgroups is to determine the degree to which the residual αIIbβ3 is functional (Table 1).

Ethnic Groups

While an estimate of 1 per million is the often given frequency of GT, it is more common in certain ethnic groups where consanguinity prevails. These include Jewish and Arab groups in Israel, French Manouche gypsies, and rural communities in India and Iran.[13,22–24] Studies on the French gypsies initially concerned the Strasbourg area in France; focussing on *ITGA2B* in view of trace amounts of β3 in WB, the authors identified a c.1544+1G>A substitution at the 5′ splice donor site of intron 15.[22] The result was an 8-bp deletion at the 3′-end of exon 15, a premature stop codon and a severely truncated αIIb. Analysis of family members revealed a strong association between a haplotype of five polymorphic loci covering a 4-cm region and the mutation suggesting a founder effect dating back to 300–400 years.[25] More recently, Zhou et al[26] screened 93 families with GT within the Chinese Han population with a lower level of consanguinity (18%) than other ethnic groups where GT is prevalent. Significantly, 74% of the patients had type I GT, 24% type II and only 2% had a variant form. A total of 43 genetic variants were identified. As had been noted earlier, patients with the same genotype sometimes presented with markedly different bleeding severities.[2,17]

Missense Mutations and Biological Heterogeneity of Type II GT

The type II subgroup is highly heterogeneous with respect to the amount of residual αIIbβ3, the genetic cause, the functionality of the residual integrin, and bleeding severity. The selected case reports detailed and summarized in Suppl Table I reflect this. The results show how the mechanism by which αIIbβ3 binds Fg after activation and its capture and storage in α-granules is clearly different. Neither is there a clear relationship between the ability of platelets to capture Fg and retract a clot, contradicting historical reports.[2,3] Also, variable is adhesion to surface-bound Fg, mostly studied using transfected cells.

Patients with ITGA2B Defects

The first patients genotyped for type II GT were independently described and concerned a homozygous p.R327H substitution in αIIb.[27,28] A twofold difference in residual platelet αIIbβ3 content between the probands suggests an influence of other undefined factors. The maturation or trafficking of pro-αIIbβ3 was shown to be impaired in transfected COS-7 or Chinese Hamster Ovary (CHO) cells. A homogeneous *ITGA2B* p.L183P mutation in a man with severe bleeding illustrates the difficulties of evaluating αIIbβ3 expression in some cases.[29] While complex-dependent MoAbs bound minimally, WB revealed 30–35% of each subunit with αIIb showing signs of proteolysis. Platelet αvβ3 was somewhat increased. Expressed in CHO cells the surface expression of the mutated αIIbβ3 was 60% as measured using subunit-specific MoAbs, whereas complex-dependent MoAbs or the activation-dependent MoAb, PAC-1, bound minimally. Both of the above mutations concerned the αIIb β-propeller.

Ambo et al[30] reported two Japanese women with platelets having residual αIIbβ3 who were homozygous for p. Q747P affecting the αIIb calf-2 domain. Platelets attached to surface-bound Fg at intermediate levels; clot retraction was near normal. In fact, p.Q747P is a founder mutation for type II GT in Japan. While other homozygous patients had a similar phenotype, compound heterozygosity combining p.Q747P and a splice site mutation leading to exon 18 skipping gave a lower 4–8% αIIbβ3 expression.[31] In CHO cells αIIbP747β3 bound Fg in the presence of an activating MoAb. Pulse-chase labeling with (35S)-methionine showed that its maturation was impaired. Next to be published was a young Spanish girl with

lifelong bleeding; her platelets had 10% residual αIIbβ3, low platelet Fg, but supported a much-reduced clot retraction.[32] She was compound heterozygous for a paternal splice site variant IVS5(+2)C->A transversion predicting a truncated protein and highly unstable mRNA, and a maternal p.C674R substitution that disrupted a disulfide bridge. Her heterozygous mother had platelets with only 30% of normal levels suggesting that her mutation had a dominant-negative effect. Expression of αIIbR674β3 in CHO cells confirmed the platelet phenotype with intracellular retention of pro-αIIbR674 by the chaperone BiP explaining a reduced transport to the Golgi apparatus and then to the surface membrane.[33]

Basani et al[34] reported type II GT linked to an *ITGA2B* p.P145L mutation reoccurring in families of American, Dutch and Chinese origins with mild to severe bleeding. Two Mennonite siblings had platelets with residual αIIbβ3 unable to bind PAC-1 and with a much-reduced Fg storage pool. Expression of αIIbβ3 was lower or absent in the Dutch and Chinese families with compound heterozygosity (5% and <1%, respectively,) again illustrating how second allele mutations help program αIIbβ3 density. For the Chinese patient, p.P145L was given by a different nucleotide change. Mutation scanning of αIIbP145 in transfected COS-1 cells showed that only p.P145K abrogated αIIb synthesis, while other mutants including p.P145L allowed pro-αIIbβ3 formation but interfered with maturation. Highly conserved, P145 locates to the upper surface of the β-propeller and is adjacent to the W3:4.1 loop identified as a potential ligand-binding site by Kamata et al[35] Mitchell et al[36] reported a boy with parents from Puerto Rico and Canada and with platelets containing 3–6% αIIbβ3 and an Italian/Sicilian family where MoAb binding showed 7–8% αIIbβ3 for four family members. As few clinical and biological details were given these cases are not included in Suppl Table I. For both families, αvβ3 was unaffected and WB confirmed residual mature αIIb and β3 in their platelets. The American proband was compound heterozygous for p.V298F near the second Ca^{2+}-binding domain in blade 5 of the αIIb β-propeller, and p.Y380* that prevents αIIb expression. The Sicilian family combined p.C674R in the αIIb calf-1 domain and a p.I374T missense mutation within the third Ca^{2+}-binding domain in blade 6 of the β-propeller. Interestingly, his father was homozygous for p.C674R while his children were compound heterozygous for both mutations. When the two β-propeller missense mutations were expressed in recombinant αIIbβ3 in 293T cells pulse-chase experiments

showed much of the mutated pro-αIIb to be retained in the ER. Jayo et al[37] described a 2-year-old girl of Chinese nationality with lifelong bleeding, no platelet aggregation or clot retraction but with platelet Fg in the normal range. Her platelets expressed <10% residual αIIbβ3 but αvβ3 was normally present. Residual Fg binding indicated a qualitative defect. Sequencing of reverse-transcribed αIIb mRNA revealed compound heterozygosity: a c.C2829T transition giving a p.P912L substitution within the αIIb light chain and a c.C1750T transition in exon 17 of *ITGA2B* that gave p.R553* predicting truncated αIIb but also nonsense-mediated decay. The object of multiple reports in Asian patients, p.R553* is clearly a mutational hotspot. Transfection in CHO cells showed that αIIbβ3L912 reaches the surface but with a fivefold reduction in the rate of expression. Enigmatically, exontrap analysis of the mutant αIIb alleles in CHO cells showed that c.C2829T was also forcing skipping of exon 28; nonetheless, despite mRNA lacking exon 28 being present in platelets, the residual platelet αIIbL912β3 only concerned the full-length transcript.

More recent studies using in silico analysis and molecular dynamics simulations of variants affecting the αIIb calf-1 domain showed dynamic allosteric effects and that were mostly long range.[38] But of the seven variants studied, only the above-mentioned p.C674R mutation gave type II GT. Pillois et al[39] also reviewed structural modifications in and around the αIIb genu, a region that is the fulcrum of the bent "resting" state of αIIbβ3. This literature survey covered mutations extending from the lower part of the β-propeller through the thigh and upper calf-1 domains identifying 37 cases involving 16 missense mutations all causal of type I GT with only p.A446P (within the seventh blade of the β-propeller) and p.C674R allowing αIIbβ3 expression and type II GT. Static in silico modeling confirmed how modifications of structuring H-bonds were the major cause of GT in the thigh domain, whereas in calf-1 long-range effects predominated.

Patients with ITGB3 Defects

The first reported β3 mutation in a type II patient concerned a Chinese girl from a consanguineous family with a lifelong history of severe bleeding.[40] Clot retraction was absent, but platelet Fg was 36% of normal. Platelet αIIbβ3 expression was reduced to 6–14% and αvβ3 was likewise reduced. Sequencing of PCR-amplified cDNA identified a homozygous p.C374Y mutation in *ITGB3*. Transient transfection in CHO cells confirmed a 85–90% reduction in the

surface expression of the mutated αIIbβ3 yet the cells retained an ability to attach to surface-bound Fg. Novel integrin β3 subunit missense mutations were then reported for unrelated Japanese patients with type II GT and lifelong mild bleeding.[41] For all patients, platelet aggregation was absent but clot retraction was in the normal range. Platelet expression of αIIbβ3 ranged from 7.5% to 20%. The first patient, an elderly woman, was homozygous for p.H280P while the second and third patients, both male, were compound heterozygous for the same mutation combined with p. G579S and p.C560F, respectively, both in the EGF-3 domain. When expressed in CHO cells there was a significantly reduced αIIbβ3 expression for p.H280P and p.G579S but surprisingly a near normal expression for p. C560F. The expression of αvβ3 in the CHO cells was also affected. Preliminary information was provided that p. C560F and p.G579S in the EGF-3 domain were activating mutations, but their contribution to the residual αIIbβ3 on the patients' platelets is unknown. Other cases in Japan have since been reported to have the p.H280P variant which may represent a founder mutation.

A girl whose mother was diagnosed with von Willebrand disease (VWD) had lifelong bleeding but normal plasma VWF levels and platelets that agglutinated with ristocetin but which failed to aggregate with physiologic agonists.[42] Her platelets bound subnormal amounts of subunit-specific MoAbs to αIIb and β3 yet bound neither AP-2, a complex-specific MoAb nor on activation did they bind Fg. Western blotting confirmed about 10% of control platelet levels of normally migrating αIIb and β3. Notwithstanding, her platelets mediated a normal clot retraction. The patient was compound heterozygous for mutations in *ITGB3* thereby showing how two inherited platelet disorders (IPDs) can occur in the same family. A paternal c.G867C868 dinucleotide deletion was predicted to give a Fs and a stop codon at p.Q267; a 50% expression of normally sized αIIbβ3 in her father's platelets confirmed the non-expression of the truncated β3. Her second mutation was a missense p.L262P substitution; the introduction of a proline (P) was predicted to introduce a β-turn and to alter β3 conformation. In transfected cells, αIIbβ3P262 was unstable and only small amounts reached the surface. Pulse-chase experiments confirmed that αIIbβ3P262 formed in the ER, but that maturation was markedly delayed. The cells retracted a fibrin clot but failed to bind to immobilized Fg although normally attaching to and spreading on Fn and Vn. Nair et al[43] described a young boy with bleeding since birth. His platelets bound

only trace amounts of MoAbs to αIIbβ3, but in WB αIIb and β3 were 10% to 30% of normal, unusually his β3 migrated as a high molecular weight band, thought to be β3 dimer as its migration normalized after disulfide reduction. Platelet Fg was <20% of normal. He was homozygous for a p.C506Y mutation in β3; this created an unpaired cysteine in the EGF-2 region. Different models proposed that C506 could link with C495 or C501 in β3.

An elderly Afghanistan woman from a consanguineous family had a lifelong history of mucocutaneous bleeding.[44] Platelet aggregation was absent and clot retraction only 10% of normal; platelet adhesion to immobilized Fg was much reduced with the residual interaction blocked by an αIIbβ3 antagonist. Her platelets expressed αIIbβ3 at 24% of normal levels by FC but failed to bind Fg or PAC-1 after activation with ADP. Thus, once more, the residual αIIbβ3 had both quantitative and qualitative defects. Platelet Fg was virtually absent. Sequencing of cDNA revealed a homozygous p.T176I mutation in *ITGA2B*. Interestingly, αIIbI176 locates to the blade 3 1:2 loop of the αIIb β-propeller suggesting that it induces conformation changes through allosteric effects rather than direct interference. Expression of the mutated αIIbβ3 in COS-7 or CHO cells was confirmed as being much reduced.

Morel-Kopp et al[45] reported a homozygous p.L196P β3 variant in a French woman with type II GT and mild bleeding. Her platelets expressed only 4100 copies of αIIbβ3 yet her intra-platelet pool of Fg was as high as 50%. A low expression of αIIbβ3P196 was confirmed in transfected CHO cells with increased cytoplasmic pro-αIIb suggesting altered pro-αIIbβ3 processing. Intriguingly, cells expressing αvβ3P196 failed to attach to surface-bound Fg, spread, form focal contacts, phosphorylate FAK (focal adhesion kinase) or retract a fibrin clot; events that were normal for cells with αIIbβ3P196. Nevertheless, αIIbβ3P196 failed to bind soluble Fg or PAC-1 when incubated with an activating MoAb. A second French family heterozygously expressing p. L196P and followed by us in Bordeaux had a similar phenotype. The proband, an elderly man with lifelong bleeding, had platelets with 8% αIIbβ3 but WB again revealed a substantial pool of Fg a finding suggestive of αIIbβ3 recycling. His mother, a sister and son, all heterozygous for β3 p.L196P had platelets with intermediate levels of αIIbβ3. A predicted second mutation in the proband was confirmed when later DNA sequencing revealed a p.C598Y substitution.[15,17] Previously reported

for a French type II patient, p.C598Y was said to be partially activated.[46] Thus, the Bordeaux patient is an example of type II GT given by compound heterozygosity for two contrasting mutations each of which contributes to residual αIIbβ3 expression.

Splice Site and Other Mutations in Type II GT

Mutations in the calf-1 and calf-2 domains of αIIb are not directly involved in ligand binding, but small discontinuous contacts link them to the EGF-3, EGF-4 and β-tail domains of β3.[47,48] A del-insert in exon 25 localizing to calf-2, and a transition in the acceptor splice site of intron 19 leading to in-frame skipping of exon 20 and affecting calf-1, resulted in type II GT.[49] In each case, the mutated pro-αIIb complexed with β3 in transfected BHK cells, but little mature αIIb resulted. Golgi mediated complex mannose glycosylation was not seen and immunolocalization confirmed that mutated αIIbβ3 was mostly retained in the ER. A homozygous c.2348+5G>C transversion in intron 23 of *ITGA2B* in an elderly male with type II GT (case 8 in the series reported by Nurden et al[17]) retained attention. Skipping of exon 23 was confirmed using a hybrid minigene transfection assay. Real-time PCR and specific primers showed major changes in spliced mRNAs together with a residual full transcript leading to the continued presence of 8–10% functional αIIbβ3 in his platelets. Although his platelets failed to aggregate, clot retraction was partial and platelet Fg was abundant and suggestive of αIIbβ3 recycling. Despite his residual αIIbβ3, he had severe bleeding when young receiving arm-to-arm transfusions in wartime. Much later, when elderly, he had severe GI bleeding. A Moroccan girl from a consanguineous marriage with frequent petechia, bruising and mucocutaneous hemorrhages possessed platelets with about 10% αIIbβ3 that bound Fg in small amounts when stimulated.[50] A homozygous c.G188A substitution at the splice donor site of intron 1 led to the use of alternative intronic donor sites leading to both a stop codon and nonsense-mediated decay and accounting for the low expression of αIIbβ3 in her platelets.

Mutations Reducing αIIbβ3 Expression and Overlapping That of Variant Forms

The classification of type I; type II and variant GT on αIIbβ3 numbers alone is purely arbitrary for borderline cases. For example, Jackson et al[51] described a girl with platelets unable to bind Fg or aggregate when stimulated but with a normal clot retraction. Her platelets expressed 27% αIIb, 16% β3 with subunit-specific MoAbs, but with minimal binding of MoAbs to complex-dependent epitopes. In WB, her platelets contained about 30% of the normal levels of each subunit. Her αIIbβ3 was unstable and failed to express LIBS-binding sites when challenged. She had a homozygous p.S162L substitution in β3 with a destabilizing effect on αIIbβ3. Highly conserved, S162 lies between the metal ion-dependent adhesion site (MIDAS) domain and a ligand regulatory loop near the so-called synergistic metal ion-binding site (SyMBS) of β3. Expression of mutated αIIbβ3L162 in COS-7 cells led to pro-αIIbβ3 synthesis but delayed maturation with only small amounts of mature αIIbβ3 and a rapid proteolysis of both subunits. This patient nicely emphasizes how the αIIbβ3 determinants responsible for platelet aggregation and clot retraction are distinct. Platelets of a young Japanese woman (Osaka-12) with moderate mucocutaneous bleeding also sustained a residual clot retraction despite failing to aggregate.[52] Her platelets expressed 36–41% αIIb and β3 as assessed using MoAbs to the subunits but bound only 13% of a complex-dependent MoAb. Her αIIbβ3 failed to bind PAC-1 when activated and aberrantly expressed LIBS epitopes. She was compound heterozygous for a p.Y143H substitution in the W3 4–1 loop of the αIIb β-propeller and a null allele; the latter largely accounting for the low αIIbβ3 density. Transfected HEK cells normally expressed αIIbH143β3 but failed to bind PAC-1 or Fg in the presence of an activating MoAb. Identical results obtained for αIIbA143β3 suggested that tyrosine is essential at position 143. Her phenotype strongly resembled that of the so-called KO variant with an Arg-Thr insertion between 160 and 161 of αIIb (Figure 1). Notwithstanding, cells transfected with αIIbH143β3 underwent a partial clot retraction, whereas the KO variant abrogated it.

Variant Forms of GT and αIIbβ3 Functioning

Activation of αIIbβ3 by "inside-out" signaling involves conformational changes in the subunit tails that when transmitted to the extracellular domains enable ligand binding.[8] This and/or clustering of αIIbβ3 initiate "outside-in" signaling and responses such as platelet spreading on Fg and clot retraction where αIIbβ3 transmits forces generated by actin and myosin. Important is

phosphorylation of the β3-cytoplasmic tail and c-Src and RhoA activities with retraction regulated by a molecular switch involving β3 dephosphorylation and cleavage at Y759.[53] Variants that mostly possess 50% or more αIIbβ3 in their platelets show loss of function and may concern one or both signaling pathways.

Mutations Affecting Extracellular Domains

Loss-of-function mutations that block the ability of αIIbβ3 to bind Fg when stimulated (Figure 1) have largely contributed to our understanding of how ligand binding occurs. The CAM variant was the first genotyped GT variant, a homozygous p.D119Y substitution causing loss of a divalent cation structured Fg-binding site within the MIDAS domain of β3.[54] Also of major importance were homozygous p.R214Q or W substitutions within the ADMIDAS (adjacent to MIDAS) domain of β3.[55,56] Here, αIIbβ3 was hypersensitive to divalent cation chelation and unable to bind Fg. In all cases, clot retraction and the platelet Fg storage pool were severely reduced. Yet treatment of αIIbβ3 with dithiothreitol restored the Fg-binding capacity. These extracellular variant forms occurred in patients with clinically severe GT. While mostly the mutations affect β3, the Japanese KO variant (also associated with severe bleeding) is an exception.[57] Here, platelets fully expressed αIIbβ3 that is unable to bind Fg or PAC-1 when stimulated and clot retraction is subnormal. In addition, αIIbβ3 was refractory to the activating MoAb PT25. A homozygous p.R,T160-161 insert on the upper face of the αIIb β-propeller localized to the C146-C167 loop; a purported ligand-binding site. Alanine substitution of oxygenated residues within this loop and expression in 293 cells revealed that only D163A abolished ligand-binding function.

Cytoplasmic Domain Mutations

Platelets of an elderly Argentinian man with a mild bleeding syndrome failed to bind Fg or aggregate with ADP despite 44% of normal levels of αIIbβ3.[58] Uniquely, he experienced severe limb deep vein thrombosis (DVT) after a long air flight.[3,19] His αIIbβ3 was predominantly in an internal platelet pool. Possessing a heterozygous β3 p. S752P cytoplasmic domain mutation his platelet Fg and clot retraction were near normal. While platelet aggregation and soluble Fg-binding remained minimal, α-granule Fg was secreted attached to αIIbβ3 of internal membranes

after platelets were challenged with thrombin.[59] An RGDS-affinity column retained detergent solubilized αIIbβ3 from his platelets confirming an intrinsic Fg-binding capacity.[58] As his daughter's platelets aggregated normally while retaining 50% αIIbβ3 expression, the father was hypothesized to have a "nul" allele. A key to the effect of this mutation is loss of binding of kindlin-3, an essential protein for αIIbβ3 activation.[8,15] A similar phenotype in a black American girl with severe bleeding and platelets expressing intermediate levels of non-functional αIIbβ3 was given by compound heterozygosity associating a null allele and a c.2268C>T, p.R724* variant giving a truncated protein missing the final 39 amino acids of the β3 cytoplasmic domain.[60] This patient lacked not only the kindlin-3 binding site but also a talin-binding domain essential for αIIbβ3 signaling.[61] CHO cells expressing αIIbβ3R724* failed to spread on Fg associated with a loss of FAK phosphorylation.

Extracellular Activating Mutations in αIIbβ3

Activating mutations within extracellular domains mostly concern cysteine residues, and β3 is rich in conserved disulfides of which 31 localize to the EGF domains. Pionering was a homozygous p.C560R substitution in the β3 EGF-3 domain of a Frenchman whose platelets expressed 20% αIIbβ3 (Figure 1).[62] He had a mild form of GT despite a long bleeding time and mild thrombocytopenia (100 to 150×10^9 platelets/L). Although platelet anisotropy was noted, platelet size changes were not striking. The patient underwent a kidney transplant when middle-aged and required multiple transfusions. The residual integrin on his platelets spontaneously bound anti-LIBS MoAbs and PAC-1. Strikingly, his circulating platelets had significant amounts of surface-bound Fg organized in clusters.[62] Yet his platelets showed no visual signs of being activated and secreted normally with thrombin when a delayed but residual platelet aggregation occurred. Clot retraction was severely reduced, but platelet Fg was normal. A stable CHO cell line expressing αIIbβ3R560 mimicked the properties of the patient's platelets, and spontaneously bound PAC-1. The cells attached readily to surface-bound Fg spreading with increased velocity. Interestingly, the patient died when elderly from a thrombotic event. Mor-Cohen et al[63] identified a founder p.C549R variant in β3 in consanguineous Jordanian Arab families with type II GT and severe

bleeding. Platelet aggregation was markedly impaired and clot retraction reduced. Transfected BHK cells showed that surface αIIbβ3 spontaneously and maximally bound PAC-1, while WB showed a higher than normal presence of pro-αIIb much of which was retained in the ER. The affected C549 forms a highly conserved disulfide with C558 in EGF-3. Kamata et al[64] investigated the role of β3 disulfides by systematically substituting cysteine with serine followed by the expression of the recombinant αIIbβ3 in CHO cells. Disruption of a single disulfide in the EGF domains commonly led to activated integrin, while disruption of only 2 of 13 disulfides outside this region led to activation.

But not all activating mutations involve cysteine residues (Figure 1). An intriguing patient was reported by Vanhoorelbeke et al[65] and involved a heterozygous p. S527F substitution in the I-EGF-3 domain of β3. The proband was a young Arab man with mild bleeding and much decreased platelet aggregation with ADP despite normal αIIbβ3 numbers. Most studies were performed using transfected CHO cells with the mutated integrin spontaneously binding Fg and PAC-1. Intriguingly, the cells formed aggregates when stirred with Fg. Anti-LIBS MoAbs bound spontaneously to αIIbβ3F527 although their binding increased when RGDS was present. β3F527 probably causes steric hindrance preventing the I-EGF-3 domain from entering a cleft necessary for the bent integrin resting conformation. A 6-month-old girl with mucocutaneous bleeding had platelets unable to aggregate or retract a clot although platelet Fg was present.[66] Amounts of αIIb and β3 were substantial although estimated as below 50%. ADP stimulated platelets failed to bind PAC-1 but strangely some Fg binding was noted. Sequencing revealed compound heterozygosity for p. D119Y and p.M124V missense mutations within the β3 MIDAS domain. Homozygous p.D119Y gives rise to the classic CAM variant (see the preceding section).[54] CHO cells transfected with αIIb and β3V124 showed increased adhesion and spreading on immobilized Fg despite a low surface expression of the mutated integrin. They also formed aggregates when agitated with soluble Fg, suggesting that αIIbβ3V124 was constitutively active. The failure of the patient's platelets to spontaneously aggregate was probably due to the dominance of the loss-of-function αIIbβ3Y119. About 50% of the recombinant αIIbβ3V124 was present as pro-αIIbβ3 in the transfected cells showing a delayed maturation.

Questions are raised as to why the platelets of patients with activating mutations do not obstruct the microcirculation. One possibility is that the Fg binds monovalently and thereby blocks platelet-to-platelet cohesion.

Macrothrombocytopenia (MTP) and a GT-Like State

The cases in the earlier section possess gain-of-function mutations with AR inheritance but without significant abnormalities in platelet count and size. In contrast, an increasingly noted group of patients associate single allele gain-of-function variants of αIIbβ3 with AD transmission and MTP (Table 1) (Figure 1). In the early 1990s, my colleagues and I reported an unique case with mild bleeding, mild thrombocytopenia ($100–160 \times 10^9$/L), some enlarged platelets and defective platelet aggregation.[67,68] Although surface αIIbβ3 was 10–20% of normal, his platelets mediated clot retraction and stored Fg; a normal αIIbβ3 internal pool suggested defective recycling to the surface. Mutation screening first highlighted a heterozygous p.R995Q mutation within the highly conserved GFFKR sequence of the αIIb cytoplasmic tail. Expression of αIIbQ995β3 in Cos-7 and CHO cells confirmed a reduced transport to the cell surface. Although the mutated αIIbβ3 did not bind Fg spontaneously, there was weak binding of PAC-1. Significantly, αIIbR995 forms an intracellular salt bridge with β3D723 and weakening or breaking this link upgrades the activation status of αIIbβ3 (Figure 1).[69] Family studies showed that R995Q came from his father, but discrepancies in their αIIbβ3 expression predicted a non-identified second maternal mutation in the son. This was later identified as a pathogenic 13bp intronic deletion near the splicing acceptor site for exon 15 of *ITGA2B* (its mechanism is discussed later in the review).[17] Subsequently, patients in five Japanese families with MTP and AD inheritance were reported with a heterozygous p.R995W substitution.[70] With a normal second allele platelet surface αIIbβ3 expression was 50% to 70% of normal. Spontaneous PAC-1 binding to platelets or transfected 293T cells confirmed that αIIbβ3 was in a partially activated conformation. Furthermore, when they were plated on Fg they spontaneously formed proplatelet-like protrusions – a finding previously reported for a patient with a heterozygous β3 p.D723H variant, D723 being the partner for R995 in the salt bridge linking the two cytoplasmic domains.[70,71] However, enigmatically this latter patient while having MTP did not have a significant platelet aggregation defect. Later, three

French families were reported with MTP, GT-like defects and heterozygous variants affecting the salt bridge.[72] Two families possessed p.R995W in αIIb and the third p. D723H in β3. In silico modeling confirmed that both variants created steric interference, weakening the intracytoplasmic ionic clasp but with secondary influences extending to other nearby amino acids. For all the above patients both the bleeding syndrome and the MTP were moderate. Nevertheless, their phenotypic variability was striking. Platelet αIIbβ3 expression ranged from 25% to 50%. Intriguingly, electron microscopy showed many enlarged round platelets often with a heterogeneous distribution of α-granules many of which showed signs of fusion. Early MK maturation was normal, but proplatelets were short and had enlarged tips. Recently, Morais et al[73] have reported more European cases with AD variants affecting the salt bridge and all with mild forms of MTP; again the authors emphasized the phenotypic variability of such cases and which extended to the degree of spontaneous activation of αIIbβ3.

Mutations in the single-pass transmembrane domains of αIIbβ3 can also give MTP. Members of two Italian families associated a moderate AD thrombocytopenia with large platelets, defective platelet function and moderate/severe bleeding.[74] The platelets associated a low surface αIIbβ3 expression with minimal spontaneous PAC-1 binding. Platelet spreading on Fg was impaired as was shear-dependent platelet adhesion to collagen. Yet clot retraction was normal. A novel heterozygous mutation in *ITGB3* led to a large in-frame deletion (p.D621-E660). Truncated β3 was noted in their platelets and a dominant negative effect was hypothesized. A heterozygous p. L718P mutation in β3 was reported in a Spanish woman with severe bleeding and MTP.[75] Platelet aggregation was much reduced and a secretion defect was found with little surface expression of P-selectin and CD63. Surface αIIbβ3 was selectively reduced. Her platelets spread poorly on Fg with defective lamellipodia. CHO cells expressing recombinant αIIbβ3P718 directly bound Fg and PAC-1 and spontaneously agglutinated in the presence of Fg. When plated on Fg, they formed long extensions often with a swelling at the tip. We have reported a heterozygous β3 p.L718del in a woman with a GT-like syndrome with MTP; her platelets did not spontaneously bind PAC-1.[76] Platelet anisotropy was again associated with enlarged "fused" α-granules. Recently, Morais et al[73] have added p.G976V in αIIb and p.T720P, p.H722P and p.R734H (p.

R760H) in β3 to the list causing MTP with GT-like functional defects (Figure 1).

These patients raise important questions. Quite clearly, the conformational status of αIIbβ3 influences MK reactivity with extracellular proteins in the bone marrow and affects platelet biogenesis, but the relationships between the changes caused by the above mutations and the step-by-step exposure of the determinants that allow soluble Fg binding remain to be determined. Furthermore, an argument can be made that "Glanzmann thrombasthenia-like macrothrombocytopenia" should define a distinct disease in the way that "platelet-like VWD" has common usage to define upregulated binding of VWF to GPIb.[77,78]

Large Series and Frequency of GT Subtypes

As sequencing capacity progressed, large cohorts of GT patients were genotyped and have considerably extended our insight into the mutation repertoire of this disease. Initially, Peretz et al[79] examined the molecular basis for GT in 40 families with a high degree of parental consanguinity from Southern India. Most patients had type I GT and 23 mutations (13 in *ITGA2B* and 10 in *ITGB3)* were identified. Founder effects were confirmed by haplotyping families with repeated p.L117W and p.Y281* mutations reflecting the closeness of the communities. Subgroups were not identified although a β3 p.R67Q variant had normal αIIbβ3 expression and clot retraction. Missense mutations affecting αIIb primarily concerned the β-propeller or thigh domains while those in β3 were distributed within the PSI, β-I and hybrid domains. Nelson et al[80] followed with a further 15 patients from the same communities. Significantly, 14 showed no αIIb in WB and 10 had no β3, while for 4 others, β3 was severely reduced. Platelet Fg was undetectable for 13 patients, severely reduced in 1; only 1 patient had normal platelet surface αIIbβ3 expression and normal Fg. Disease-causing mutations were identified for 11 patients with type I GT predominating.

Jallu et al[81] examined phenotype/genotype relationships in 24 Paris patients with 19 classed as type I and 3 as type II. They identified 29 mutations, their validation included expression in COS-7 cells, in silico analysis and mRNA processing. Noteworthy was a p.Q595H substitution that failed to change αIIbβ3 expression in COS-7 cells despite its absence from platelets; in fact, the causal c.1878G>T transversion led to altered mRNA splicing

and skipping of exon 18 in megakaryocytes. One type II patient was compound heterozygous for an *ITGA2B* c.3060+2T>C transition leading to deletion of exon 29. For another, only a heterozygous *ITGA2B* p.R946* nonsense mutation was detected with a predicted but non-identified second allele mutation allowing residual expression of αIIbβ3. Two type II patients had platelets with substantial pools of Fg (51% and 83%, respectively,). Sandrock-Lang et al[82] in Germany reported 19 GT patients including 11 type I and 2 type II. They found 27 mutations. One type II patient, a male Arab with severe bleeding, was compound heterozygous for the *ITGA2B* p.L183P missense mutation and a c.3092delT within exon 30 of *ITGA2B* introducing a Fs and a protein prolonged in the cytoplasmic tail. The second patient combined a p.I154M missense mutation and a known p.R597X stop codon within *ITGA2B*. Both missense mutations affected the αIIb β-propeller.

In Bordeaux, we led a large international consortium that genotyped members of 76 affected families.[17] Sequencing in a national sequencing center (Genoscope) identified 78 disease-causing variants; in parallel 4 large deletions or duplications were found using quantitative real-time PCR. Most families were from France; others were from Argentina, Canada, Morocco, Spain, Switzerland and the USA. Many had a history of blood transfusions or rFVIIa in response to bleeding or preventively. Occasionally bleeding was mild and wide variability was highlighted. Phenotyping assigned 58 families with type I GT, 9 with type II and 8 had variant forms. *ITGA2B* missense mutations mainly affected the β-propeller and caused type I GT but gave type II in three families. Of the calf-1 and calf-2 domain mutations, p.V903F re-occurred in two nonrelated French families with type II GT and compound heterozygosity; expression studies confirmed an altered maturation of pro-αIIbF903β3. An elderly French male with type II GT associated an *ITGA2B* p.G792E missense variant with a stop codon. Heterozygous p.C674R associated with a null allele in a patient with type II GT was first reported with homogeneous expression in Spanish patients with type II GT (Suppl Table I).[32] Noteworthy were missense p.Q595H and p.F160V mutations given by nucleotide transitions that also interfere with splicing as previously noted for p. Q595H by Jallu et al.[81] While homozygous β3 mutations mostly gave type I GT, a heterozygous p.R37C variant associated with p.R143* in a patient with type II GT. When expressed in COS-7 cells, intracellular pro-

αIIbβ3C37 predominated. While most β-I domain mutations gave type I GT, the type II subgroup was occasionally seen. Four missense mutations localized to the disulfide-rich β3 EGF-3 and EGF-4 domains but gave type I GT. Multiple stop codons and splice site variations affected both genes. Particularly interesting was a French patient heterozygous for the Manouche gypsy mutation with 50% αIIbβ3 but no platelet aggregation and who was also homozygous for a *FERMT3* mutation.[83] *FERMT3* encodes kindlin-3 and its absence rendered the residual αIIbβ3 non-functional. Clinically, his severe bleeding phenotype was associated with mild immunodeficiency characteristic of leukocyte adhesion deficiency type III (LAD-III) syndrome given by mutations of *FERMT3*. A Jamaican patient with normal αIIbβ3 and no mutations in *ITGA2B* and *ITGB3* was later found by my group to be homozygous for a mutation in *RASGRP2* abrogating CaLDAG-GEFI function.[84] Careful phenotyping is needed to exclude *RASGRP2* and *FERMT3* defects in cases with variant GT (Table 1).

Exon skipping occurred in three families with *ITGA2B* variants and in five families with *ITGB3* variants. Enigmatic was a heterozygous 13-bp single allele deletion in intron 14 first reported by Jallu et al[81] and affecting seven families with type I GT and no obvious ancestral links. These included three Catalan family members confirming its inheritance. A hybrid minigene transfection assay in COS-7 cells showed that it invoked a new cryptic 5′ splice site causing a 2-bp deletion in the mRNA, a reading-frame shift, and a premature stop codon after 105 aberrant amino acids. The abnormal mRNA was also predicted to be a target for nonsense-mediated decay.

GT Platelets and Fibrin

While early studies confirmed a role for αIIbβ3 in fibrin-dependent clot retraction, the wide diversity in the mutation-dependent responses in type II and variant GT imply that Fg and fibrin interact with different sites on αIIbβ3. Podolnikova et al[85] showed how a cluster of newly exposed amino acids in the Fg γ370-381 sequence mediate the interaction of immobilised Fg or fibrin with αIIbβ3. Much later, the same group revealed how this cluster had several potential contact sites within the αIIb β-propeller.[86] These differ from the activation-dependent sites on αIIbβ3 that mediate binding of the γ404GAKQAGDV411 peptide or RGDS sequences of Fg and which respectively localize to the extremity of the αIIb β-propeller and to the β3 β-I domain.[8,35] The situation clarified when the GPVI collagen

receptor on platelets was also shown to recognize fibrin and surface-bound Fg.[87,88] Thus, not only does GPVI participate in platelet binding to the vessel wall, it also intervenes as a more generalized signaling receptor in thrombus formation. GPVI not only may explain the accumulation of platelets of type I GT patients on fibrin, it may also intervene in the microthrombus formation seen for some patients. Nonetheless, the genetic absence of GPVI does not abrogate clot retraction and recent studies show that GPVI and αIIbβ3 play complementary non-redundant roles.[89,90] GPVI may also have a physiologic role in inflammatory states, cancer and other acquired states in GT.[19,91]

Platelet Proteome

Some 40 years ago, McGregor et al[92] studied platelet membrane glycoproteins in GT by associating carbohydrate-specific or protein-specific labeling procedures with high-resolution two-dimensional gel electrophoresis. Glycosylation defects of residual GPIIb and GPIIIa extended to other components of the platelet surface in type I and type II GT; suggesting an unexplored source of functional heterogeneity. Loroch et al[93] investigated platelet function and the platelet proteome in type I GT patients with homozygous Fs mutations in *ITGA2B* leading to premature stop codons. Mass spectroscopy validated about 3% αIIb and 5% β3 in platelets of the two patients studied. Signaling proteins such as kindlin-3, CalDAG-GEFI, and Src were all normally present as were αv, GPIb-IX and GPVI. Downregulated in platelets were Fg, FXIIIB, carboxypeptidase-B2 (a fibrinolysis inhibitor) and plasminogen despite normal plasma levels. FcγRIIA and laminin-α4 were upregulated. Megakaryocytes synthesize FXIIIA that is stored in platelets but FXIIIB is taken up – perhaps in association with Fg. Plasminogen interacts with carboxypeptidase B suggesting a common endocytic pathway. FcγRIIA increase was linked to increased dense granule secretion in response to immunoglobulin complexes. Recently, Blair et al[94] used state-of-the-art mass spectrometry (MS) and metal-tagged antibodies to study activation-dependent changes on GT platelets. They highlighted the elevated levels of CD9, CD42a and CD63 while the density of CD21, CD154 and GPVI was low. CD9 and CD63 are granule markers that were increased, whereas the normal expression of P-selectin, another granule membrane protein, was unexplained. But perhaps more significant is that the bulk of the platelet proteome appeared to be unchanged. It would be interesting to examine a wider range of patients including variant forms.

Diagnosis and High-Throughput Sequencing

The advent of high-throughput sequencing (HTS) for diagnosis of IPDs has greatly accelerated gene identification and mutation analysis as well as posing new sets of challenges.[77,78,95,96] Precise genotyping in GT is required for optimal clinical management. It also provides insight into αIIbβ3 structure–function relationships, facilitates family planning and assists in the development of new treatments including gene therapy. Targeted sequencing (TS), whole-exome sequencing (WES) or whole-genome sequencing (WGS) are all viable options with TS against a selected gene panel including *ITGA2B* and *ITGB3* the initial and most economic choice for GT. Potential users should consult the annually updated curated gene panel from the ISTH SSC.[95] The gene panel should also include *RASGRP2* encoding CaLDAG-GEFI and *FERMT3* encoding kindlin-3 whenever the clinical profile suggests GT variant-like platelet function. While WES can cover intronic flanking regions and splice sites its basic advantage is to allow the detection of new genes. A negative result in TS and WES should be followed by WGS when possible, but both WES and WGS require extensive computer-based facilities and technical back up.[96] Key is the classification of variants into those with known pathogenicity, likely pathogenic and those with uncertain pathogenicity, while copy number variations represent a separate challenge as do variants deep within introns (reviewed in Ver Donck et al[96]). Particularly interesting is that for some patients bleeding results from combinations of homozygous or single allele mutations on two or more different genes that may or may not involve *ITGB3* or *ITGA2B*. Variant analysis should also include those of modest pathogenicity that on their own do not cause a sufficient inhibition of platelet function to disturb hemostasis but which may nonetheless modulate disease severity. The identification of gene modifiers that influence disease pathogenesis and penetrance in GT is a major future challenge.

Extent That Mutations of *ITGA2B* and *ITGB3* are Found in the General Population

In a pioneering study, Buitrago et al[97] analysed next-generation sequencing data (WES and WGS strategies) from five international databases and assessed the frequency of missense mutations affecting αIIb and β3 in 16,108 normal individuals and compared their findings to

111 missense mutations reported in the literature as causing GT. Their survey identified 114 missense variants in *ITGA2B* (affecting 11% of the amino acids) and 68 in *ITGB3* (affecting 9% of the amino acids). Of these, 96% had minor allele frequencies (MAF) of <0.1% while a significant proportion were predicted to be deleterious. Strikingly, none of the missense mutations known to cause GT were present. This study showed that 1.3% of the population has a variant of *ITGA2B* or *ITGB3*. The authors concluded that on the basis of their rarity, the bulk of the amino acid substitutions had entered the population recently. We hypothesized that if this applied to the GT-causing mutations in our international cohort, then many patients would share non-damaging synonymous and non-synonymous SNPs in both genes.[17,98] We therefore quantified non-disease causing genetic variants including human platelet alloantigen (HPA) polymorphisms within the sequenced regions of *ITGA2B* and ITGB3.[98] Our results show that many damaging and GT-causing mutations share SNP haplotypes with little genetic variation in unrelated families of wide geographical origins: a result that agrees with many GT-causing mutations being of recent origin. It is probable that a high proportion of private mutations outside ethnic groups and closed communities will disappear due to natural selection and that the mutation profile in GT is constantly changing.

Treatment

If local measures fail, GT patients receive platelet transfusions or recombinant factor VIIa (rFVIIa) given with or without anti-fibrinolytics. George et al[3] evaluated treatment for 112 patients followed in Paris and reported that a majority had received such measures at least once. However, the extent of bleeding was difficult to predict and had no dependence on whether the patient had type I, type II or variant GT. This has been confirmed many times; bleeding may vary considerably in severity and frequency even among members of the same family.[17,26] One situation where treatment is adapted is in the presence of isoantibodies to αIIbβ3 with rFVIIa advised for patients with inhibitors and refractoriness.[99,100] Occasional adverse events linked to rFVIIa have mostly involved DVT.[19] Al-Battat et al[101] showed that platelets from type I patients compete with and hinder the hemostatic efficacy of transfused platelets and emphasized the importance of maintaining a high proportion of transfused platelets. But the major risk of platelet transfusion remains immune sensitization when the integrin is lacking in type I GT.[102,103] In

this respect, genotyping and biological testing helps choice of treatment.

Pregnancy is rare in women with type I GT both through individual choice and the long-term use of hormonal therapy. Apart from bleeding risk, pregnancy may lead to immunization following passage of fetal cells into the mother's circulation.[103] Transplacental passage of maternal antibodies may cause thrombocytopenia in the fetus. Leticée et al[104] described pregnancy in a woman with type I GT and isoantibodies following blood transfusions. As the husband's platelets fully expressed αIIbβ3, the fetus was an obligate heterozygote. Fetal death occurred at 31 weeks due to intracranial hemorrhage. Although absent from platelets when αIIbβ3 and αvβ3 are lacking, HPA-systems on β3 are a factor on platelets and other cells that express αvβ3 when the gene defects affect *ITGA2B* as well as in type II GT and for variant GT. The risk of antibody formation will remain in the event of alloantigen incompatibility. Santoso et al[105] found that intracranial hemorrhage in mothers with fetal/neonatal alloimmune thrombocytopenia (FNAIT) caused by anti-HPA-1a antibodies, was predominately due to their reactivity with αvβ3 in endothelial cells. Evidence for anti-HPA-1 alloantibodies reactive with αvβ3 in GT was provided by Fiore et al[106] who detected them in polytransfused patients with the French Manouche gypsy mutation (on *ITGA2B*) with αvβ3 in platelets and other cells expressing β3 homozygous for the rare HPA-1b allele. Thus HPA typing is recommended in GT and especially for affected women.

How Other Gene Defects Can Modify the GT Phenotype

High-throughput gene screening allows the simultaneous evaluation of a wide variety of gene variants that can influence the GT phenotype. In addition to the examples already illustrated in this review and in the literature for other IPDs,[96] it allowed us to find a heterozygous p.G146R mutation in *TUBB1* encoding β-tubulin in a patient who combined MTP and type I GT caused by compound heterozygosity of p.P189S and p.C210S in *ITGB3*.[107] It is becoming more and more clear that bleeding in GT depends not only on the mutations that define the pathology but involves variants of a wide variety of genes involving coagulation factors and the vasculature as previously discussed for other IPDs.[77,78] For example, Deshpande et al[108] identified a combination of GT and

mild FVII deficiency in a 13-yr-old Indian boy with GT and a moderately severe bleeding syndrome. His platelets lacked αIIbβ3 due to compound heterozygosity for p. Q132K and p.K650T in *ITGB3*. Three polymorphisms (two in the promoter region) of *F7* were predicted to cause the FVII deficiency. In a pioneering study, Owaidah et al[109] performed targeted sequencing of 72 family members of GT patients in Saudi Arabia and as well as identifying 17 mutations within *ITGA2B* and *ITGB3* they found possible disease-influencing variants in *ITGA2, VWF* and *F8*. Notwithstanding, the genes analyzed should also include variants potentially protectrice against severe bleeding such as Factor V Leiden but for which the jury is out at the moment with regard to their influence in GT.[19,95]

Human Stem Cell Transplants (HSCT) and Gene Therapy

Restoring hemostasis in GT patients with life-threatening bleeding by HSCT has proved remarkably successful. Pioneering studies were performed in Paris, first on a brother and later on his sister with severe type I GT and antibodies to αIIbβ3 making them refractory to transfusions.[110] Both received bone marrow transplants (BMT) from an asymptomatic sibling but under evolving conditions. Platelet function and αIIbβ3 expression was restored and remained stable for years. Significantly antibodies to αIIbβ3 were no longer present. Flood et al[111] performed BMT on three type I patients with severe bleeding. Two donors were matched family members but the third was unrelated. A conditioning regimen consisting of busulfan, cyclophosphamide and fludarabine met with isolated episodes of graft versus host disease (GVHD); thrombocytopenia due to antibodies to αIIbβ3 was successfully treated by immunosuppressive therapy and intravenous gammaglobulin (IVIgG). Complete and long-term donor engraftment was observed. Poon et al[100] reviewed data from an international registry for BMTs in GT composed of 43 patients. Despite variable conditioning regimes and donor graft sources, there was an 81% survival at a median 47 months. Clearly, HSCT can restore the quality of life in GT; notwithstanding, it remains a last resort due to the possible complications and especially GVHD. The future for treating GT patients with severe bleeding remains gene therapy, yet remarkably this remains at an experimental stage. David Wilcox et al in Milwaukee have been pioneers. First, they transduced BM

cells using a lentivirus vector containing a cDNA cassette encoding human β3 and transplanted them into irradiated β3-/- mice.[112] Human β3 complexed with murine αIIb but platelets in the mice only expressed around 12% of the normal levels of functional αIIbβ3. Notwithstanding their platelet Fg normalized and their tail bleeding times improved. Antibodies to αIIbβ3 were observed in one mouse, but perfusion of IVIgG slowed platelet clearance. Studies followed on dogs with an *ITGA2B* mutation and type I GT.[113] Bleeding was corrected, but the expression of chimeric αIIbβ3 in the platelets again remained low with only about 10% platelets expressing around 5000 copies of αIIbβ3. One of four dogs formed antibodies to αIIbβ3 that were eliminated by IVIgG. However, questions are raised as platelets of patients with type II GT and uniformly expressing 10% of functional αIIbβ3 can have a serious bleeding syndrome. Clearly improved protocols are required. Two alternative procedures are promising. The first is the use of patient-derived induced pluripotent stem cells (iPSCs). Sullivan et al[114] designed a specific construct to reprogram monocytes from two patients with type I GT to produce iPSCs with restored synthesis of αIIbβ3. Their procedure led to corrected αIIbβ3 expression and function in MKs. Hu et al[115] generated iPSCs from skin fibroblasts from a boy with type I GT and compound heterozygosity in *ITGA2B*. Differentiation of the iPSCs into MKs and restoration of the defective gene led to platelets expressing αIIbβ3; nonetheless, the difficulties encountered led the authors to suggest that blood cells were a better source for iPSCs. Notwithstanding, CRISPR/Cas technology has opened new concepts for directly correcting gene defects in rare diseases and GT will be an obvious choice. As proof of concept, Zhang et al[116] edited the genome of heterologous cells and then iPScs to change HPAs. More precisely, iPSCs expressing HPA-1a (β3Leu33) were converted to HPA-1b (β3Pro33) and then differentiated into progenitor cells. The advantage of CRISPR/Cas procedures is that the correction is made on the patient's own cells; therefore, the question of immune tolerance is of lesser importance. This field offers much scope for future years.

Conclusions

Looking back over a long career that nears 50 years has enabled me to review the progress made and to offer thoughts for the future. Clearly, an improved point-of-care test to better evaluate bleeding risk under the flow conditions of the microcirculation is needed as is a spot

test to allow the rapid detection of antibodies to αIIbβ3 prior to surgery or delivery. While the classification of GT patients into type I, type II and variant subgroups has served a useful purpose, genotyping has confirmed that GT as a whole is not divided into subgroups but consists of a range of genetic defects leading to an absence of αIIbβ3 or residual amounts that vary in quantity and function with no clear boundaries between them. An international consensus is required to determine if AD GT-like MTP should be given separate status in a similar way to platelet-type VWD. The place of genotyping and the use of high-throughput sequencing procedures will gain in importance and their early use will allow for more rapid and targeted biological testing. Finally and most importantly, while gene mutations define the disease, bleeding severity depends on a range of other factors, both epidemiological and genetic and defining these must be the priority in coming years as personalized medicine and gene therapy become options for patient care. Notwithstanding, differential diagnosis and biological studies will remain an essential part of accurate patient phenotyping and patient management, especially in third-world countries.[117] The place of new technologies both in platelet function testing and in genotyping will need continued and constant evaluation.

Note

Human Genome Variation Society (HGVS) nomenclature is used throughout for cDNA and protein numbering. For nucleotide numbering, the A nucleotide of the ATG start codon was designated +1 (cDNA *ITGA2B* and *ITGB3* GenBank accession numbers NM_000419.3 and NM_000212.2, respectively). For amino acid numbering +1 corresponds to the initiating Met with signal peptide included. However, as the numbering for the mature protein was used for the crystal structure of αIIbβ3 [see Xiao et al[10] and Nurden et al[15]], mature protein numbering is used here for missense and other mutations affecting protein structure. This involves subtracting 31 amino acids from the HGVS numbering of αIIb and 26 amino acids for β3.

Acknowledgments

Disclosure

The author has no disclosures to declare.

References

1. Caen JP, Castaldi PA, Leclerc JC, et al. Congenital bleeding disorders with long bleeding time and normal platelet count. I. Glanzmann's thrombasthenia (Report of 15 patients). *Am J Med*. 1966;41(1):4–26. doi:10.1016/0002-9343(66)90003-9
2. George JN, Caen JP, Nurden AT. Glanzmann thrombasthenia: the spectrum of clinical disease. *Blood*. 1990;75(7):1383–1395.
3. Bellucci S, Caen J. Molecular basis of Glanzmann's thrombasthenia and current strategies in treatment. *Blood Rev*. 2002;16 (3):193–202. doi:10.1016/S0268-960X(02)00030-9
4. Nurden AT, Caen JP. An abnormal platelet glycoprotein in three cases of Glanzmann's thrombasthenia. *Br J Haematol*. 1974;28 (2):233–253. doi:10.1111/j.1365-2141.1974.tb06660.x
5. Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. *J Clin Invest*. 1977;60(3):535–545. doi:10.1172/JCI108805
6. Hagen I, Nurden A, Bjerrum OJ, Solum NO, Caen J. Immunochemical evidence for protein abnormalities in platelets from patients with Glanzmann's thrombasthenia and Bernard-Soulier syndrome. *J Clin Invest*. 1980;65(3):722–731. doi:10.1172/JCI109719
7. Kunicki TJ, Pidard D, Rosa JP, Nurden AT. The formation of Ca2+-dependent complexes in solution as determined by crossed immunoelectrophoresis. *Blood*. 1981;58(2):268–278. doi:10.1182/blood.V58.2.268.268
8. Coller BS, Shattil SJ. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists and even a bend. *Blood*. 2008;112(8):3011–3025. doi:10.1182/blood-2008-06-077891
9. Carrell NA, Fitzgerald LA, Steiner B, Ericksen HP, Phillips DR. Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. *J Biol Chem*. 1985;260 (3):1743–1749. doi:10.1016/S0021-9258(18)89656-9
10. Xiao T, Takagi J, Coller BS, Wang JH, Springer TA. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature*. 2004;432(7013):59–67.
11. Fitzgerald LA, Steiner B, Rall SC Jr, Lo SS, Phillips DR. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarly to "integrin". *J Biol Chem*. 1987;262(9):3936–3939. doi:10.1016/S0021-9258(18)61290-6
12. Thornton MA, Poncz M, Korostishevsky M, et al. The human platelet alphaIIb gene is not closely linked to its integrin partner beta3. *Blood*. 1998;94(6):2039–2047. doi:10.1182/blood. V94.6.2039
13. Newman PJ, Seligsohn U, Lyman S, Coller BS. The molecular genetic basis of Glanzmann thrombasthenia in the Iraqi-Jewish and Arab populations in Israel. *Proc Natl Acad Sci USA*. 1991;88 (8):3160–3164. doi:10.1073/pnas.88.8.3160
14. French DL, Coller BS. Hematologically important mutations: glanzmann thrombasthenia. *Blood Cells Mol Dis*. 1997;23 (3):39–51. doi:10.1006/bcmd.1997.0117
15. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood*. 2011;118(23):5996–6005. doi:10.1182/blood-2011-07-365635
16. Nurden AT, Pillois X. ITGA2B and ITGB3 gene mutations associated with Glanzmann thrombasthenia. *Platelets*. 2018;29 (1):98–101. doi:10.1080/09537104.2017.1371291
17. Nurden AT, Pillois X, Fiore M, et al. Expanding the mutation spectrum affecting αIIbβ3 integrin in Glanzmann thrombasthenia: screening of the ITGA2B and ITGB3 genes in a large international cohort. *Hum Mutat*. 2015;36(5):548–561. doi:10.1002/humu.22776

18. Coller BS, Seligsohn U, West SM, Scudder LE, Norton KJ. Platelet fibrinogen and vitronectin in Glanzmann thrombasthenia: evidence consistent with specific roles for glycoprotein IIb/IIIa and alphavbeta3 integrins in platelet protein trafficking. *Blood*. 1991;78(10):2603–2610. doi:10.1182/blood.V78.10.2603.2603

19. Nurden AT. Should studies on Glanzmann thrombasthenia not be telling us more about cardiovascular disease and other major illnesses? *Blood Rev*. 2017;31(5):287–299. doi:10.1016/j.blre.2017.03.005

20. De Cuyper IM, Meinders M, van de Vijver E, et al. A novel flow cytometry-based platelet aggregation assay. *Blood*. 2013;121(10): e70–e80. doi:10.1182/blood-2012-06-437723

21. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063–2065. doi:10.1111/j.1538-7836.2010.03975.x

22. Schlegel N, Gayet O, Morel-Kopp M-C, et al. The molecular genetic basis of Glanzmann's thrombasthenia I a gypsy population in France: identification of a new mutation on the αIIb gene. *Blood*. 1995;86(3):977–982. doi:10.1182/blood.V86.3.977.977

23. Kannan M, Ahmad F, Yadav BK, Kumar R, Choudhry VP, Saxena R. Molecular defects in ITGA2B and ITGB3 genes in patients with Glanzmann thrombasthenia. *J Thromb Haemost*. 2009;7(11):1878–1885. doi:10.1111/j.1538-7836.2009.03579.x

24. Farsinejad A, Farajollahi MM, Kazemi A, Saemi N, Faranoush M. Different biochemical expression pattern of platelet surface glycoproteins suggests molecular diversity of Glanzmann's thrombasthenia in Iran. *Blood Coagul Fibrinolysis*. 2013;24(6):613–618. doi:10.1097/MBC.0b013e328360a558

25. Fiore M, Pillois X, Nurden P, Nurden AT, Austerlitz F. Founder effect and estimation of the age of the French gypsy mutation associated with Glanzmann thrombasthenia in Manouch families. *Eur J Hum Genet*. 2011;19(9):981987. doi:10.1038/ejhg.2011.61

26. Zhou L, Jiang M, Shen H, et al. Clinical and molecular insights into Glanzmann's thrombasthenia in China. *Clin Genet*. 2018;94 (2):213–220. doi:10.1111/cge.13366

27. Wilcox DA, Paddock CM, Lyman S, Gill JC, Newman PJ. Glanzmann thrombasthenia resulting from a single amino acid substitution between the second and third calcium-binding domains of GPIIb. *J Clin Invest*. 1995;95(4):1553–1560. doi:10.1172/JCI117828

28. Ferrer M, Fernandez-Pinel M, Gonzalez-Manchon C, Gonzalez J, Ayuso MS, Parrilla R. A mutant (Arg327->His) GPIIb associated to thrombasthenia exerts a dominant negative effect in stably transfected CHO cells. *Thromb Haemost*. 1996;76(3):292–301. doi:10.1055/s-0038-1650574

29. Grimaldi CM, Chen F, Wu C, Weiss HJ, Coller BS, French DL. Glycoprotein IIb Leu214Pro mutation produces Glanzmann thrombasthenia with both quantitative and qualitative abnormalities in GPIIb/IIIa. *Blood*. 1998;91(5):1562–1571. doi:10.1182/blood.V91.5.1562

30. Ambo H, Kamata T, Handa M, et al. Novel point mutations in the αIIb subunit (Phe289->Ser, Glu324->Lys, Gly747Pro) causing thrombasthenic phenotypes in four Japanese patients. *Br J Haematol*. 1998;102(3):829–840. doi:10.1046/j.1365-2141.1998.00824.x

31. Tadokoro S, Tomiyama Y, Honda S, et al. A Gln747->pro substitution in the αIIb subunit is responsible for a moderate αIIbβ3 deficiency in Glanzmann thrombasthenia. *Blood*. 1998;92 (8):2750–2758. doi:10.1182/blood.V92.8.2750

32. Gonzalez-Manchon C, Fernandez-Pinel M, Arias-Salgado EG, et al. Molecular genetic analysis of a compound heterozygote for the glycoprotein (GP) IIb gene associated with Glanzmann's thrombasthenia: disruption of the 674–687 disulfide bridge in GPIIb prevents surface exposure of GPIIb-IIIa complexes. *Blood*. 1999;93(3):866–875. doi:10.1182/blood.V93.3.866

33. Arias-Salgado EG, Butta N, Gonzalez-Manchon C, Larrucea S, Ayuso MS, Parrilla R. Competition between normal (674C) and mutant (674R)GPIIb subunits: role of the molecular chaperone BiP in the processing of GPIIb-IIIa complexes. *Blood*. 2001;97 (9):2640–2647. doi:10.1182/blood.V97.9.2640

34. Basani RB, French DL, Vilaire G, et al. A naturally occurring mutation near the amino terminus of αIIb defines a new region involved in ligand binding to αIIbβ3. *Blood*. 2000;95(1):180–188. doi:10.1182/blood.V95.1.180

35. Kamata T, Tieu KK, Irie A, Springer TA, Takada Y. Amino acid residues in the αIIb subunit that are critical for ligand binding to integrin αIIbβ3 are clustered in the β-propeller model. *J Biol Chem*. 2001;276(47):44275–44283. doi:10.1074/jbc.M107021200

36. Mitchell WB, Li JH, Singh F, et al. Two novel mutations in the αIIb calcium-binding domains identify hydrophobic regions for αIIbβ3 biogenesis. *Blood*. 2003;101(6):22682276. doi:10.1182/blood-2002-07-2266

37. Jayo A, Pabon D, Lastres P, Jimenez V, Gonzalez-Manchon C. Type II Glanzmann thrombasthenia in a compound heterozygote for the αIIb gene. A novel missense mutation in exon 27. *Haematologica*. 2006;91(10):1352–1359.

38. Goguet M, Narwani TJ, Petermann R, Jallu V, de Brevern AG. In silico analysis of Glanzmann variants of Calf-1 domain of αIIbβ3 integrin revealed dynamic allosteric effect. *Sci Rep*. 2017;7 (1):8001. doi:10.1038/s41598-017-08408-w

39. Pillois X, Peters P, Segers K, Nurden AT. In silico analysis of structural modifications in and around the integrin αIIb genu caused by ITGA2B variants in human platelets with emphasis on Glanzmann thrombasthenia. *Mol Genet Genomic Med*. 2018;6 (2):249–260. doi:10.1002/mgg3.365

40. Grimaldi CM, Chen F, Scudder LE, Coller BS, French DL. A Cys374Tyr homozygous mutation of platelet glycoprotein IIIa (β3) in a Chinese patient with Glanzmann's thrombasthenia. *Blood*. 1996;88(5):1666–1675. doi:10.1182/blood.V88.5.1666.1666

41. Ambo H, Kamata T, Handa M, et al. Three novel integrin β3 subunit missense mutations (H280P, C560F and G579S) in thrombasthenia, including one (H280P prevalent in Japanese patients. *Biochem Biophys Res Commun*. 1998;251(3):763–768. doi:10.1006/bbrc.1998.9526

42. Ward CN, Kestin AS, Newman PJ. A Leu262Pro mutation in the integrin β3 subunit results in an αIIbβ3 complex that binds fibrin but not fibrinogen. *Blood*. 2000;96(1):161–169. doi:10.1182/blood.V96.1.161

43. Nair S, Li J, Mitchell WB, Mohanty D, Coller BS, French DL. Two new beta3 integrin mutations in Indian patients with Glanzmann thrombasthenia: localization of mutations affecting cysteine residues in integrin beta3. *Thromb Haemost*. 2002;88 (3):503–509. doi:10.1160/TH2002880503

44. Westrup D, Santoso S, Follert-Hagendorff K, et al. Glanzmann thrombasthenia Frankfurt I is associated with a point mutation Thr176Ile in the N-terminal region of αIIb subunit integrin. *Thromb Haemost*. 2004;92(5):1040–1051. doi:10.1160/TH04-03-0170

45. Morel-Kopp M-C, Melchior C, Chen P, et al. A naturally occurring point mutation in the β3 integrin MIDAS-like domain affects differently αvβ3 and αIIbβ3 receptor function. *Thromb Haemost*. 2001;86(6):1425–1434. doi:10.1055/s-0037-1616746

46. Chen P, Melchior C, Brons NHC, Schlegel N, Caen J, Kieffer N. Probing conformational changes in the I-like domain and the cysteine-rich repeat of human β3 integrins following disulfide bond disruption by cysteine mutations. Identification of cysteine 598 involved in αIIbβ3 activation. *J Biol Chem*. 2001;276 (42):38628–38635. doi:10.1074/jbc.M105737200

47. Xiong JP, Stehle T, Diefenbach B, et al. Crystal structure of the extracellular segment of the integrin αvβ3. *Science*. 2001;294 (5541):339–345. doi:10.1126/science.1064535

596

Journal of Blood Medicine 2021:12

48. Takagi J, Petre B, Walz T, Springer TA. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell*. 2002;110(5):599–601. doi:10.1016/S0092-8674(02)00935-2

49. Rosenberg N, Yatuv R, Sobolev V, Peretz H, Zivelin A, Seligsohn U. Major mutations in calf-1 and calf-2 domains of glycoprotein IIb in patients with Glanzmann thrombasthenia enable GPIIb/IIIa complex formation but impair its transport from the endoplasmic reticulum to the Golgi apparatus. *Blood*. 2003;101(6):4808–4815. doi:10.1182/blood-2002-08-2452

50. Gonzalez-Manchon C, Arias-Salgado EG, Butta N, et al. A novel homozygous splice junction mutation in GPIIb associated with alternative splicing, nonsense-mediated decay of GPIIb-mRNA, and type II Glanzmann's thrombasthenia. *J Thromb Haemost*. 2003;1(5):1071–1078. doi:10.1046/j.1538-7836.2003.00204.x

51. Jackson DE, White MM, Jennings LK, Newman PJ. A Ser162->Leu mutation within glycoprotein (GP) IIIa (integrin αIIbβ3) results in an unstable αIIbβ3 complex that retains partial function in a novel form of type II Glanzmann thrombasthenia. *Thromb Haemost*. 1998;80(1):42–48. doi:10.1055/s-0037-1615136

52. Kiyoi T, Tomiyama Y, Honda S, et al. A naturally occurring Tyr143HisαIIb mutation abolishes αIIbβ3 function for soluble ligands but retains its ability for mediating cell adhesion and clot retraction: comparison with other mutations causing ligand-binding defects. *Blood*. 2003;101(9):3485–3491. doi:10.1182/blood-2002-07-2144

53. Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. *J Cell Biol*. 2007;179(3):553–565. doi:10.1083/jcb.200703185

54. Loftus JC, O'Toole TE, Plow EF, Glass A, Frehlinger AL 3rd, Ginsberg MH. A β3 integrin mutation abolishes ligand binding and alters divalent-cation dependent conformation. *Science*. 1990;249(4971):915–918. doi:10.1126/science.2392682

55. Bajt ML, Ginsberg MH, Frelinger AL, Berndt MC, Loftus JC. A spontaneous mutation of integrin αIIbβ3 (platelet glycoprotein IIb-IIIa) helps define a ligand binding site. *J Biol Chem*. 1992;267(6):3789–3794. doi:10.1016/S0021-9258(19)50595-6

56. Lanza F, Stierle A, Fournier D, et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein IIIa [214]Arg->[214]Trp mutation. *J Clin Invest*. 1992;89(6):1995–2004. doi:10.1172/JCI115808

57. Honda S, Tomiyama Y, Shiraga M, et al. A two-amino acid insertion in the Cys146-Cys167 loop of the αIIb subunit is associated with a variant of Glanzmann thrombasthenia. *J Clin Invest*. 1998;102(6):1183–1192. doi:10.1172/JCI3206

58. Chen YP, Djaffar I, Pidard D, et al. Ser-752-Pro mutation in the cytoplasmic domain of integrin beta3 subunit and defective activation of platelet integrin alphaIIbbeta3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc Natl Acad Sci USA*. 1992;89(21):10169–10173. doi:10.1073/pnas.89.21.10169

59. Nurden P, Poujol C, Winckler J, Combrié R, Caen JP, Nurden AT. A Ser752->Pro substitution in the cytoplasmic domain of beta3 in a Glanzmann thrombasthenia variant fails to prevent interactions between the alphaIIbbeta3 integrin and the granule pool of fibrinogen. *Br J Haematol*. 2002;118(4):1143–1151. doi:10.1046/j.1365-2141.2002.03758.x

60. Wang R, Shattil SJ, Ambruso DR, Newman PJ. Truncation of the cytoplasmic domain of beta3 in a variant form of Glanzmann thrombasthenia abrogates signaling through the integrin alpha(IIb)beta3 complex. *J Clin Invest*. 1997;100(9):2393–2403. doi:10.1172/JCI119780

61. Vinogradova O, Velyvis A, Velyvienne A, et al. A structural mechanism of integrin alpha(IIb)beta(3) "inside-out" activation as regulated by its cytoplasmic face. *Cell*. 2002;110(5):587–597. doi:10.1016/S0092-8674(02)00906-6

62. Ruiz C, Liu C-Y, Sun Q-H, et al. A point mutation in the cysteine-rich domain of glycoprotein (GP) IIIa results in the expression of a GPIIb-IIIa (αIIbβ3) integrin receptor locked in a high-affinity state and a Glanzmann thrombasthenia-like phenotype. *Blood*. 2001;98(8):2432–2441. doi:10.1182/blood.V98.8.2432

63. Mor-Cohen R, Rosenberg N, Peretz H, et al. Disulfide bond disruption by a β3-Cys549Arg mutation in six Jordanian families with Glanzmann thrombasthenia causes diminished production of constitutively active αIIbβ3. *Thromb Haemost*. 2007;98:1257–1265. doi:10.1160/TH07-04-0248

64. Kamata T, Ambo H, Puzon-McLaughin W, et al. Critical cysteine residues for regulation of integrin αIIbβ3 are clustered in the epidermal growth factor domains of the β3 subunit. *Biochem J*. 2004;378(3):1079–1082. doi:10.1042/bj20031701

65. Vanhoorelbeke K, De Meyer SF, Pareyn I, et al. The novel S527F mutation in the integrin β3 chain induces a high affinity αIIbβ3 receptor by hindering adoption of the bent conformation. *J Biol Chem*. 2009;284(22):14914–14920. doi:10.1074/jbc.M809167200

66. Gonzalez-Manchon C, Butta N, Larrucea S, et al. A variant thrombasthenic phenotype associated with compound heterozygosity of integrin β3-subunit (Met124Val)β3 alters the subunit dimerization rendering a decreased number of constitutive active αIIbβ3 receptors. *Thromb Haemost*. 2004;92(6):1377–1386. doi:10.1160/TH04-06-0380

67. Hardisty R, Pidard D, Cox A, et al. A defect of platelet aggregation associated with an abnormal distribution of glycoprotein IIb-IIIa complexes within the platelet: the cause of a lifelong bleeding disorder. *Blood*. 1992;80(3):696–708. doi:10.1182/blood.V80.3.696.696

68. Peyruchaud O, Nurden AT, Milet S, et al. An Arg995 [R] to Gln (Q) aminoacid substitution in the GFFKR sequence of the cytoplasmic domain of the integrin [IIb] subunit in a patient with an unusual variant form of Glanzmann's thrombasthenia. *Blood*. 1998;92(11):4178–4187. doi:10.1182/blood.V92.11.4178

69. Hughes PE, Diaz-Gonzalez F, Leong L, et al. Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *J Biol Chem*. 1996;271(12):6571–6574. doi:10.1074/jbc.271.12.6571

70. Kunishima S, Kashiwagi H, Otsu M, et al. Heterozygous ITGA2B R995W mutation inducing constitutive activation of the alphaIIbbeta3 receptor affects proplatelet formation and causes congenital macrothrombocytopenia. *Blood*. 2011;117(2):5479–5484. doi:10.1182/blood-2010-12-323691

71. Ghevaert C, Salamann A, Watkins NA, et al. A nonsynonymous SNP in the ITGB3 gene disrupts the conserved membrane proximal cytoplasmic salt bridge in the alphaIIbbeta3 integrin and cosegregates dominantly with abnormal proplatelet formation and the macrothrombocytopenia. *Blood*. 2008;111(7):3407–3414. doi:10.1182/blood-2007-09-112615

72. Favier M, Bordet J-C, Favier R, et al. Mutations of the αIIb/β3 intracytoplasmic salt bridge cause macrothrombocytopenia and enlarged platelet α-granules. *Am J Haematol*. 2017;93(2):195–204. doi:10.1002/ajh.24958

73. Morais S, Oliveira S, Lau C, et al. αIIbβ3 variants in ten families with autosomal dominant macrothrombocytopenia: expanding the mutational and clinical spectrum. *PLoS One*. 2020;15(12):e0235136. doi:10.1371/journal.pone.0235136

74. Gresele P, Falcinelli E, Giannini S, et al. Dominant inheritance of a novel integrin beta3 mutation associated with a hereditary macrothrombocytopenia and platelet dysfunction in two Italian families. *Haematologica*. 2009;94(5):663–669. doi:10.3324/haematol.2008.002246

75. Jayo A, Conde I, Lastres P, et al. L718P mutation in the membrane-proximal cytoplasmic tail of beta3 promotes abnormal alphaIIbbeta3 clustering and lipid domain coalescence and associates with a thrombasthenia-like phenotype. *Haematologica*. 2010;95(7):1158–1166. doi:10.3324/haematol.2009.018572

76. Nurden P, Bordet JC, Pillois X, Nurden AT. An intracytoplasmic β3 Leu718 deletion in a patient with a novel platelet phenotype. *Blood Adv.* 2017;1(8):494–499. doi:10.1182/bloodadvances.2016002808

77. Nurden AT, Nurden P. Inherited thrombocytopenias: history advances and perspectives. *Haematologica.* 2020;105 (8):2004–2019. doi:10.3324/haematol.2019.233197

78. Nurden P, Stritt S, Favier R, Nurden AT. Inherited platelet diseases with normal platelet count: phenotypes, genotypes and diagnostic strategy. *Haematologica.* 2021;106(2):337–350. doi:10.3324/haematol.2020.248153

79. Peretz H, Rosenberg N, Landau M, et al. Molecular diversity of Glanzmanns thrombasthenia in Southern India: new insights into mRNA splicing and structure-function correlations of alphaIIbbeta3 integrin (ITGA2B, ITGB3). *Hum Mutat.* 2006;27 (4):359–369. doi:10.1002/humu.20304

80. Nelson EJR, Nair SC, Peretz H, et al. Diversity of Glanzmann thrombasthenia in Southern India: 10 novel mutations identified among 15 unrelated patients. *J Throm Haemost.* 2006;4 (8):1730–1737. doi:10.1111/j.1538-7836.2006.02066.x

81. Jallu V, Dusseaux M, Panzer S, et al. αIIbβ3 integrin: new allelic variants in Glanzmann thrombasthenia. Effects on *ITGA2B* and *ITGB3* mRNA splicing, expression, and structure-function. *Hum Mutat.* 2009;31(3):237–246. doi:10.1002/humu.21179

82. Sandrock-Lang K, Oldenburg J, Wiegering V, et al. Characterization of patients with Glanzmann thrombasthenia and identification of 17 novel mutations. *Thromb Haemost.* 2015;113:782–791. doi:10.1160/TH14-05-0479

83. Robert P, Canault M, Farnarier C, et al. A novel leukocyte adhesion deficiency III variant: kindlin-3 deficiency results in integrin and non-integrin related defects in late steps of leukocyte adhesion. *J Immunol.* 2011;186(9):5273–5283. doi:10.4049/jimmunol.1003141

84. Desai A, Bergmeier W, Canault M, et al. Phenotype analysis and clinical management in a large family with a novel truncating mutation in RASGRP2, the CaLDAG-GEFI encoding gene. *Res Pract Thromb Haemost.* 2017;1(1):128–133. doi:10.1002/rth2.12019

85. Podolnikova NP, Gorkun OV, Loreth RM, Yee VC, Lord ST, Ugarova TP. A cluster of basic amino acid residues in the γ370–380 sequence of fibrinogen comprises a binding site for platelet integrin αIIbβ3 (GPIIb/IIIa). *Biochemistry.* 2005;44 (51):1620–2630. doi:10.1021/bi051581d

86. Podolnikova NP, Yakovlev S, Yakubenko VP, Wang X, Gorkun OV, Ugarova TP. The interaction of integrin αIIbβ3 with fibrin occurs through multiple binding sites in the αIIb β-propeller domain. *J Biol Chem.* 2014;289(4):2371–2383. doi:10.1074/jbc.M113.518126

87. Mammadova-Bach E, Ollivier V, Loyau S, et al. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood.* 2015;126:683–691. doi:10.1182/blood-2015-02-629717

88. Alshehri OM, Hughes CE, Montague S, et al. Fibrin activates GPVI in human and mouse platelets. *Blood.* 2015;126:1601–1608. doi:10.1182/blood-2015-04-641654

89. Onsalaer M-B, Hardy AT, Wilson C, et al. Fibrin and D-dimer bind to monomeric GPVI. *Blood Adv.* 2017;1(19):1495–1504. doi:10.1182/bloodadvances.2017007732

90. Perrella G, Huang J, Provenzale I, et al. Nonredundant roles of platelet glycoprotein VI and integrin αIIbβ3 in fibrin-mediated microthrombus formation. *Arterioscler Thromb Vasc Biol.* 2021;41(2):e97–e111. doi:10.1161/ATVBAHA.120.314641

91. Nurden AT. Clinical significance of altered collagen receptor functioning in platelets with emphasis on GPVI. *Blood Rev.* 2019;38:100592. doi:10.1016/j.blre.2019.100592

92. McGregor JL, Clemetson KJ, James E, et al. Glycoproteins of platelet membranes from Glanzmann's thrombasthenia. A comparison with normal using carbohydrate-specific or protein-specific labeling techniques and high-resolution two-dimensional gel electrophoresis. *Eur J Biochem.* 1981;116(2):379–388. doi:10.1111/j.1432-1033.1981.tb05346.x

93. Loroch S, Trabold K, Gambaryan S, et al. Alterations of the platelet proteome in type I Glanzmann thrombasthenia caused by different homozygous delG frameshift mutations in ITGA2B. *Thromb Haemost.* 2017;117(3):556–569. doi:10.1160/TH16-07-0515

94. Blair TA, Michelson AD, Frelinger AL III. Mass cytometry reveals distinct platelet subtypes in healthy subjects and novel alterations in surface glycoproteins in Glanzmann thrombasthenia. *Sci Rep.* 2018;8(1):10300. doi:10.1038/s41598-018-28211-5

95. Megy K, Downes K, Simeoni I, et al. Curated disease-causing genes for thrombotic, and platelet disorders; Communication from the SSC of the ISTH. *J Thromb Haemost.* 2019;17 (8):1253–1260. doi:10.1111/jth.14479

96. Ver Donck F, Downes K, Freson K. Strengths and limitations of high-throughput sequencing for the diagnosis of inherited bleeding and platelet disorders. *J Thromb Haemost.* 2020;18 (8):1839–1845. doi:10.1111/jth.14945

97. Buitrago L, Rendon A, Liang Y, et al.,;. αIIbβ3 variants defined by next-generation sequencing: predicting variants likely to cause Glanzmann thrombasthenia. *Proc Natl Acad Sci USA.* 2015;112: E1898–1907. doi:10.1073/pnas.1422238112

98. Pillois X, Nurden AT. Linkage disequilibrium amongst ITGA2B and ITGB3 gene variants in patients with Glanzmann thrombasthenia confirms that most disease-causing mutations are recent. *Br J Haematol.* 2016;175(4):686–695. doi:10.1111/bjh.14283

99. Di Minno G, Zotz RB, d'Oiron R, et al. The international, prospective Glanzmann Thrombasthenia Registry: treatment modalities and outcomes of non-surgical bleeding episodes in patients with Glanzmann thrombasthenia. *Haematologica.* 2015;100 (8):1031–1037. doi:10.3324/haematol.2014.121475

100. Poon MC, Di Minno MN, d'Oiron R, Zotz RB. New insights into the treatment of Glanzmann thrombasthenia. *Transfus Med Rev.* 2016;30(2):92–99. doi:10.1016/j.tmrv.2016.01.001

101. Al-Battat S, Rand ML, Bouskill V, et al. Glanzmann thrombasthenia platelets compete with transfused platelets, reducing the haemostatic impact of platelet transfusions. *Br J Haematol.* 2018;81 (3):410–413. doi:10.1111/bjh.14623

102. Fiore M, Firah N, Pillois X, Nurden P, Heilig R, Nurden AT. Natural history of platelet antibody formation against αIIbβ3 in a French cohort of Glanzmann thrombasthenia patients. *Haemophilia.* 2012;18(3):201–209. doi:10.1111/j.1365-2516.2011.02744.x

103. Nurden AT. Acquired antibodies to αIIbβ3 in Glanzmann thrombasthenia; From transfusion and pregnancy to bone marrow transplants and beyond. *Transfus Med Rev.* 2018;S0887-7963(18)30037–3. doi:10.1016/j.tmrv.2018.05.002

104. Leticée N, Kaplan C, Lémery D. Pregnancy in mother with Glanzmann's thrombasthenia and isoantibody against GP IIb-IIIa: is there a foetal risk? *Eur J Obstet Gynecol Reprod Biol.* 2005;121(2):138–142. doi:10.1016/j.ejogrb.2005.02.011

105. Santoso S, Wihadmadyatami H, Bakchoul T, et al. Anti-endothelial αvβ3 antibodies are a major cause of intracranial bleeding in fetal-neonatal alloimmune thrombocytopenia. *Arterioscler Thromb Vasc Biol.* 2016;36(8):1517–1524. doi:10.1161/ATVBAHA.116.307281

106. Fiore M, Bayat B, Phuangtham R, et al. Immunization against both αIIbβ3 and αvβ3 in Glanzmann thrombasthenia patients carrying the French gypsy mutation. *J Thromb Haemost.* 2021;19(1):255–261. doi:10.1111/jth.15117

598

Journal of Blood Medicine 2021:12

107. Guillet B, Bayart S, Pillois X, Nurden P, Caen JP, Nurden AT. A Glanzmann thrombasthenia family associated with a TUBB1-related macrothrombocytopenia. *J Thromb Haemost*. 2019;17(12):2221. doi:10.1111/jth.14622

108. Deshpande R, Shanbhag S, Jadii A, Shetty S. A rare case of Glanzmann's thrombasthenia and factor VII deficiency due to a combination of pathogenic and non-pathogenic gene variants. *Haemophilia*. 2020;26(1):e26–e27. doi:10.1111/hae.13898

109. Owaidah T, Saleh M, Baz B, et al. Molecular yield of targeted sequencing for Glanzmann thrombasthenia patients. *NPJ Genom Med*. 2019;4:4. doi:10.1038/s41525-019-0079-6

110. Bellucci S, Damaj G, Boval B, et al. Bone marrow transplantation in severe Glanzmann's thrombasthenia with antiplatelet alloimmunization. *Bone Marrow Transplant*. 2000;25(3):327–330. doi:10.1038/sj.bmt.1702139

111. Flood V, Johnson FL, Boshkov LK, et al. Sustained engraftment post bone marrow transplant despite anti-platelet antibodies in Glanzmann thrombasthenia. *Pediatr Blood Cancer*. 2005;45(7):971–975. doi:10.1002/pbc.20365

112. Fang J, Hodivala-Dilke K, Johnson BD, et al. Therapeutic expression of the platelet-specific integrin, αIIbβ3, in a murine model for Glanzmann thrombasthenia. *Blood*. 2005;106(8):2671–2679. doi:10.1182/blood-2004-12-4619

113. Fang J, Jensen ES, Boudreaux MK, et al. Platelet gene therapy improves hemostatic function for integrin αIIbβ3-deficient dogs. *Proc Natl Acad Sci USA*. 2011;108(23):9583–9588. doi:10.1073/pnas.1016394108

114. Sullivan KA, Mills JA, Koukouritaki SB, et al. High-level transgene expression in induced pluripotent stem-cell derived megakaryocytes: correction of Glanzmann thrombasthenia. *Blood*. 2013;124(5):753–757.

115. Hu L, Du L, Zhao Y, et al. Modeling Glanzmann thrombasthenia using patient specific iPSCs and restoring platelet aggregation function by CD41 overexpression. *Stem Cell Res*. 2017;2:14–20. doi:10.1016/j.scr.2017.02.003

116. Zhang N, Zhi H, Curtis BR, et al. CRISPR/Cas9-mediated conversion of human platelet alloantigen allotypes. *Blood*. 2016;127(6):675–680. doi:10.1182/blood-2015-10-675751

117. Engin MMN. Bleeding disorders associated with abnormal platelets: glanzmann thrombasthenia and Bernard-Soulier syndrome. *Platelets*. 2020. doi:10.5772/intechopen.9329

Journal of Blood Medicine

The Escalation of Osteosarcoma Stem Cells Apoptosis After the Co-Cultivation of Peripheral Blood Mononuclear Cells Sensitized with Mesenchymal Stem Cells Secretome and Colony Stimulating Factor-2 in vitro

Ferdiansyah Mahyudin, Fachrizal Arfani Prawiragara, Mouli Edward, Dwikora Novembri Utomo, Mohammad Hardian Basuki, Yunus Abdul Bari, Alexander Patera Nugraha & Fedik Abdul Rantam

ORIGINAL RESEARCH

The Escalation of Osteosarcoma Stem Cells Apoptosis After the Co-Cultivation of Peripheral Blood Mononuclear Cells Sensitized with Mesenchymal Stem Cells Secretome and Colony Stimulating Factor-2 in vitro

Ferdiansyah Mahyudin [1]
Fachrizal Arfani Prawiragara[1]
Mouli Edward [1]
Dwikora Novembri Utomo [1]
Mohammad Hardian Basuki [1]
Yunus Abdul Bari[1]
Alexander Patera Nugraha [2]
Fedik Abdul Rantam [3]

[1]Orthopedic and Traumatology Department, Faculty of Medicine, Dr Soetomo General Hospital, Airlangga University, Surabaya, Indonesia; [2]Orthodontics Department, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia; [3]Laboratory of Virology and Immunology, Microbiology Department, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia

Introduction: Peripheral blood mononuclear cells (PBMCs) sensitized with mesenchymal stem cells (MSCs) secretome and/or colony stimulating factor-2 (CSF-2) as an immunotherapy candidate may escalate osteosarcoma stem cells (OS-SCs) apoptosis. This study aimed to investigate the escalation of osteosarcoma stem cells' apoptosis after the co-cultivation with PBMCs sensitized by MSCs secretome with/or CSF-2 and it was completed by analyzing the level of serum tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) and tumor necrosis factor-α (TNF-α) level, annexin V binding, caspase-3 and caspase-8 expression in vitro.
Methods: OS-SCs were derived from a single human osteosarcoma sample with its high grade and osteoblastic essential clinical characteristics obtained from a biopsy before the chemotherapy treatment. They were then isolated and cultured confirmed by the cluster of differentiation-133 (FITC) by applying immunofluorescence analysis with fluorescein iso-thiocyanate (FITC) labeled. MSCs secretome was obtained with cells extracted from the bone marrow of a healthy patient. Furthermore, enzyme linked immunosorbent assay (ELISA) was utilized to analyze sTRAIL and TNF-α level in each group. The expression of caspase-3, caspase-8, and annexin V assay in each group was examined by applying the immunofluorescence labeled with FITC. The comparison analysis between treatment groups and the control group was performed by utilizing the analysis of variance (ANOVA) and continued with Tukey Honest Significant Difference (HSD) (p<0.05).
Results: There was a significant difference in the upregulation of sTRAIL and TNF-α level indicated by the increased annexin V, caspase-3, and caspase-8 expression binding between groups (p<0.05).
Conclusion: MSCs Secretome and CSF-2 could significantly increase the activity of PBMCs through the improvement of sTRAIL and TNF-α levels which could lead to the escalation of OS-SCs apoptosis through an enhanced expression of caspase 3, caspase 8 and annexin V binding in vitro.
Keywords: cancer, caspase, osteosarcoma, peripheral blood mononuclear cells, mesenchymal stem cells, tumor necrosis factor

Introduction

As the most frequent primary bone tumor, osteosarcoma (OS) has the prevalence for approximately 4–5 out of 1.000.000 per year with higher prevalence in men than

Correspondence: Ferdiansyah Mahyudin
Email ferdiansyah@fk.unair.ac.id

in women.[1,2] Osteosarcoma is a neoplasm that can be diagnosed based on the histological examination of osteoid production associated with the aggressive malignant mesenchymal cells that tend to metastasize early and it has several types conventionally categorized by the cell types such as osteoblastic, chondroblastic and fibroblastic.[3,4]

In Indonesia, the incidence of bone tumors at Central Hospital Cipto Mangunkusumo, Jakarta was 1.2%, whereas the incidence of malignant bone tumors was 1.3%. The prevalence of osteosarcoma was one of the top five cancer cases found at the age group of 1–17 years old. The profile evaluation of bone tumors in children at Central Hospital Cipto Mangunkusumo in Jakarta during 1995–2004 showed that 73.7% of the cases were confirmed as osteosarcoma.[5]

In curing osteosarcoma, the existing therapy that has been used is the conventional therapies including surgery, chemotherapy, and radiotherapy.[6] Prior investigation exhibited that homologous recombination fixes defects contained within the designed BRCAness genomic signature of high-grade osteosarcomas and apoptosis. There may be a link between caspase activation and the poly (ADP-ribose) polymerase (PARP). Furthermore, the diminished cell viability is uncovered by administrating Talazoparib on the MNNG/HOS cell line, but there is no sign in the study of apoptosis.[7] The growing quality of life in the osteosarcoma patients has only been seen as the incremental changes in survival. In addition, the immune environment of the bone is highly specialized, and several immune signaling pathways have major contribution in bone homeostasis. Thus, the alternative therapy targeting deoxyribonucleate (DNA) that leads to DNA fragmentation known as apoptosis is still urgently needed.[8]

Moreover, apoptosis can occur through two different pathways, which are the extrinsic pathway and intrinsic pathway. The first pathway can be completed with an external stressor that initiates the apoptosis by activating Fas-associated death domain (FADD)/TNFRSF1A Associated Via Death Domain (TRADD) resulting in the pro-caspase activation.[9,10] It is categorized into transduction pathways, oxygen reactive pathways, growth factor pathways, and cytokine pathways. Meanwhile, the intrinsic pathway is an apoptotic process involving mitochondrial dependent cytochrome C, caspase 3, caspase 9, apoptotic protease activating factor-1 (Apaf-1), Bcl-2, BAX, and BAK.[11] The various cell death processes mentioned above are most likely becoming the option for

osteosarcoma treatment by employing peripheral blood mononuclear cells (PBMCs) as an immunotherapy.

In addition, immunotherapy can be benefited for cancer therapy by applying cancer-specific immunoglobulins including monoclonal antibodies, cancer vaccines, and T-cell therapy. PBMCs consist of several cells such as lymphocytes like monocytes, natural Killer (NK) cells, dendritic cells (DC), T-helper cells (Th1, Th2, Th17), and macrophages which have different phenotypes and activations in the immune system.[12] PBMCs significantly contribute in controlling the immune system through cytokine networks or interleukins, secretions and expressions such as fas ligand, perforin, granzyme B, and soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) which can generate cancer cell necrosis through extrinsic and intrinsic routes.[13] Although in many researches, the results revealed that the activation of PBMCs can be raised by administering antioxidants, lipopolysaccharides, or other types of herbs containing tannins.[14] There is another compound which has not been widely studied to sensitize and activate PBMCs, that is mesenchymal stem cells (MSCs) secretome.

A secretome of MSCs is a combination of many secreted molecules which includes interleukins, growth factors and any of secreted extracellular vesicles like microvesicles, apoptotic bodies, ectosomes, and exosomes. MSCs secretome might contribute a significant capacity in the physiological activation of PBMCs both in vivo and in vitro.[15] Additionally, another molecule that may stimulate and activate the PBMCs is the colony stimulating factor-2 (CSF-2). CSF-2 entails molecules that have the essential part in the transcription factors and immune cell proliferation consisting NK-cells, cytotoxic T lymphocyte (CTL), and monocytes that can expand the activity of mononuclear cells through the secretion of tumor necrosis factor-α (TNF-α), interleukin (IL)-2, and IL-6 with paracrine effect.[16]

Furthermore, TNF-α is one of the pro-inflammatory cytokines produced by peripheral blood mononuclear cells covering macrophages, T cells, eosinophils, mast cells, NK cells and some nerve cells. TNF-α also has a fundamental part in regulating the immune system which entails inducing the cell death through apoptosis.[17] In addition, launched by several cell types, serum tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) is qualified in interlacing the transmembrane pro-apoptotic death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 in human. Therefore, the initial marker

of pro-apoptotic induction molecule is the levels of TNF-α and TRAIL which reflect the activated extrinsic pathway. The sTRAIL level is influenced by the time, which means that the more it is secreted, the more it can affect the micro environment.[18]

Significantly, the enhancement of TNF-α and sTRAIL from PBMCs sensitized by CSF-2 and/or MSCs secretome may induce the apoptosis of cancer cells through extrinsic pathway. The biomarker which can be used to detect the apoptosis via extrinsic pathway along with annexin V, caspase-3 and caspase-8.[19] Known as one of cellular proteins within the annexin group, annexin V is often employed to identified an apoptosis by tying up phosphatidylserine outside the plasma membrane. Annexin V which binds the uppermost layer of the cellular membrane's phosphatidylserine could detect the process of cell damage or apoptosis.[20] Meanwhile, caspase-3 is the part of protease enzyme family which contributes essentially and fundamentally in the apoptosis and caspase 8 is the initiator to cleave Atg3 during receptor-mediated cell death and induces apoptosis.[19]

As the cell targets, osteosarcoma stem cells (OS-SCs) can be generated from the patient's osteosarcoma tissues. Besides that, not only that osteosarcoma tissues are the resource of cancer stem but these tissues can also produce the extracted osteosarcoma associated stromal cells which are non-tumoral mesenchymal stem cell-like cells.[20,21] However, the isolated OS-SCs must be confirmed by utilizing the markers of CD133, SOX2 and CD44. The enhanced expression of SOX2 may directly contribute in the growth and initiation of tumor.[22] Moreover, SOX2 expression in sarcoma patients is associated with the tumor grade, differentiation, invasive potential and lower patient survival.[23] Meanwhile, in osteosarcoma, the features of CD117 and STRO-1 for the most lethal characteristics of the disease-metastasis and drug resistance, these markers offer candidates for the drug delivery of cancer stem cells aiming in eradicating the osteosarcoma.[24]

The combination of PBMCs with MSCs secretome, or PBMCs with CSF-2, or PBMCs with MSCs secretome and CSF-2 as mentioned above can lead to the discovery of immunotherapy for osteosarcoma treatment. Thus, this study's main goal was to inquire the escalation of osteosarcoma stem cells (OS-SCs) apoptosis after the co-cultivation with PBMCs sensitized by MSCs secretome with/or colony stimulating factor-2 completed by analyzing sTRAIL and TNF-α level, annexin V binding, caspase-3 and caspase-8 expression in vitro.

Materials and Methods
Study Design
This scientific investigation was an in vitro study and a true experimental post-test only control group design. The size of this study sample was seven samples for each group. The sample was then selected blind-randomly and then assigned into each group. This study was carried out in Dr Soetomo General Hospital (RSUD. Dr Soetomo) and the Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia. Related to the study protocol, the ethical health committee of Dr Soetomo General Hospital, Surabaya, East Java, Indonesia, had granted the research ethics authorization for this study.

Isolation, Culture, Sub-Culture of Osteosarcoma Stem Cells
In this study, OS-SCs were derived from a single human osteosarcoma sample with its high-grade and osteoblastic essential clinical characteristics obtained from biopsy before the chemotherapy treatment. OS-SCs were isolated from osteosarcoma tissue with a 3–5 cm excision from the osteosarcoma patient treated at the department of Orthopedics and Traumatology in Dr Soetomo General Hospital, Surabaya, Indonesia. The excision was performed by surgeons after obtaining a written informed consent from the patient for the osteosarcoma tissue collection. The isolated osteosarcoma tissues were then cleaned by washing them with phosphate-buffered saline (PBS) (Sigma Aldrich, US) entailing 5% penicillin/streptomycin (Gibco, US). The specimens of the tissue were chopped in small pieces by applying a sterile scissor then placed into Erlenmeyer tube consisted of 5 mL Type I collagenase 0.075% within PBS containing 2% of penicillin/streptomycin. Twenty percent heat-inactivated fetal bovine serum (FBS) (Gibco, US) contained in 5 mL of α-minimal essential medium (Gibco, US) were blended into the tube to counterbalance the Type I collagenase activity. In addition, samples were then refined afterward by pouring them into the beaker glass wrapped with 3 plies of sterile gauze. The resulted supernatant of filtration was later centrifuged in 2000 rpm for 5 min, then disposed, and its pellet was resuspended by mixing it with PBS for pellet wash. The pellet was rewashed repeatedly and eventually resuspended with the complete medium. For the final step of this procedure, those suspensions were cultivated on the

culture plate and then stored inside the incubator with 37°C and 5% CO_2.[25]

Characterization of Osteosarcoma Stem Cells

To start this characterization, the filtration of digested OS-SCs was cultured in the sterile 10 cm petri dish and a complete 10 mL growth medium was added. Then, OS-SCs were incubated in a 5% CO_2 incubator with temperature at 37°C for 5 days. The characterization of confluent OS-SCs was carried out by immunofluorescence staining with immunofluorescence isothiocyanate (FITC) labeled using the specific markers of OS-SCs such as cluster of differentiation (CD) CD133+, SOX2, and CD44+.[22–25]

Isolation and Sensitization of PBMC with BM-MSCs Secretome and/or CSF-2

The collection of peripheral blood sample from healthy volunteers was performed that had been examined for HIV and Hepatitis-B after obtaining the written informed consent from those volunteers. PBMCs isolation was done by employing the Ficoll system with a gradient of 1.077. A sample of 10 mL from the whole blood was mixed with EDTA, then washed with PBS and centrifugation at a speed of 1600 rpm. Next, the supernatant was removed and then the blood was taken with a micropipette and put into a 15-mL tube containing 5 mL Ficoll. Furthermore, 1600 rpm centrifugation was carried out at a temperature of 20°C. The buffy coat was slowly separated with a pipette and placed in sterile PBS. After centrifugation, the pellets were resuspended with a complete growing medium (RMPI 1640, 10% FBS) added with BM-MSCs secretomes or CSF-2 (Sigma Aldrich, US).

Moreover, bone marrow mesenchymal stem cells (BM-MSCs) secretome was generated from healthy volunteer, and the examination of HIV and Hepatitis-B was performed after obtaining the written informed consent from this volunteer. Two growth media were selected for producing mesenchymal stem cells conditioned medium (MSC-CM). Firstly, the chemically defined low glucose DMEM (DMEM-LG) (Sigma Aldrich, US) was selected as a conventionally used medium for MSC culture. Secondly, among the commercially available media designed to specifically support the growth of undifferentiated MSC, a defined, xeno-free, serum-free MSCs NutriStem medium was preferred for this process (Biological Industries, Israel). It was fundamental to note

that only basal media without nutrimental supplements (FBS or NutriStem supplement) were used for MSC conditioning.[26] BM-MSCs secretomes were generated from the Stem Cells Research and Development Center, Universitas Airlangga, Surabaya, Indonesia, and finally incubated at a 37°C incubator for 2 days.

Co-Cultivation of Osteosarcoma Stem Cells with Sensitized Peripheral Blood Mononuclear Cells

At this step, co-cultivation between the sensitized PBMCs and OS-SCs with a ratio of 5:1 was cultured in a 10 cm petri dish and then 10 mL MEM alpha medium with 20% FBS was administered. Finally, the sensitized PBMCs and OS-SCs were incubated in 5% CO_2 incubator at a temperature of 37°C for 5 days.[25]

The Level of sTRAIL and TNF-α Analysis by Indirect Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assay (ELISA) was employed to analyze sTRAIL (E-EL-H1593, Elabscience, US) and TNF-α levels (E-EL-H0109, Elabscience, US) in each group. The first step was 100 μL supernatant medium coated in 96 well microplate by applying a coating buffer with a ratio of 1:10 for 24 h at 4°C. After an antigen coating, 3 times washing with 0.02% tween-X was performed, then continued with the blocking using 1% BSA. After the washing, each antibody was added in the ratio of 1:1500 and then incubated in an incubator at 37°C for 2 h. In the post-washing process, it was reacted with the secondary conjugate antibody labeled with alkaline phosphatase, combined with PNpp and incentivized in a dark room for 15 min, then the reaction was stopped with 1 N H_2O_2 or 1N HCl. Finally, the analysis was carried out with an ELISA reader with a 450 wavelength.[25]

Immunofluorescence Analysis on Annexin V, Caspase-3 and Caspase-8 Expression

After being co-cultivated with sensitized PBMCs after 5 days, OS-SCs were washed with sterile PBS, preserved with acetone at −20°C for 3–5 min, and then rewashed 3 times with PBS. In addition, blocking was performed with 1% serum. In addition, anti-caspase 8, anti-caspase 3, and anti-annexin V FITC-conjugated antibodies as much as 100 μL with a ratio of 1:500 were combined and incubated

for 1 h at a 37°C. The comparison was done between normal cells and cells stained with 4,6-diamidino-2-phenylindole (DAPI). The sample was then observed under fluorescent inverted microscope with 20x magnification.[25]

Data Analysis

All data were collected, then processed, and statistically tested by using 20.0 version of Statistical Program for Social Science (SPSS) for Windows (IBM Corporation, Illinois, Chicago, US). The data was described as an average in each group and the normality data test ($p > 0.05$) was carried out. The comparison analysis between the treatment groups and the control group was done by utilizing the analysis of variance (ANOVA) continued with Tukey Honest Significant Difference (HSD) ($p < 0.05$). The analysis of statistics was employed to investigate the effect of times (2 days, 4 days, and 6 days) and the TNF-α and sTRAIL level, and the expression of caspase-3, caspase-8 and annexin V examination.

Results

OS-SCs were successfully isolated and cultured from a patient's osteosarcoma tissue with the high grade and osteoblastic essential clinical characteristics showed the morphology of the OS-SCs (Figure 1A–D). The OS-SCs were sub-cultured until the 6th passage and then characterized with CD133+ and CD44+ markers (Figure 1E–H). Moreover, the morphology of PBMCs isolated from healthy people was further sensitized with MSCs secretome and CSF-2 for two days that can be seen in Figure 2A. As a result, the sensitized PBMCs cocultivated with OS-SCs showed a reactivity that can be seen in Figure 2B–D.

The highest TNF-α level was found in PBMCs + CSF-2 group as a significant difference was also discovered in TNF-α level in 2 days between PBMCs + CSF-2 group and PBMCs alone, and PBMCs + MSCs secretome and PBMCs alone ($p < 0.05$). However, there was no significant difference in TNF-α level between PBMCs + CSF-2 group and PBMCs + MSCs secretome group in 2 days ($p > 0.05$) (Figure 3A).

The highest sTRAIL level was expressed in PBMCs +OS-SCs+ MSCs secretome (6d) group. There was a significant difference located in sTRAIL level between OS-SCs group, PBMCs+MSCs secretome (2d) group, PBMCs+OS-SCs+MSCs secretome (2d) group and PBMCs +OS-SCs+MSCs secretome (6d) group ($p < 0.05$). In addition, the significant difference was also shown in sTRAIL

secretion between PBMCs+OS-SCs+MSCs secretome (6d) group and OS-SCs (6d) group ($p < 0.05$) (Figure 3B). The highest sTRAIL level was found in PBMCs+OS-SCs+CSF2 group. A significant difference was also presented in sTRAIL level between OS-SCs (6d) group, PBMCs+CSF2 (2d) group, PBMCs+OS-SCs+CSF2 (2d) group, PBMCs +OS-SCs+CSF2 (4d) group and PBMCs+OS-SCs+CSF2 (6d) group ($p < 0.05$). Moreover, a significant difference was also found between PBMCs+OS-SCs+CSF2 (6d) group and OS-SCs (6d) group ($p < 0.05$) (Figure 3C). Annexin V binding was positively detected in OS-SCs of each group which can be seen in Figure 4A–C. Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretome can be seen in Figure 4A1 and A2. Before and after the co-cultivation of OS-SCs and PBMCs sensitized with CSF-2 can be seen in Figure 4B1 and B2. Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretome and CSF-2 can be seen in Figure 4C1 and C2. The highest Annexin V expression was uncovered in OS-SCs+PBMCs+MSCs Secretome +CSF2 group. Correspondingly, a significant difference was exhibited in Annexin V binding between groups (OS-SCs; OS-SCs+PBMCs; OS-SCs+PBMCs+Scrt; OS-SCs +PBMCs+Scrt+CSF) ($p < 0.05$) (Figure 4D).

The expression of caspase-3 was exhibited positively in OS-SCs of each group, which can be seen in Figure 5A–C. Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretomes shown in Figure 5A1 and A2. Before and after the co-cultivation of OS-SCs and PBMCs and sensitized with CSF-2 can be seen in Figure 5B1 and B2. Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretomes and CSF-2 shown in Figure 5C1 and C2. The highest expression of caspase-3 was revealed in the OS-SCs+PBMCs+ MSCs secretome +CSF2 group. In a same manner, there was a significant difference was in caspase-3 expression between groups of OS-SCs, OS-SCs+PBMCs, OS-SCs+PBMCs+Scrt, OS-SCs+PBMCs+CSF, OS-SCs+PBMCs+Scrt+CSF ($p < 0.05$). However, an insignificant difference was found between OS-SCs+PBMCs+Scrt group, OS-SCs +PBMCs+CSF group, and OS-SCs+PBMCs+Scrt+CSF group ($p > 0.05$) (Figure 5D).

The expression of caspase-8 was revealed positively in OS-SCs of each group (Figure 6A–C). Before and after OS-SCs co-cultivated with PBMCs and sensitized with MSCs secretomes shown in Figure 6A1 and A2. Before and after OS-SCs co-cultivated with PBMCs
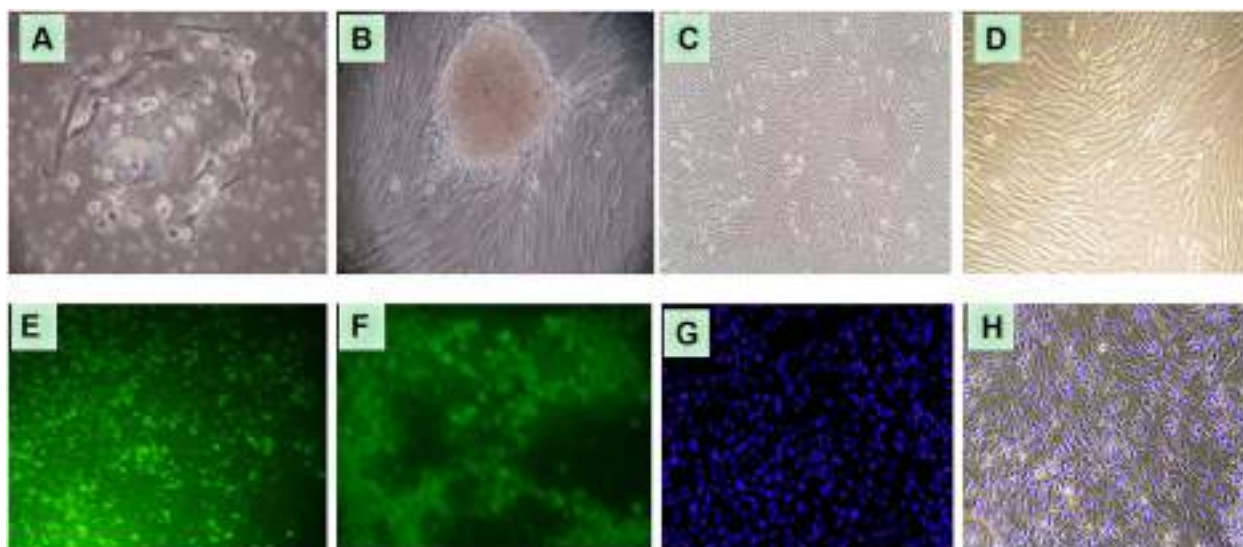
Figure 1 Growth of OS-SCs isolated from a single human osteosarcoma sample with its high grade and osteoblastic essential clinical characteristics obtained from biopsy before the chemotherapy treatment. (**A**) OS-SCs fibroblast-like cells grow 60% in 3rd passage; (**B**). OS-SCs fibroblast-like cells grow 90% confluent in 4th passage; (**C**) 90% confluent cell growth of OS-SCs in 5th passage; (**D**) 90% growth of OS-SCs in 6th passage; (**E**) OS-SCs positively expressed by CD133+ expression; (**F**) OS-SCs positively expressed by CD44+ expression; (**G**) OS-SCs stained with DAPI; (**H**) The comparison between normal cells and cells stained with DAPI.
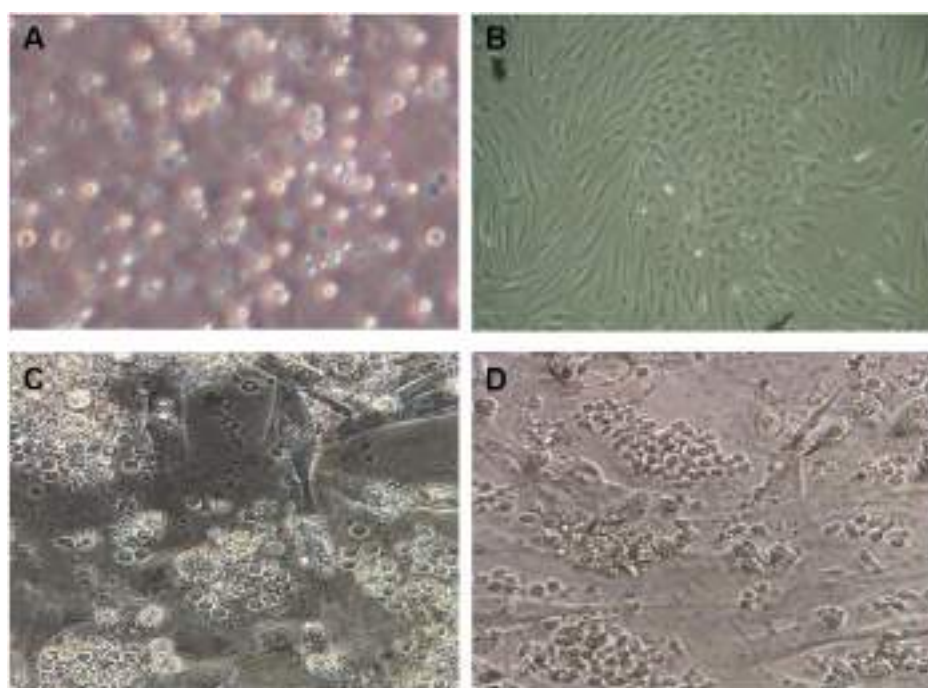
Figure 2 The sensitization of PBMCs. (**A**) Sensitization of PBMCs with MSCs secretome and CSF-2 after 2 days; (**B**) OS-SCs co-cultivated with PMBCs; (**C**) Co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretome showed by the appearance of endocytosis; (**D**) Sensitized PBMCs with CSF-2 after 6 days OS-SCs induced apoptosis.

and sensitized with CSF-2 shown in Figure 6B1 and B2. Before and after OS-SCs co-cultivated with PBMCs and sensitized with MSCs secretomes and CSF-2 Figure 6C1 and C2. The highest expression of caspase-8 was found in OS-SCs+PBMCs+Scrt+CSF group. Moreover, a significant difference was expressed in caspase-8 between groups (p<0.05) (Figure 6D).

606
Journal of Blood Medicine 2021:12
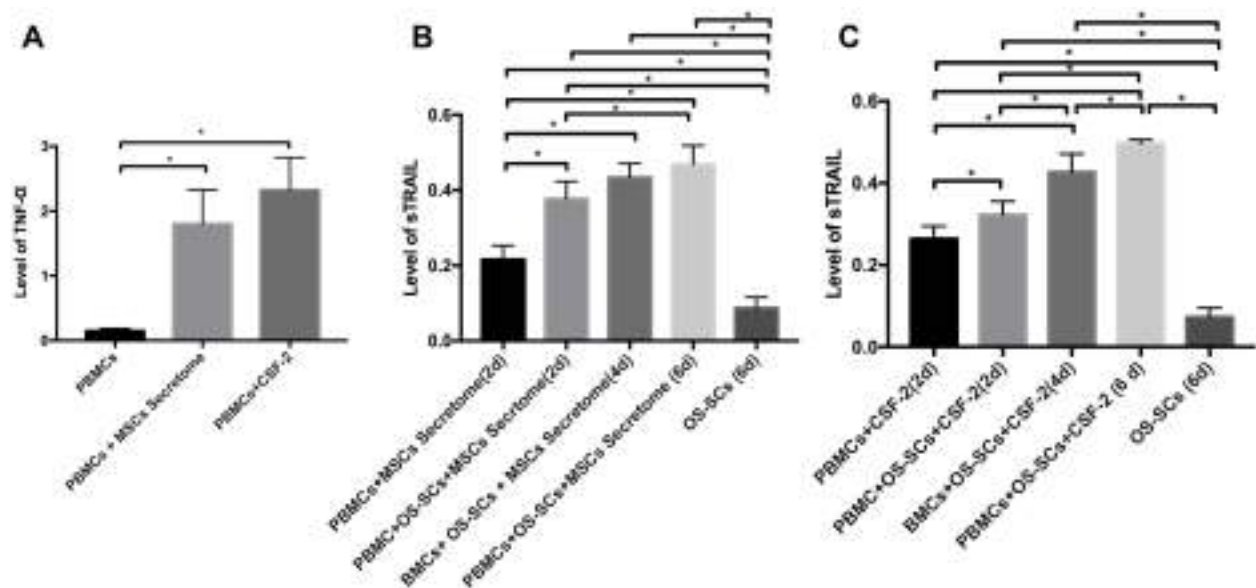
Figure 3 Co-cultivation of OS-SCs with sensitized PBMCs with MSCs secretome or CSF-2. (**A**) The level of TNF-α in each group in 2 days; (**B**) The level of sTRAIL in each group of PBMCs sensitized with MSCs secretome; (**C**) The level of sTRAIL in each group of PBMCs sensitized with CSF-2. *Information: signification p-value at p<0.05.
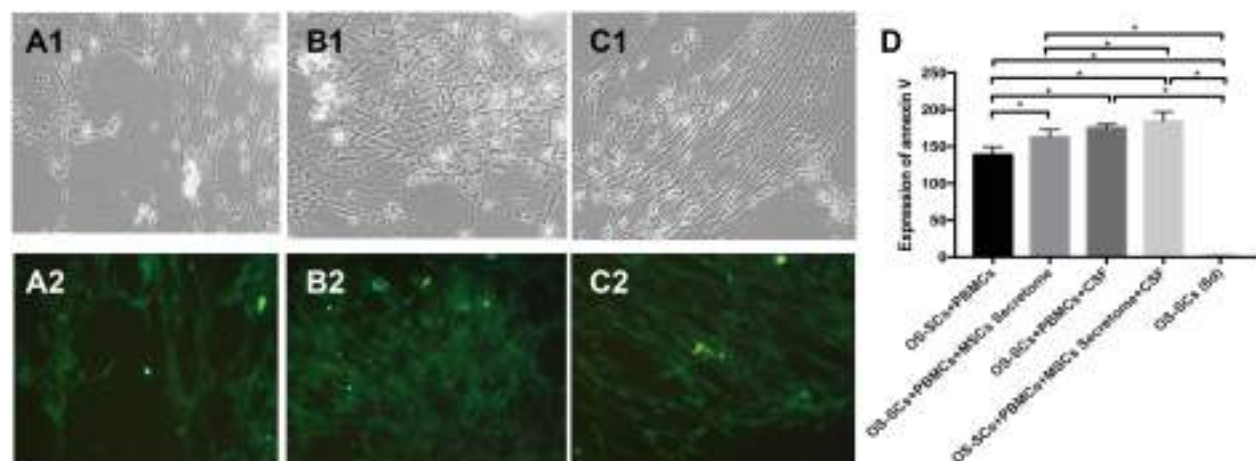
Figure 4 The result of Annexin V labeled with FITC binding was positively detected in OS-SCs in each group. (**A1** and **A2**) Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretome; (**B1** and **B2**) Before and after the co-cultivation of OS-SCs and PBMCs sensitized with CSF-2; (**C1** and **C2**) Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretome and CSF-2. The observation was carried out by utilizing the light inverted microscope with 20x magnification; (**D**) The cell quantification of Annexin V binding in each group. *Information: significant at p<0.05.

Discussion

This investigation explored and closely examined the activity of sensitized PBMCs co-cultivated with OS-SCs. The sensitized PBMCs may secreted cytokine that is beneficial against osteosarcoma in vitro such as the production of memory T cells and regulatory T cells (CD25+) NK cells. The active initiation of NK cells is fundamental to induce OS-SCs apoptosis. The presence of activated CD25 + and NK cells can lead to the resistant type of T cells to the cell-mediated suppression of newly activated myeloid stem cells (MDSCs), and protect the body against tumor development and relapse.[27]

The sensitized PBMCs with MSCs secretome and/or CSF-2 indicate the presence of activated lymphocytes, namely CD25+, NK cells as well as CD4+ and CD8+ adaptive immune cells.[25] The possibility that MSCs secretomes contain IL-2, IL-7 and IL-15 has been widely reported to support the homeostatic T-cell proliferation as well as the increased NK cell function, the maturation of IL-2 terminal NKT cells, and the expanded size of CD8+.[28] Meanwhile,
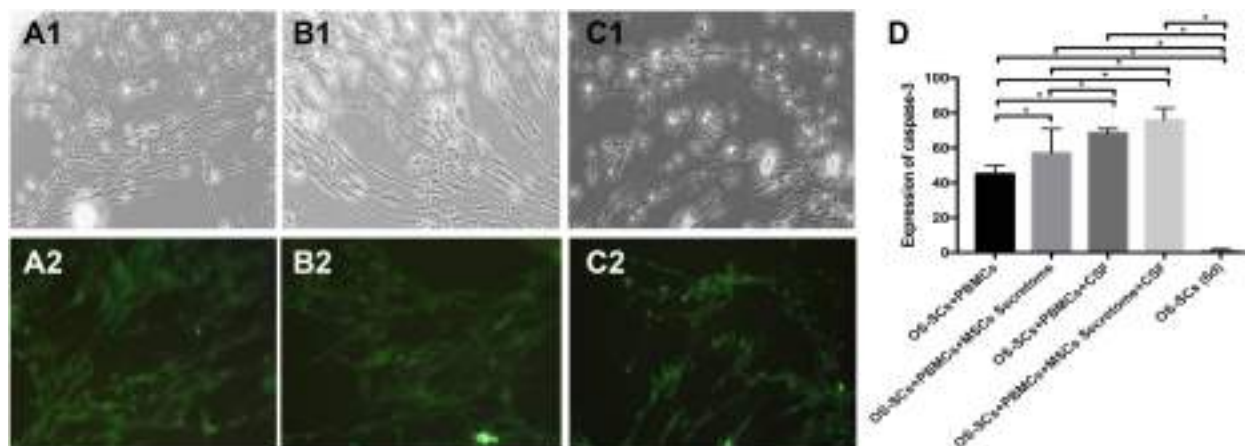
Figure 5 The result of caspase-3 expression labeled with FITC was expressed positively in OS-SCs in each group. (**A1** and **A2**) Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretomes; (**B1** and **B2**) Before and after the co-cultivation of OS-SCs and PBMCs and sensitized with CSF-2; (**C1** and **C2**) Before and after the co-cultivation of OS-SCs and PBMCs sensitized with MSCs secretomes and CSF-2. The observation was carried out by utilizing the light inverted microscope with 20x magnification. (**D**) The cell quantification that expressed caspase-3 in each group. *Information: significant at $p<0.05$.
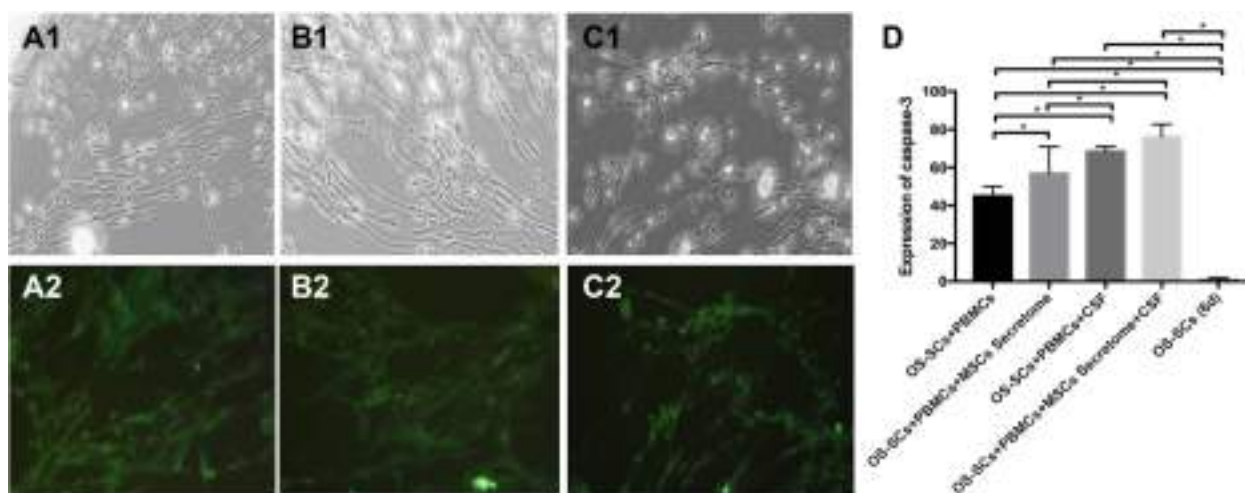
Figure 6 The result of caspase-8 expression labeled with FITC was demonstrated positively in OS-SCs in each group. (**A1** and **A2**) Before and after OS-SCs co-cultivated with PBMCs and sensitized with MSCs secretomes; (**B1** and **B2**) Before and after OS-SCs co-cultivated with PBMCs and sensitized with CSF-2; (**C1** and **C2**) Before and after OS-SCs co-cultivated with PBMCs and sensitized with MSCs secretomes and CSF-2. The observation was carried out by employing the light inverted microscope with 20x magnification. (**D**) The cell quantification that expressed caspase-8 in each group. *Information: significant at $p<0.05$.

CSF-2 stimulates the activity of macrophages, NK cells, and cytotoxic T cells.[16] Consequently, the sensitization of PBMCs by MSCs secretome and/or CSF-2 may enhance NK cells, CD4+ and CD8+.

Furthermore, TNF-α level in PBMCs improves after the sensitization of PBMCs through molecule signaling in macrophages. In this study, the result revealed that PBMCs that have been activated with MSCs secretomes or CSF-2 initiate the activation of macrophages to secrete TNF-α. The highest TNF-α level was expressed in PBMCs + CSF-2 group after 2 days. There was a significant difference

exhibited in TNF-α level between groups. In addition, TNF-α is a molecule that contributes majorly in regulating the inflammatory factors and as a master in regulating cytokine production.[17] In producing various kinds of cytokines and enzymes, macrophages can go through complex processes, which are both passive and active action. Macrophage activation comes from the signaling molecules lymphokine, chemokines, and pathogen associated molecular patterns (PAMPs) and then secretes the products like IL-1, IL-12, TNF-α, IL-10, nitric oxide (NO) and reactive oxidative stress (ROS), IL-18, and IL-17.[29]

In this study, the highest sTRAIL level was discovered in PBMCs + OS-SCs + MSCs secretome (6d) group. Consistently, a significant difference was demonstrated in sTRAIL levels between groups and in sTRAIL secretion between PBMCs + OS-SCs + MSCs secretome (6d) group and OS-SCs (6d) group. In addition, OS-SCs co-cultivated with PBMCs and sensitized with CSF-2 were significantly different in sTRAIL levels between groups. Furthermore, there was a display of significant difference between PBMCs + OS-SCs + CSF2 (6d) group and OS-SCs (6d) group. Therefore, 6 days was applied as a time frame reference in this study. In fact, TRAIL-3 and TRAIL-4 can be suppressed by receptor activator nuclear kappa beta ligand (RANKL) to induce apoptosis.[30]

Correspondingly, TNF-α and sTRAIL secreted by PBMCs are molecules that can be used as the indicators in PBMCs activation for osteosarcoma immunotherapy marked by the escalation of OS-SCs apoptosis. Furthermore, the signaling molecule from death-ligand-domain interaction can influence the prevalence of apoptosis via the gathering of death-inducing signaling complex (DISC). It is configurated by the FADD which enhances the autocatalytic caspase processing, caspase activation, and apoptosis. In line with this study, the ability of TRAIL to eliminate cancer cells was investigated by developing a recombinant form of TRAIL or TRAIL receptor agonist for cancer therapy such as receptor-specific monoclonal antibody.[31]

In this present study, the highest Annexin V binding in OS-SCs + PBMCs + MSCs Secretome + CSF2 was uncovered as a significant difference and found in Annexin V binding between groups. The addition of MSCs secretome and CSF-2 is possible to inhibit the secretion of IL-10; thus, the dominant secretion of pro-inflammatory cytokine occurs.[32] The result of this study also revealed that the highest expression of caspase-8 was exhibited in OS-SCs + PBMCs + MSCs secretome + CSF group. Likewise, a significant difference was featured in caspase-8 expression between groups in this study.

Moreover, MSCs Secretome is a molecular complex that maintains the tissue's microenvironment in a homeostasis condition. Meanwhile, CSF-2 is a pure molecule with the functions in increasing the activity of macrophages to response the imbalance condition by inducing, initiating, and signaling the macrophage's activation. Subsequently, the activated macrophage will proliferate, differentiate and polarize the macrophage rapidly.[33] Additionally, the activation of caspase-8 is stimulated the continuous signal from extrinsic factors to endogenous ligands.[34] At the same time, the activation of macrophages that produces TNF-α and TRAIL which can activate endogenous ligands. The increased level of TNF-α and TRAIL depends on the time of production which can affect quickly or not in inducing the apoptosis. The extrinsic factor of apoptosis is influenced by the time and the possibility of inductor concentration against endogenous ligands. The extrinsic pathway of apoptosis is strongly influenced by the activity of caspase-3.[35] In this present study, the highest expression of caspase-3 was found in the OS-SCs + PBMCs + MSCs secretome + CSF2 group. Accordingly, there was a significant difference demonstrated in caspase-3 expression between groups. This was in line with the previous study that investigated Shikonin from medical herbs that can enhance the osteosarcoma apoptosis through the up-regulation of caspase-3 and caspase-8.[36] The presence of interactions between molecules is also influenced by the time, the concentration and the microenvironment which is very dominant in facilitating the level of TRAIL and TNF-α as the initial inductor that can trigger annexin V, caspase-8 and caspase-3 activation. The major limitation in this study is these cells that were derived only from a single human osteosarcoma tissue and a single healthy human BM-MSCs secretome.

Conclusion

Based on our study, MSCs secretome and CSF-2 can increase the activity of PBMCs through the enhancement of sTRAIL and TNF-α levels that can lead to the escalation of OS-SCs apoptosis via a raised expression of caspase 3 and caspase 8 which can be indicated by the increased annexin V binding in vitro. Further study is still needed to examine the apoptosis signaling pathway of OS-SCs co–cultivated by PBMCs sensitized with CSF-2 and/or MSCs secretome in vitro and in vivo.

Data Sharing Statement

Please contact the correspondence author with requests for data supporting reported results.

Institutional Review Board Statement

The study was performed accordingly based on the guidelines derived from the Declaration of Helsinki and the Ethics Committee of Dr Soetomo Regional General Hospital Ethical confirm and approve for these study protocols.

Informed Consent Statement

Written informed consent has been obtained from the patient(s) to publish this paper.

Acknowledgments

We would like to send our gratitude to the Faculty of Medicine, Airlangga University and Dr Soetomo Regional General Hospital for their tremendous support to our study.

Disclosure

The authors declare no conflicts of interest for this work.

References

1. Fletcher CDM, Bridge JA, Hogendoorn P, Mertens F. *WHO Classification of Tumours of Soft Tissue and Bone*. 4th ed. Lyon, France: IARC Press; 2013.
2. Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. *Cancer Treat Res*. 2009;152:3–13.
3. Ritter J, Bielack SS. Osteosarcoma. *Ann Oncol*. 2010;21(7):320–325. doi:10.1093/annonc/mdq276
4. Picci P, Manfrini M, Fabbri N, Gambarotti M, Vanel D, Atlas of Musculoskeletal Tumors and Tumorlike Lesions [Internet]. Cham: Springer International Publishing; 2014 [cited 2017 September 12.]. Available from: http://link.springer.com/10.1007/978-3-319-01748-8. Accessed June 28, 2021.
5. Khan S, Barwar N, Kumar V. Surface osteosarcomas: diagnosis, treatment and outcome. *Indian J Orthop*. 2014;48(3):255. doi:10.4103/0019-5413.132503
6. Biazzo A, De Paolis M. Multidisciplinary approach to osteosarcoma. *Acta Orthop Belg*. 2016;82(4):690–698.
7. Kager L, Tamamyan G, Bielack S. Novel insights and therapeutic interventions for pediatric osteosarcoma. *Future Oncol*. 2017;13 (4):357–368. doi:10.2217/fon-2016-0261
8. Kansara M, Teng MW, Smyth MJ, Thomas DM. Translational biology of osteosarcoma. *Nat Rev Cancer*. 2014;14(11):722–735.
9. Goldar S, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pac J Cancer Prev*. 2015;16(6):2129–2144. doi:10.7314/APJCP.2015.16.6.2129
10. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. *Nat Rev Clin Oncol*. 2020;17(7):395–417.
11. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ*. 2000;7(12):1166–1173. doi:10.1038/sj.cdd.4400783
12. Fang F, Xiao W, Tian Z. Challenges of NK cell-based immunotherapy in the new era. *Front Med*. 2018;12(4):440–450. doi:10.1007/s11684-018-0653-9
13. Horinaka M. Application of molecular-targeting cancer prevention to tumor immunity. *Nihon Eiseigaku Zasshi*. 2014;69(1):8–14. doi:10.1265/jjh.69.8
14. Xie Y, Chen B, Wang H, Wang K, Li R. Advances in the research of peripheral blood mononuclear cell-derived multipotential cell. *Zhonghua Shao Shang Za Zhi*. 2016;32(12):762–764. doi:10.3760/cma.j.issn.1009-2587.2016.12.013
15. Lötvall J, Rajendran L, Gho YS, et al. The launch of Journal of Extracellular Vesicles (JEV), the official journal of the International Society for Extracellular Vesicles - about microvesicles, exosomes, ectosomes and other extracellular vesicles. *J Extracell Vesicles*. 2012;16;1. doi:10.1016/0006-2952(75)90016-7
16. Arellano M, Waller K. Granulocyte-macrophage-colony-stimulating factor and other cytokines: as adjuncts to cancer immunotherapy, stem cell transplantation, and vaccines. *Curr Hematol Rep*. 2004;3 (6):424–431.
17. van Horssen R, Ten Hagen TL, Eggermont AM. TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. *Oncologist*. 2006;11(4):397–408. doi:10.1634/theoncologist.11-4-397
18. von Karstedt S, Montinaro A, Walczak H. Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat Rev Cancer*. 2017;17(6):352–366.
19. Chaudhry GE, Jan R, Naveed Zafar M, Mohammad H, Muhammad TST. Vitex Rotundifolia Fractions Induced Apoptosis in Human Breast Cancer T-47D Cell Line via Activation of Extrinsic and Intrinsic Pathway. *Asian Pac J Cancer Prev*. 2019;20 (12):3555–3562. doi:10.31557/APJCP.2019.20.12.3555
20. Brune JC, Tormin A, Johansson MC, et al. Mesenchymal stromal cells from primary osteosarcoma are non-malignant and strikingly similar to their bone marrow counterparts. *Int J Cancer*. 2011;129 (2):319–330. doi:10.1002/ijc.25697
21. Le Nail LR, Brennan M, Rosset P, et al. Comparison of Tumor- and Bone Marrow-Derived Mesenchymal Stromal/Stem Cells from Patients with High-Grade Osteosarcoma. *Int J Mol Sci*. 2018;19 (3):707. doi:10.3390/ijms19030707
22. Skoda J, Nunukova A, Loja T, et al. Cancer stem cell markers in pediatric sarcomas: sox2 is associated with tumorigenicity in immunodeficient mice. *Tumour Biol*. 2016;37(7):9535–9548. doi:10.1007/s13277-016-4837-0
23. Adhikari AS, Agarwal N, Wood BM, et al. CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. *Cancer Res*. 2010;70(11):4602–4612. doi:10.1158/0008-5472.CAN-09-3463
24. Menendez ST, Rey V, Martinez-Cruzado L, et al. SOX2 Expression and Transcriptional Activity Identifies a Subpopulation of Cancer Stem Cells in Sarcoma with Prognostic Implications. *Cancers*. 2020;12(4):964. doi:10.3390/cancers12040964
25. Mahyudin F, Yazid H, Edward M, Basuki MH, Bari YA, Rantam FA. The enhancement apoptosis of osteosarcoma mesenchymal stem cells co-cultivation with peripheral blood mononuclear cells sensitized by secretome and granulocyte macrophage colony-stimulating factor. *J Adv Pharm Technol Res*. 2020;11:213–219. doi:10.4103/japtr. JAPTR_52_20
26. Sagaradze G, Grigorieva O, Nimiritsky P, et al. Conditioned Medium from Human Mesenchymal Stromal Cells: towards the Clinical Translation. *Int J Mol Sci*. 2019;20(7):1656. doi:10.3390/ijms20071656
27. Anderson J, Toh ZQ, Reitsma A, Do LAH, Nathanielsz J, Licciardi PV. Effect of peripheral blood mononuclear cell cryopreservation on innate and adaptive immune responses. *J Immunol Methods*. 2019;465:61–66. doi:10.1016/j.jim.2018.11.006
28. Maumus M, Jorgensen C, Noël D. Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: role of secretome and exosomes. *Biochimie*. 2013;95(12):2229–2234. doi:10.1016/j.biochi.2013.04.017
29. Shapouri-Moghaddam A, Mohammadian S, Vazini H, et al. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol*. 2018;233(9):6425–6440. doi:10.1002/jcp.26429

30. Secchiero P, Rimondi E, Agnoletto C. C-Reactive protein downregulates TRAIL expression in human peripheral monocytes via an Egr-1-dependent pathway. *Clin Cancer Res*. 2013;19(8):1949–1959. doi:10.1158/1078-0432.CCR-12-3027

31. Kamohara H, Matsuyama W, Shimozato O, Abe K, Galligan C, Yoshimura T. Regulation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and TRAIL receptor expression in human neutrophils. *Immunology*. 2004;111(2):186–194. doi:10.1111/j.0019-2805.2003.01794.x

32. Munoz LE, Franz S, Pausch F. The influence on the immunomodulatory effects of dying and dead cells of Annexin V. *J Leukoc Biol*. 2007;81(1):6–14. doi:10.1189/jlb.0306166

33. Zhou D, Huang C, Lin Z, et al. Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. *Cell Signal*. 2014;26(2):192–197. doi:10.1016/j.cellsig.2013.11.004

34. Fritsch M, Günther SD, Schwarzer R, et al. Caspase-8 is the molecular switch for apoptosis, necroptosis and pyroptosis. *Nature*. 2019;575(7784):683–687. doi:10.1038/s41586-019-1770-6

35. Wang W, Zhu M, Xu Z, et al. Ropivacaine promotes apoptosis of hepatocellular carcinoma cells through damaging mitochondria and activating caspase-3 activity. *Biol Res*. 2019;52(1):36. doi:10.1186/s40659-019-0242-7

36. Yang Q, Li S, Fu Z, et al. Shikonin promotes adriamycin-induced apoptosis by upregulating caspase-3 and caspase-8 in osteosarcoma. *Mol Med Rep*. 2017;16(2):1347–1352. doi:10.3892/mmr.2017.6729

Efficacy of rFIXFc versus rIX-FP for the Treatment of Patients with Hemophilia B: Matching-Adjusted Indirect Comparison of B-LONG and PROLONG-9FP Trials

Jan Astermark, Piotr Wojciechowski, Samuel Aballéa, Zalmai Hakimi, Jameel Nazir & Robert Klamroth

ORIGINAL RESEARCH

Efficacy of rFIXFc versus rIX-FP for the Treatment of Patients with Hemophilia B: Matching-Adjusted Indirect Comparison of B-LONG and PROLONG-9FP Trials

Jan Astermark[1]
Piotr Wojciechowski ⓘ[2]
Samuel Aballéa[3]
Zalmai Hakimi[4]
Jameel Nazir[4]
Robert Klamroth ⓘ[5]

[1]Department of Translational Medicine, Lund University, and Department of Hematology, Oncology and Radiation Physics, Skåne University Hospital, Malmö, Sweden; [2]Creativ-Ceutical, Krakow, Poland; [3]Creativ-Ceutical, Rotterdam, the Netherlands; [4]Swedish Orphan Biovitrum AB, Stockholm, Sweden; [5]Department of Internal Medicine, Hemophilia Treatment Centre, Vivantes Klinikum im Friedrichshain, Berlin, Germany

Purpose: In patients with hemophilia B, treatment with extended half-life (EHL) recombinant factor IX allows for longer dosing intervals while providing equal or superior bleeding protection compared with standard half-life products. This enables flexible, individualized treatment schedules, which reduce the burden of prophylaxis and improve patient outcomes. This analysis compared the efficacy of recombinant factor IX Fc fusion protein (rFIXFc) and recombinant factor IX albumin fusion protein (rIX-FP), two EHL therapies approved for prophylaxis and treatment of bleeding in hemophilia B.

Patients and Methods: Matching-adjusted indirect treatment comparison (MAIC) was used to adjust the between-treatment differences in baseline characteristics. Individual patient data for rFIXFc (B-LONG) were matched to aggregated data for rIX-FP (PROLONG-9FP) followed by statistical comparison for estimated annualized bleeding rate (ABR) using a Poisson regression model with adjustment for over dispersion. Data were analyzed according to treatment regimen prior to study entry: prior prophylaxis (rFIXFc, n=48; rIX-FP, n=40) or prior episodic treatment (n=43 and n=19, respectively). Relative treatment effects are presented as incidence rate ratios (IRR) with 95% confidence intervals (CI).

Results: After adjustment for baseline characteristics, estimated ABR observed for rFIXFc and rIX-FP was not significantly different in patients on prior prophylaxis (1.87 versus 1.58; IRR 1.18, 95% CI 0.67–2.10) or prior episodic (2.25 versus 2.22; IRR 1.01 95% CI 0.40–2.57) regimens.

Conclusion: This MAIC analysis shows that the estimated ABR for rFIXFc-treated patients from B-LONG was similar to that of rIX-FP-treated patients from PROLONG-9FP and, therefore, indicates that the two EHL therapies provide similar efficacy when used as prophylaxis for patients with hemophilia B. Trough levels differ between the two products (1–3% [targeted] versus 20% [observed], respectively), suggesting that trough level is not a surrogate indicator when ABR is used as a criterion for clinical efficacy when comparing these FIX products in hemophilia B.

Keywords: annualized bleeding rate, comparative effectiveness research, factor IX deficiency, factor IX Fc fusion protein, rIX-FP fusion protein, treatment outcome

Introduction

Routine prophylaxis is the optimal standard of care for the management of patients with severe hemophilia B. Prophylaxis with recombinant factor IX (rFIX), especially when initiated early in life, has been shown to result in favorable clinical outcomes.[1] Compared with on-demand therapy, regular

Correspondence: Robert Klamroth
Email robert.klamroth@vivantes.de

613

prophylaxis provides a consistent decrease in both total bleeds and hemarthrosis (joint bleeds), reducing joint deterioration (arthropathy) and consequently improving patients' quality of life.[1] Despite these benefits, the relatively short half-life of standard rFIX products typically necessitates frequent intravenous dosing, at least twice weekly for severe hemophilia B, which represents a substantial burden to patients and their caregivers and families.[2] Extended half-life (EHL) rFIX products provide a mean half-life extension 3–5 times that of standard products, which allows for longer dosing intervals while providing equal or superior bleeding protection to standard care.[2] This enables the use of flexible treatment schedules to meet individual patient needs, decreases the burden of prophylaxis, and potentially further improves patient outcomes.[3]

The aim of treating hemophilia is to prevent joint bleeds and, ultimately, arthropathy.[4] In hemophilia B, a trough level that prevents all bleeding has not been established and is likely to vary from patient to patient based on a number of factors, including joint status and age.[5] Correlation between time spent under target trough levels and bleed rates, including spontaneous, traumatic and joint bleeds, has been demonstrated in patients with severe hemophilia B treated with recombinant FIXFc fusion protein (rFIXFc); as trough level increased, the predicted bleed rate reduced and the predicted probability of being bleed-free improved.[6] However, other contributors to the variation in hemostatic and clinical outcomes and response to treatment should be considered when determining the most appropriate prophylaxis regimen,[7] including the pharmacokinetic (PK) characteristics of the replacement factor and the patient's individual PK profile.[3] Ideally, the regimen should be individualized, taking the patient's lifestyle into account, in order to reduce the known heterogeneity in bleeding patterns.[3] Furthermore, it is important to remember that, as FIX can enter the extravascular space, trough levels may not reflect FIX tissue levels and, consequently, FIX bleeding prevention ability.[8]

rFIXFc is an EHL replacement therapy indicated for the treatment and prophylaxis of bleeding episodes, including perioperative management of bleeding, in patients with hemophilia B of all ages.[9,10] Approval was based on two Phase 3 studies assessing the efficacy and safety of rFIXFc in previously treated pediatric (<12 years; Kids B-LONG)[11] or adolescent/adult (≥12 years; B-LONG)[12] patients with hemophilia B.

In both Kids B-LONG and B-LONG, rFIXFc prophylaxis, adjusted to maintain a trough level of 1–3 IU/dL, resulted in low annualized bleeding rates (ABRs).[11,12] These results were confirmed in the long-term extension study (B-YOND); in the majority of patients, low ABRs were maintained and extended dosing intervals were sustained for up to 5 years, with a cumulative duration up to 6.5 years.[13] Furthermore, a post hoc interim analysis of data from patients (≥12 years) who received rFIXFc prophylaxis with a ≥14-day dosing interval at any time during B-LONG or B-YOND showed that most patients remained well controlled, with ABRs consistent with those observed in the overall study population.[14] Therefore, a dosing interval of ≥14 days is an option for some patients,[10] offering a broader flexibility of dosing interval and further reducing treatment burden for patients while maintaining bleed protection.

Recombinant FIX albumin fusion protein (rIX-FP) is also indicated for the treatment and prophylaxis of bleeding episodes, including perioperative management of bleeding, in patients of all ages with hemophilia B.[15,16] Prophylaxis with rIX-FP has been shown to be effective for bleed prevention and treatment in both pediatric and adolescent/adult patients with hemophilia B, with weekly dosing intervals in children and up to 14-day dosing in adolescents/adults (PROLONG-9FP).[17,18] These results were confirmed in long-term extension studies, which also showed that adequate bleed protection could be achieved with extended dosing intervals of 10 or 14 days and 21 days in selected pediatric and adolescent/adult patients, respectively.[19,20]

Both rFIXFc and rIX-FP have been shown to be effective for the prevention and treatment of bleeds in patients with hemophilia. However, there are no direct comparative studies and in the absence of head-to-head trials an indirect comparison can be made using established methods, such as network meta-analysis[21] or matching-adjusted indirect comparison (MAIC).[22] MAIC is a widely used, validated method for the comparison of outcomes when there is no common comparator or the comparative studies are not sufficiently homogenous. MAIC matches patient-level data from clinical trials of one treatment with published aggregate data from clinical trials of another treatment, thus reducing observed differences between the trials and providing a balanced patient population for comparison.[22] The aim of this analysis was to apply MAIC to compare the efficacy of rFIXFc and rIXFP, two EHL therapies approved for prophylactic treatment of patients with hemophilia B.

Methods

Data Sources and Sample Selection

Source data for the MAIC analysis were extracted from the pivotal phase 3 trials (B-LONG for rFIXFc and PROLONG-9FP for rIX-FP), which provided efficacy and safety data for market authorization. Comparisons were based on the approved dosing regimens for each product (Table 1). The study design and results of these trials have been described in detail elsewhere.[12,17] Briefly, both were non-randomized, open-label studies in previously treated male adolescent/adult patients (≥12 years) with severe hemophilia B. In B-LONG, patients (N=119) were treated with one of four rFIXFc regimens: weekly dose-adjusted prophylaxis (group 1: starting at 50 IU/kg; n=63), interval-adjusted prophylaxis (group 2: starting at 100 IU/kg every 10 days; n=29), on-demand treatment as needed for bleeding episodes and dose adjusted according to bleeding severity (group 3: 20–100 IU/kg, n=27) and treatment for perioperative care (group 4).[12]

In PROLONG-9FP, patients (N=63) were treated with one of two rIX-FP regimens.[17] Group 1 (n=40) received 35–50 IU/kg once weekly during the first 26 weeks and were then allowed to switch to 75 IU/kg every 10 or 14 days if they had no spontaneous bleeds for ≥4 weeks before switching and were receiving ≤40 IU/kg or ≤50 IU/kg rIX-FP in order to switch to the 14- or 10-day interval, respectively. Group 2 (n=19) received on-demand treatment during the first 26 weeks followed by a fixed dose of 35–50 IU/kg once weekly for ≥26 weeks as determined by the physician.

Several differences between the two trials are worth noting. In B-LONG, but not PROLONG-9FP, patients entering the prophylaxis treatment arms could have been on a previous on-demand regimen; 27.5% of the prophylaxis group in PROLONG-9FP had received previous prophylaxis with rIX-FP before study entry.[12,17] In groups 1 and 2 of B-LONG, dose and interval, respectively, were adjusted to maintain a plasma trough level of 1–3 IU/dL or higher in participants who had two breakthrough spontaneous bleeding episodes in a rolling 3-month period. In PROLONG-9FP, the dose could be increased or decreased based on assessment of bleeding phenotype by the treating physician, level of physical activity, or clinical outcome.[20] A mean trough FIX activity of 20 and 12 IU/dL was maintained with rIX-FP prophylaxis using 40 IU/kg weekly and 75 IU/kg every 2 weeks, respectively. Lastly, in PROLONG-9FP, after 26 weeks of treatment, patients receiving weekly prophylaxis were evaluated for their eligibility to switch to a longer treatment interval (10 or 14 days) and all patients in the on-demand group switched to weekly prophylaxis for at least an additional 26 weeks.[17] In B-LONG, patients received the regimens assigned at enrolment throughout the entire study period.[12]

Outcome Assessment

The efficacy outcome assessed was mean ABR, a clinically relevant treatment outcome typically evaluated in clinical studies of hemophilia. Median duration of treatment was 51.6 and 58.3 weeks in the dose- and interval-adjusted groups of B-LONG, respectively. In PROLONG-9FP, median duration of treatment was 34.3–55.1 weeks in group 1 and in group 2 was 26.7 weeks during the first 26 weeks of on-demand treatment and 45.1 weeks after switching to a weekly prophylaxis regimen for the remaining time on study.

Table 1 Approved Dosing Regimens for Long-Term Prophylaxis with rFIXFc or rIX-FP in Adolescent/Adult Patients (≥12 Years) with Hemophilia B

Prophylaxis Regimen	rFIXFc	rIX-FP
Weekly dose-adjusted	50 IU/kg once weekly to start[9,10] Adjust dose based on individual response	35–50 IU/kg once weekly;[16] 25–40 IU/kg every 7 days[15]
Interval-adjusted	100 IU/kg once every 10 days to start[9,10] Adjust interval based on individual response Patients who are well controlled on a once every 10 days regimen may be treated on an interval of 14 days or longer	Up to 75 IU/kg once every 10 or 14 days[16] For patients >18 years, further extension of the treatment interval may be considered Patients well controlled on an every 7 day regimen may be switched to a 14-day interval at 50–75 IU/kg[15]

Abbreviations: rIX-FP, rFIX albumin fusion protein; rFIXFc, recombinant factor IX-Fc fusion protein.

Data Analysis

MAIC methodology was used to compare estimated ABRs for rFIXFc and rIX-FP, according to recommendations from the National Institute for Health and Clinical Excellence Decision Support Unit (NICE DSU).[23] Individual patient data from the B-LONG study were weighted to match the mean baseline characteristics reported for patients in the PROLONG-9FP study, with regard to age (mean, standard deviation [SD]), body weight (mean, SD), ethnicity (proportion of white patients) and mean (SD) number of bleeding events prior to study enrollment. The characteristics selected were the only variables available for comparison due to limited reporting of baseline characteristics across the studies. Matching between two populations with different baseline characteristics always results in a loss of information. The size of the population after matching can be estimated according to the principle provided by the NICE DSU.[23] After weighting, the adjusted baseline characteristics of B-LONG participants were the same as those in the population of the PROLONG-9FP study. Adjusted ABRs for rFIXFc were estimated using a Poisson regression model with adjustment for overdispersion and weights calculated through MAIC. Adjusted ABRs can be interpreted as estimates of bleeding frequency if rFIXFc were administered to patients with similar baseline characteristics to those patients recruited in the PROLONG-9FP trial. Finally, estimated ABRs for rFIXFc were compared with the results reported for rIX-FP in the PROLONG-9FP study, and relative treatment effects were presented as incidence rate ratios (IRR) with 95% confidence intervals (CIs). Statistical comparisons were conducted in R v.3.5.5 [https://www.r-project.org/]).

Results

Baseline Characteristics Before Matching

In the current analysis, baseline characteristic data for rFIXFc were extracted from the weekly dose-adjusted (n=63) and interval-adjusted prophylaxis groups (n=29), while those for rIX-FP, which were not reported separately for each arm, were taken from the overall patient population (N=59; 4 of the 23 patients in group 2 received on-demand treatment only and were excluded from the analysis). Data were analyzed according to treatment regimen prior to study entry: prior prophylaxis (rFIXFc, n=48; rIX-FP, n=40) or prior on-demand treatment (n=43 and n=19, respectively).

Patient baseline characteristics from the B-LONG and PROLONG-9FP studies are summarized in Table 2. In the weekly dose-adjusted and interval-adjusted prophylaxis groups of B-LONG, the median age was 28 and 33 years, respectively. The treatment regimen prior to study entry was prophylaxis in 53.2% and 51.7% of patients, respectively, and the proportion of white patients was 65.1% and 62.1%. Furthermore, at baseline, endogenous FIX levels were <1 IU/dL in 79.4% and 75.9% of patients and 1–2 IU/dL in 20.6% and 24.1% of patients, in the weekly dose-adjusted and interval-adjusted prophylaxis groups of B-LONG, respectively.[12] Median duration of treatment was 51.6 and 58.3 weeks in the weekly dose-adjusted and interval-adjusted prophylaxis arm, respectively, and the median weekly dose for patients receiving weekly prophylaxis was 45 IU/kg. In the prophylaxis group of PROLONG-9FP, the mean age was 31.6 years, and the proportion of white patients was 82.5%. In the on-demand group, the mean age was 35.3 years, and the proportion of white patients was 82.6%. Endogenous FIX levels were ≤1 IU/dL in 87.5% and 87.0% of patients assigned to the prophylaxis or on-demand groups, respectively, and ≤2 IU/dL in 100% of patients.[17] Median duration of treatment was 34.3–55.1 weeks and median consumption was 162.3 and 194.7 IU/kg per month in patients receiving 14-day and 7-day prophylaxis, respectively.

Matching of Baseline Characteristics

Individual patient data from the weekly dose-adjusted and interval-adjusted prophylaxis groups of B-LONG were matched to aggregated data for the participants of PROLONG-9FP regarding baseline age, weight, prior bleeding frequency and ethnicity (Tables 3 and 4). Matching was conducted separately in subsets of patients, who were receiving either prophylaxis or on-demand regimens prior to study enrollment. After matching, the populations of both studies were well balanced regarding all baseline variables and the estimated effective sample size for B-LONG was 26 (63%) for patients who received prior prophylaxis (Table 3) and 10 (26%) for patients who received prior on-demand (Table 4).

Annualized Bleeding Rate

After adjusting for age, weight, prior bleeds, and ethnicity, estimated ABR in patients who received prior prophylaxis was 1.87 for rFIXFc and 1.58 for rIX-FP. The difference

Table 2 Baseline Characteristics in the B-LONG[12] and PROLONG-9FP[17] Phase 3 Studies

	B-LONG		PROLONG-9FP	
	Weekly Dose-Adjusted Prophylaxis (n=63)	Interval-Adjusted Prophylaxis (n=29)	Prophylaxis (n=40)	On-Demand (n=23)
Age, years	28 (12–71)[a]	33 (12–62)[a]	31.6 (15.2)[b]	35.3 (11.1)[b]
Weight, kg	70.2 (45.2–186.7)[a]	76.0 (50.0–128.0)[a]	69.6 (14.4)[b]	75.1 (20.7)[b]
Ethnicity, n (%)				
White	41 (65.1)	18 (62.1)	33 (82.5)	19 (82.6)
Asian	7 (11.1)	2 (6.9)	6 (15.0)	4 (17.4)
Black/African American	7 (11.1)	7 (24.1)	1 (2.5)	0
Other	8 (12.7)	2 (6.9)	–	–
Endogenous FIX level, n (%)				
<1 IU/dL	50 (79.4)	22 (75.9)		
≤1 IU/dL	–	–	35 (87.5)	20 (87.0)
1–2 IU/dL	13 (20.6)	7 (24.1)	–	–
≤2 IU/dL	–	–	40 (100)	23 (100)
Pre-study FIX therapy, n (%)				
Prophylaxis	33 (53.2)	15 (51.7)	40 (100)	0
On-demand	29 (46.8)	14 (48.3)	0	23 (100)
Bleeding episodes in prior 12 months				
Prior prophylaxis	2.5 (0–21)[a]	2.0 (0–7)[a]	2.0 (0–4.5)[a, c]	–
Prior on-demand	23.0 (6–70)[a]	25.0 (10–100)[a]	–	23.5 (22–28)[a, d]

Notes: [a]Median (range); [b]Mean (SD); [c]Patients assigned to the prophylaxis arm of PROLONG-9FP; [d]Patients assigned to the on-demand arm of PROLONG-9FP, only patients who were previously receiving on-demand treatment were eligible for this group.
Abbreviations: FIX, factor IX; SD, standard deviation.

between the two treatment groups was not statistically significant (IRR 1.18; 95% CI 0.67–2.10; Figure 1).

Similarly, after adjustment, the difference in estimated ABR in patients who received prior on-demand was not statistically significant between rFIXFc and rIX-FP (2.25 versus 2.22, respectively; IRR 1.01, 95% CI 0.40–2.57; Figure 1).

Discussion

The results of this MAIC analysis show that the estimated ABR for rFIXFc-treated patients from B-LONG was similar to that of rIX-FP-treated patients from PROLONG-9FP and, therefore, provides evidence of similar bleed protection between the two EHL FIX products in patients with hemophilia B. This is despite the large difference between target trough levels for rFIXFc (1–3 IU/kg)[12] and measured trough levels with rIX-FP (20.0 IU/dL at day 7 during once weekly prophylaxis with 40 IU/kg; 12.4 IU/kg at day 14

during 14-day prophylaxis with 75 IU/kg).[17] Higher FIX trough levels may be needed for rIX-FP to achieve similar protection from bleeds as rFIXFc at target trough levels of 1–3 IU/kg, which might be attributed to differences in their physiological (PK/pharmacodynamic) profiles, including extravascular distribution. However, it should be noted that the relationship between targeted and measured trough levels and clinical outcomes cannot be directly compared, owing to differences in the PK characteristics of the two products.

EHL FIX products, such as rFIXFc and rIX-FP, facilitate the use of prophylactic treatment in patients with hemophilia B, by maintaining or improving bleed protection while reducing the injection frequency, versus standard half-life products.[2] Therefore, they have the potential to decrease the burden of prophylaxis, improve health outcomes, and allow for a more active lifestyle.[2,3] The standard trough level for bleed prevention is generally regarded as 1%, and is based on data, largely

Table 3 Balance of Baseline Characteristics and Effective Sample Size Following Matching of Patients on a Prior Prophylactic Regimen from the B-LONG Weekly Dose-Adjusted and Interval-Adjusted Prophylaxis Groups and Group 1 of PROLONG-9FP

	Prior to Matching		rFIXFc Adjusted Population	
	rIX-FP (n=40)	rFIXFc Unadjusted Population (n=45)[a]	Estimate (SD)	ESS, n (%)
Mean (SD) age, years	31.6 (15.2)	34.2 (14.8)	31.6 (15.2)	41.7 (92.6)
Mean (SD) weight, kg	69.6 (14.4)	76.8 (17.4)	69.6 (14.4)	37.4 (83.1)
Mean (SD) prior bleeds	3.4 (3.8)	4.8 (5.9)	3.4 (3.8)	37.9 (92.4)
Proportion of white patients, %	83	67	83	40.2 (89.3)
Final adjusted population				
Mean (SD) age, years	–	–	31.6 (15.2)	25.6 (62.6)
Mean (SD) weight, kg	–	–	69.6 (14.4)	
Mean (SD) prior bleeds	–	–	3.4 (3.8)	
Proportion of white patients, %	–	–	83	

Note: [a]Data missing for ABR and treatment duration (n=3).
Abbreviations: ABR, annualized bleeding rate; ESS, effective sample size; rIX-FP, rFIX albumin fusion protein; rFIXFc recombinant factor IX-Fc fusion protein; SD standard deviation.

Table 4 Balance of Baseline Characteristics and Effective Sample Size Following Matching of Patients on a Prior on-Demand Regimen from the B-LONG Weekly Dose-Adjusted and Interval-Adjusted Prophylaxis Groups and Group 2 of PROLONG-9FP

	Prior to Matching		rFIXFc Adjusted Population	
	rIX-FP (n=23)	rFIXFc Unadjusted Population (n=42)[a]	Estimate (SD)	ESS, n (%)
Mean (SD) age, years	35.3 (11.1)	31.3 (13.9)	35.3 (11.1)	32.0 (76.1)
Mean (SD) weight, kg	75.1 (20.7)	75.1 (24.3)	75.1 (20.7)	41.4 (98.5)
Mean (SD) prior bleeds	24.3 (7.3)	28.2 (18.3)	24.3 (7.3)	28.0 (71.7)
Proportion of white patients, %	83	57	83	33.2 (79.1)
Final adjusted population				
Mean (SD) age, years	–	–	35.3 (11.1)	10.2 (26.1)
Mean (SD) weight, kg	–	–	75.1 (20.7)	
Mean (SD) prior bleeds	–	–	24.3 (7.3)	
Proportion of white patients, %	–	–	83	

Note: [a]Data missing for ABR and treatment duration (n=2).
Abbreviations: ABR, annualized bleeding rate; ESS, effective sample size; rIX-FP, rFIX albumin fusion protein; rFIXFc, recombinant factor IX-Fc fusion protein; SD standard deviation.

from experience with hemophilia A, showing that patients with factor activity levels >1% tend to have fewer joint bleeds and less arthropathy during prophylaxis.[24] However, data have shown that some patients experience joint bleeds with trough levels >3%,[25] indicating that FIX levels should be determined on a person-to-person basis, and in consideration of the specific molecular characteristics of the FIX product being used.

Current guidelines provide broad recommendations for calculating dosing regimens;[26] however, they do not account for inter-patient variability in PK parameters, bleeding phenotype, levels of physical activity, lifestyle and joint status.[3] Importantly, although trough level can be used as a surrogate marker for monitoring the efficacy of a prophylaxis regimen, it cannot be compared across
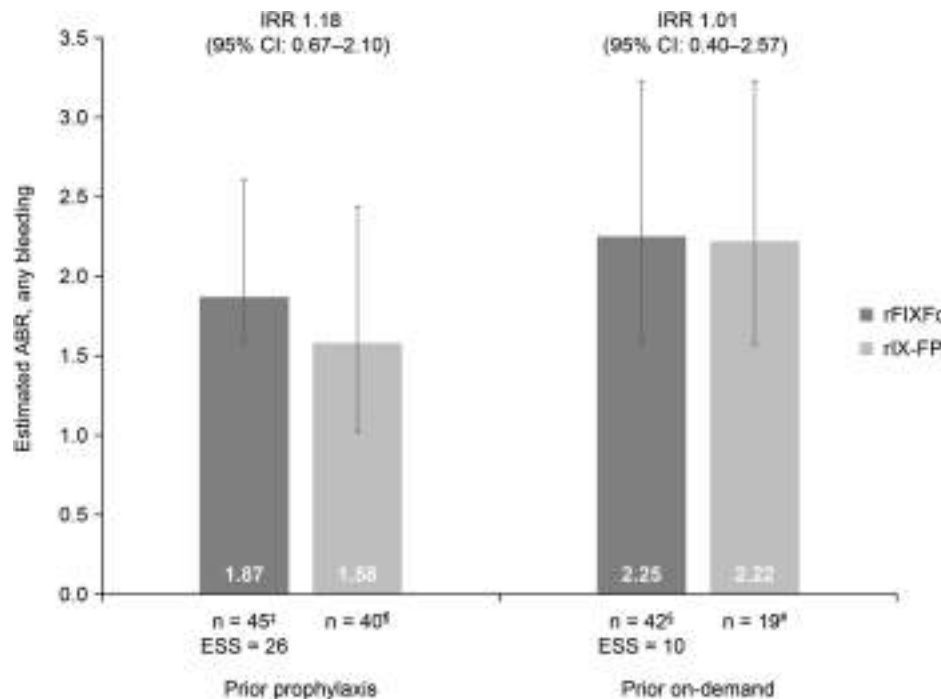
Figure 1 Estimated ABR, any bleeding, after matching for all selected baseline variables[†].
Notes: [†]Age, weight, prior bleeds and ethnicity (proportion of white patients); [‡]Data missing for ABR and treatment duration (n=3); [¶]Data from PROLONG-9FP; [§]Data missing for ABR and treatment duration (n=2); [#]Data from PROLONG-9FP, of the 23 patients receiving prior on-demand only 19 patients transitioned to prophylaxis.
Abbreviations: ABR, annualized bleeding rate; CI, confidence interval; ESS, effective sample size; IRR, incidence rate ratio; rIX-FP, rFIX albumin fusion protein; rFIXFc, recombinant factor IX-Fc fusion protein.

patients or products, especially products with different moieties/modifications to extend half-life. Other parameters, such as clinical outcomes (eg, bleed rates, assessment of joint health) should be considered, and therapy tailored to individual patient needs may be the best approach to achieve optimal bleed protection. In addition, long-term effects of these treatments should also be considered; in the rIX-FP extension study, the most frequently reported treatment-emergent adverse event was arthralgia (25 events in 19 [32.2%] patients).[20]

The study has the following limitations. Although MAIC is the recommended method, as it can account for population differences when comparing treatments assessed in disconnected studies, the method cannot adjust for all possible differences across trials, such as undocumented differences at baseline. Furthermore, the lack of randomization with a placebo arm in B-LONG and PROLONG-9FP hampers adjustments for residual confounding. Another limitation is that estimated sample sizes following assignment of weights were low, and, therefore, the amount of information for the comparison between rFIXFc and rIX-FP

was limited, which should be considered when interpreting the results.

Despite the limitations, this indirect treatment comparison provides a useful measure of relative efficacy between these two EHL FIX products for the treatment of patients with hemophilia B.

Conclusions

This MAIC analysis shows that the estimated ABR for rFIXFc-treated patients from B-LONG was similar to that of rIX-FP-treated patients from PROLONG-9FP and, therefore, provides no evidence of a difference in efficacy between rFIXFc and rIX-FP when used as prophylaxis in patients with hemophilia B. This is despite the large difference in trough levels between the two products (target [1–3 IU/dL] versus obtained [20 IU/dL], respectively), suggesting that trough level is not a surrogate indicator when ABR is used as a criterion for clinical efficacy when comparing these FIX products in hemophilia B.

Compliance with Ethics Guidelines

Ethical approval was not required for this analysis as it was based on data from two previously published Phase III trials

(B-LONG and PROLONG-9FP). Both the B-LONG and PROLONG-9FP studies were conducted in accordance with the Declaration of Helsinki and local regulations. The protocols were approved by the authorities and the institutional review board/ethics committee at each participating center, and signed informed consent was obtained from all patients. Informed consent for this analysis was not required given the de-identified nature of the B-LONG individualized patient-level data, and the use of aggregated, previously published data from PROLONG-9FP.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. The manuscript was reviewed by Sobi and Sanofi before publishing.

Disclosure

J. Astermark reports research support from Sobi, CSL Behring, Takeda/Shire and Bayer; honoraria for consulting from Octapharma, Novo Nordisk, Pfizer, Bayer, Sobi, CSL Behring, Takeda/Shire, BioMarin, uniQure and Spark Therapeutics; and speaker bureau fees from Octapharma, Novo Nordisk, Pfizer, Bayer, Sobi, CSL Behring, Takeda/ Shire and BioMarin. R. Klamroth: reports research funding and honoraria for consulting and lectures from Bayer, BioMarin, Biotest, CSL Behring, Novo Nordisk, Octapharma, Pfizer, Roche, Takeda/Shire and Sobi. Piotr Wojciechowski and S. Aballéa are employees of Creativ-Ceutical, a consultancy company that received funding from Sobi for this research. Z. Hakimi and J. Nazir are employees of Sobi. The authors report no other conflicts of interest in this work.

References

1. Iorio A, Marchesini E, Marcucci M, Stobart K, Chan AK. Clotting factor concentrates given to prevent bleeding and bleeding-related complications in people with hemophilia A or B. *Cochrane Database Syst Rev.* 2011;(9):CD003429.
2. Lambert T, Benson G, Dolan G, et al. Practical aspects of extended half-life products for the treatment of haemophilia. *Ther Adv Hematol.* 2018;9(9):295–308. doi:10.1177/2040620718796429
3. Castaman G. The benefits of prophylaxis in patients with hemophilia B. *Expert Rev Hematol.* 2018;11(8):673–683. doi:10.1080/17474086.2018.1489719
4. Jiménez-Yuste V, Auerswald G, Benson G, et al. Achieving and maintaining an optimal trough level for prophylaxis in haemophilia: the past, the present and the future. *Blood Transfusion.* 2014;12(3):314–319.
5. Walsh C, Coppens M, Escobar M, Wang M. Optimal trough levels in haemophilia B: raising expectations. *Haemophilia.* 2020;26(6):e334–e336. doi:10.1111/hae.14098
6. Shapiro A, Potts J, Li SV, et al. Association of bleeding tendency with time under target FIX activity levels in severe hemophilia B Patients treated with recombinant factor IX Fc fusion protein [abstract]. *Blood.* 2013;122(21):2349. doi:10.1182/blood.V122.21.2349.2349
7. Tjärnlund-Wolf A, Lassila R. Phenotypic characterization of haemophilia B - Understanding the underlying biology of coagulation factor IX. *Haemophilia.* 2019;25(4):567–574.
8. Iorio A, Fischer K, Blanchette V, et al. Tailoring treatment of haemophilia B: accounting for the distribution and clearance of standard and extended half-life FIX concentrates. *Thromb Haemost.* 2017;117 (6):1023–1030. doi:10.1160/TH16-12-0942
9. Sanofi Genzyme. Alprolix Prescribing Information; Published 2017. Available from: http://products.sanofi.us/Alprolix/alprolix.html. Accessed November 2020.
10. European Medicines Agency. Alprolix Summary of Product Characteristics; Published 2020. Available from: https://www.ema. europa.eu/en/documents/product-information/alprolix-epar-product-information_en.pdf. Accessed November 2020.
11. Fischer K, Kulkarni R, Nolan B, et al. Recombinant factor IX Fc fusion protein in children with haemophilia B (Kids B-LONG): results from a multicentre, non-randomised phase 3 study. *Lancet Haematol.* 2017;4(2):e75–e82. doi:10.1016/S2352-3026(16)30193-4
12. Powell JS, Pasi KJ, Ragni MV, et al. Phase 3 study of recombinant factor IX Fc fusion protein in hemophilia B. *N Engl J Med.* 2013;369 (24):2313–2323. doi:10.1056/NEJMoa1305074
13. Pasi KJ, Fischer K, Ragni M, et al. Long-term safety and sustained efficacy for up to 5 years of treatment with recombinant factor IX Fc fusion protein in subjects with haemophilia B: results from the B-YOND extension study. *Haemophilia.* 2020;26(6):e262–e271. doi:10.1111/hae.14036
14. Shapiro AD, Pasi KJ, Ozelo MC, et al. Extending recombinant factor IX Fc fusion protein dosing interval to 14 or more days in patients with hemophilia B. *Res Pract Thromb Haemost.* 2019;3(1):109–113. doi:10.1002/rth2.12163
15. CSL Behring. Idelvion Prescribing Information; Published 2020. Available from: https://www.ema.europa.eu/en/documents/product-information/idelvion-epar-product-information_en.pdf. Accessed November 2020.
16. European Medicines Agency. Idelvion Summary of Product Characteristics; Published 2020. Available from: https://www.ema. europa.eu/en/documents/product-information/idelvion-epar-product-information_en.pdf. Accessed November 2020.

17. Santagostino E, Martinowitz U, Lissitchkov T, et al. Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: results of a phase 3 trial. *Blood*. 2016;127 (14):1761–1769. doi:10.1182/blood-2015-09-669234

18. Kenet G, Chambost H, Male C, et al. Long-acting recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in children. Results of a phase 3 trial. *Thromb Haemost*. 2016;116 (4):659–668. doi:10.1160/TH16-03-0179

19. Kenet G, Chambost H, Male C, et al. Long-term safety and efficacy of recombinant coagulation factor ix albumin fusion protein (rIX-FP) in previously treated pediatric patients with hemophilia B: results from a Phase 3b extension study. *Thromb Haemost*. 2020;120 (4):599–606. doi:10.1055/s-0040-1705116

20. Mancuso ME, Lubetsky A, Pan-Petesch B, et al. Long-term safety and efficacy of rIX-FP prophylaxis with extended dosing intervals up to 21 days in adults/adolescents with hemophilia B. *J Thromb Haemost*. 2020;18(5):1065–1074. doi:10.1111/jth.14778

21. Chaimani A, Caldwell DM, Higgins JPT, et al. Chapter 11: undertaking network meta-analyses. In: Higgins JPT, Thomas J, Chandler J, editors. *Cochrane Handbook for Systematic Reviews of Interventions. Version 6.1 Ed*. Cochrane; 2020.

22. Signorovitch JE, Sikirica V, Erder MH, et al. Matching-adjusted indirect comparisons: a new tool for timely comparative effectiveness research. *Value Health*. 2012;15(6):940–947. doi:10.1016/j. jval.2012.05.004

23. NICE Decision Support Unit. NICE DSU Technical Support Document 18: methods for population-adjusted indirect comparisons in submissions to NICE; Published 2016. Available from: http:// nicedsu.org.uk/technical-support-documents/population-adjusted-indirect-comparisons-maic-and-stc/. Accessed November 2020.

24. Ahlberg A. Haemophilia in Sweden. VII. Incidence, treatment and prophylaxis of arthropathy and other musculo-skeletal manifestations of haemophilia A and B. *Acta Orthop Scand Suppl*. 1965;Suppl 77:73–132.

25. Ahnstrom J, Berntorp E, Lindvall K, Bjorkman S. A 6-year follow-up of dosing, coagulation factor levels and bleedings in relation to joint status in the prophylactic treatment of haemophilia. *Haemophilia*. 2004;10(6):689–697. doi:10.1111/j.1365-2516.2004.01036.x

26. Srivastava A, Santagostino E, Dougall A, et al. WFH Guidelines for the Management of Hemophilia, 3rd edition. *Haemophilia*. 2020;26 (Suppl 6):1–158. doi:10.1111/hae.14046

Leukapheresis Does Not Improve Early Survival Outcome of Acute Myeloid Leukemia with Leukostasis Patients – A Dual-Center Retrospective Cohort Study

Ikhwan Rinaldi, Resti Mulya Sari, Vanya Utami Tedhy & Kevin Winston

Published online: 14 Jul 2021.

Submit your article to this journal

Article views: 303

View related articles

View Crossmark data

Citing articles: 15 View citing articles

ORIGINAL RESEARCH

Leukapheresis Does Not Improve Early Survival Outcome of Acute Myeloid Leukemia with Leukostasis Patients – A Dual-Center Retrospective Cohort Study

Ikhwan Rinaldi[1]
Resti Mulya Sari[2]
Vanya Utami Tedhy[3]
Kevin Winston [iD][3]

[1]Division of Hematology and Medical Oncology, Department of Internal Medicine, Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [2]Division of Hematology and Medical Oncology, Department of Internal Medicine, Dharmais National Cancer Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia; [3]Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Introduction: Leukostasis is a medical emergency with high mortality which often occurs in acute myeloid leukemia patients with hyperleukocytosis. One of the therapies that can be used for leukostasis in acute myeloid leukemia is leukapheresis. However, whether leukapheresis can provide better survival benefit when compared with patients not receiving leukapheresis is still unclear. Hence, we aimed to evaluate the effect of chemotherapy plus leukapheresis combination versus chemotherapy only on 28-day survival of acute myeloid leukemia patients with leukostasis.

Methods: This study was a dual-center retrospective cohort using secondary data from medical records collected from November 2018 to March 2019. Inclusion criteria were adult patients aged 18 years old or above, diagnosed with acute leukemia with hyperleukocytosis status defined by WBC count greater than 100,000/uL, and with symptoms of leukostasis. One-month survival analysis was conducted using Kaplan–Meier curve method. Univariate and multivariate analyses were then conducted using Cox proportional hazards model to obtain value of hazard ratio (HR) with a 95% confidence interval (CI).

Results: A total of 38 patients were obtained for analysis. The median overall survival was 25 days (95% CI: 17.001–32.999 days) in the chemotherapy only group and 20 days (95% CI: 1.497–38.503) in the chemotherapy with leukapheresis group. The use of leukapheresis did not affect 28-day survival (HR: 1.140; 95% CI: 0.396–3.283; p value: 0.809) and 7-day survival (HR: 1.073; 95% CI: 0.277–4.152; p value: 0.919). In the multivariate analysis, age ≥60 years, blast percentage ≥90%, creatinine ≥1.4 mg/dL, and presence of disseminated intravascular coagulation were associated with worse 28-day survival.

Conclusion: AML patients with leukostasis who received both chemotherapy and leukapheresis did not have better 28-day survival and 7-day survival when compared with patients receiving chemotherapy only. Old age, high blast percentage, high creatinine, and presence of disseminated intravascular coagulation were prognostic factors for worse 28-day survival.

Keywords: leukemia, hyperleukocytosis, leukostasis, leukapheresis, chemotherapy, survival

Introduction

Leukostasis is a medical emergency which can occur in acute leukemia due to obstruction of small blood vessels by malignant blast cells which result in tissue and organ ischemia with high potential for mortality and morbidity.[1–3] Diagnosis of leukostasis is clinically based on manifestations arising from tissue hypoxia in the target organs such as respiratory distress, impaired kidney function, central nervous system disorders, and coagulopathy, after excluding other possible etiologies.[2,3]

Correspondence: Ikhwan Rinaldi
Division of Hematology and Medical Oncology, Department of Internal Medicine, Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
Tel +628111001758
Email ikhwan.rinaldi@ui.ac.id

623

Leukostasis generally starts to occur in conditions such as hyperleukocytosis where the leucocyte count is above 100,000 cells/μL in acute myeloid leukemia (AML) and in range of around 200,000/μL to 400,000/μL in acute lymphoblastic leukemia (ALL).[4–6] The difference in the cut-off numbers of leukocytes between AML and ALL is due to the fact that blast cells from myeloid progenitor have bigger size and lower deformability than blast cells from lymphoid progenitor.[3] However, leukostasis may also occur in leucocyte count below 100,000 cells/μL.[3]

The mortality from leukostasis in acute leukemia reaches about 40%, which usually occurs within a few weeks of diagnosis.[3] Hence, prompt treatment of acute leukemia patients with leukostasis is important to reduce mortality and prevent complications. The aim of leukostasis therapy is to reduce the number of blast cells, which can be achieved by using either chemotherapy with or without leukapheresis.[2,7] Chemotherapy works by causing blast cells to undergo apoptosis in infiltrated organs, peripheral circulation, and bone marrow, thereby reducing the total burden of leukocytes and resolving leukostasis.[3] The type of chemotherapy given is specific according to the type of leukemia, hence the administration is usually postponed until the type of leukemia is confirmed. One of the chemotherapies that can be used is hydroxyurea.[3] However, the issue with chemotherapy in acute leukemia patients with leukostasis is the potential risk of tumor lysis syndrome (TLS) after chemotherapy due to large number of blast cells undergoing lysis simultaneously.[3,8,9] Additionally, not all patients are clinically suitable to receive chemotherapy, such as due to old age or frailty.

Another treatment modality for leukostasis is leukapheresis, in which the blast cells from the circulation are removed through a filter of the leukapheresis machine.[10] Leukapheresis can reduce the number of blast cells faster but the procedure is associated with the risk of blast cells' rebound because the removal of blast cells is not accompanied by destruction of blast cells in the bone marrow which can quickly replace the removed blast cells in peripheral circulation.[11,12] Other disadvantages of leukapheresis procedure are the expensive cost, the requirement of the availability of special facilities, insertion of central venous catheter, and the need for experienced staff.[2,13] The main advantage of leukapheresis is being able to overcome metabolic disorders and coagulation via plasma and electrolyte administration during the procedure.[2,13]

Whether adding leukapheresis treatment in conjunction with chemotherapy can produce mortality reduction in acute leukemia patients with leukostasis is currently still unclear and contradictory in several studies.[3,10,12–18] For example, there were several studies supporting the use of leukapheresis such as a study conducted in 2017 by Nan et al, which observed that the use of leukapheresis decreased 28-day-mortality rate in AML patients with hyperleukocytosis when compared with patients not receiving leukapheresis (30.8% vs 57.7%, p: 0.022).[19] Another study by Bug et al also stated that leukapheresis improved survival in the first 3 weeks of AML patients (16% vs 32%, p: 0.015).[16] Meanwhile other studies found no benefit of early mortality from the use of leukapheresis such as the study by Malkan et al, Porcu et al, Giles et al, and Stahl et al.[13,15,20,21]

The difference between the results of various studies indicates that there is still unclear evidence that supports the use of leukapheresis in reducing mortality of acute leukemia patients with leukostasis. Additionally, many studies were different in the measured survival endpoint. Therefore, this research was conducted with the aim to analyze and compare 7-day survival, 14-day survival, 21-day survival, and 28-day survival outcomes of acute myeloid leukemia patients with leukostasis who received leukapheresis compared to those who did not receive it.

Methods
Study Design
This was a dual-center retrospective cohort study using secondary data from medical records to compare 7-day, 14-day, 21-day, and 28-day survival outcome of acute myeloid leukemia patients with leukostasis based on the type of treatments received. The treatments were divided into leukapheresis plus chemotherapy group and chemotherapy only group.

The research was conducted at Cipto Mangunkusumo National General Hospital and Dharmais National Cancer Hospital using medical record data of inpatient acute myeloid leukemia patients with leukostasis during 2007–2018. Both hospitals are tertiary hospital and national referral hospital in Indonesia. Medical record data were searched and gathered by the authors starting from November 2018 to March 2019.

Patients
The target population was Indonesian acute myeloid leukemia patients who underwent treatment at Cipto Mangunkusumo National General Hospital and Dharmais National Cancer Hospital during 2007–2018. Inclusion

criteria used were adult patients aged 18 years old or above suffering from acute myeloid leukemia with hyperleukocytosis of WBC count greater than 100,000/uL, with symptoms of leukostasis. Exclusion criteria for the study were incomplete medical records, acute leukemia type other than AML, AML without hyperleukocytosis, AML without leukostasis, palliative leukemia patients, and refractory leukemia patients.

The type of leukemia was diagnosed from examination of bone marrow biopsy. Hyperleukocytosis status was diagnosed from blood leukocyte count. Leukostasis status was diagnosed clinically by the attending physicians in our centers based on the clinical manifestations associated with organ ischemia in patients with hyperleukocytosis. Patients suspected of having leukostasis were also given a series of diagnostic tests such as chest X-ray, blood cultures, brain imaging, cerebrospinal fluid analysis, liver enzymes, lactic dehydrogenase, and coagulation tests to exclude other etiologies before making leukostasis diagnosis. In this study, the chemotherapy used for AML patients consisted of either hydroxyurea or cytarabine.

Demographic and clinical data of patients were taken from medical records for baseline characteristics. Baseline characteristics presented in this study included age, gender, BMI, clinical manifestations type of leukostasis, presence of chronic comorbidities, and hematological parameters. Hematological parameters taken included hemoglobin, leukocytes, platelet count, creatinine, and blast percentage. Chronic comorbidities included tuberculosis infection, chronic liver disease, diabetes mellitus, history of stroke, chronic heart failure, hypertension, and chronic kidney disease. Tumor lysis syndrome events and disseminated intravascular coagulation events prior to treatments were also recorded.

Procedure of Leukapheresis
Patients were first informed about leukapheresis procedure and possible adverse effects that may arise from the procedure. The patients were then given time to ask questions or reconsider his or her decision of undergoing leukapheresis. Written informed consent was then obtained prior to procedure. In our centers, central venous catheter placement was conducted on all patients. No peripheral venous catheter was used for the procedure. The leukapheresis procedures for all patients were conducted using continuous-flow blood cell separator (Haemonetics®, MCS+®, LN 9000 apheresis machine). The collection speed was 20–300 cc/minute. An average of 15% to 20% of a patient's

total blood volume was processed during a single leukapheresis session with a total average leukapheresis duration of five hours or until reaching target of buffy coat. During the procedure, acid citrate dextrose solution A (ACD-A) was used as an anticoagulant with concomitant administration of intravenous calcium gluconate.

Endpoints
Primary endpoint of this study was 7-day, 14-day, 21-day and 28-day survival after starting treatments. Possible confounding factors were analyzed by multivariate analysis to determine their association with survival.

Ethics
The research protocol was approved by the Faculty Health Research Ethics Committee Medicine, University of Indonesia Cipto Mangunkusumo National General Hospital (FKUI-RSCM) No. 1209/UN2.F1/ETIK/2018. All patients' medical record data entered into research were protected to maintain confidentiality. Since this was a retrospective cohort study, no informed consent was needed. Finally, this study is in compliance with Declaration of Helsinki.

Statistical Analysis
All the statistical analyses were performed using SPSS (SPSS 21, IBM) and STATA (MP 14.2, Stata Corp LP, College Station, TX, USA). Data on the baseline characteristics of the subjects at the time of the intervention were described in the tables. Numerical data with a normal distribution were displayed as means and standard deviation while numerical data with skewed distribution were displayed as median with minimum-maximum range value. Normal distribution of data was assessed with Kolmogorov–Smirnov test. Meanwhile, categorical data were described as percentage. Differences in baseline characteristics were assessed using Chi-squared test or Fisher's exact test for categorical variables and t-test or Mann–Whitney U test for continuous variables.

Survival analysis and comparison of 7-day, 14-day, 21-day, and 28-day survival of AML patients with both hyperleukocytosis and leukostasis based on type of treatments received were analyzed using Kaplan–Meier survival curve method. Differences in survival curves were then measured with log rank (Mantel-Cox) and Breslow (Generalized Wilcoxon) tests. P values of <0.05 were considered to be statistically significant.

For the main variables which were associated with one-month survival in this study, univariate analysis was

conducted using Cox proportional hazards model to obtain value of hazard ratio (HR) with a 95% confidence interval (CI). Variables selected for univariate cox regression analysis were filtered with proportional hazard assumption tests. The type of proportional hazard assumption tests used for the study consisted of Ln Ln survival test and global test. Variables fulfilled proportional hazard assumption test if there was no intersect on Ln Ln survival test and had p value of > 0.05 on global test. Subsequently, variables analyzed in univariate analysis that had p value of ≤0.25 were selected for multivariate analysis.

Results
Baseline Characteristics
A total of 80 adult acute leukemia patients were initially identified from the medical records. However, 18 patients were excluded due to lack of leukostasis symptoms. Additionally, 6 patients were excluded due to lack of bone marrow biopsy data and another 18 patients were excluded due to being ALL type. As a result, the number of subjects used in this study was 38 AML patients with leukostasis (Table 1).

From the 38 AML patients with leukostasis, a total of 11 patients (28.9%) received chemotherapy with leukapheresis while the other 27 patients (71.1%) received chemotherapy only. The baseline characteristics of AML patients can be seen in Table 1.

Mortality rates within the first month were compared weekly. Results showed that there was no association between 7-day mortality and the type of treatment given (p: 1.000). A total of 7 patients died in the first week and eleven in the fourth week for the chemotherapy group. Meanwhile, in the chemotherapy plus leukapheresis group, a total of three patients died in the first week and five in the fourth week. Moreover, no statistically significant differences in mortality were observed in the second to fourth week as well.

Table 1 Baseline Characteristics of AML Patients

Characteristics	Total n=38	Leukapheresis + Chemotherapy n=11	Chemotherapy Only n=27	P value
Age (years) (SD)	42.45 (15.52)	40.82 (8.67)	43.11 (17.67)	0.685
Gender Male (%) Female (%)	 17 (44.7%) 21 (55.3%)	 4 (36.4%) 7 (63.6%)	 13 (48.1%) 14 (51.9%)	0.762
Body Mass Index (Kg/m^2) (median)	21.125 (11.98–36.57)	21.96 (11.98–30.08)	21.71 (12.33–36.57)	0.552
Hemoglobin (g/dl) (SD)	7.527 (2.227)	6.694 (2.165)	7.866 (2.200)	0.144
MCV (Femtolitre) (SD)	87.458 (5.689)	87 (7.1255)	87.644 (5.140)	0.756
Leukocyte (mm^3) (median)	205,835 (105,000–847,000)	353,830 (143,360–847,000)	170,930 (105,000–369,100)	0.003
Thrombocyte (mm^3) (median)	39,550 (6000–665,000)	57,900 (6000–125,000)	34,000 (7540–665,000)	0.509
Blast (%) (median)	90 (42–97)	90 (54–97)	90 (42–96)	0.485
Creatinine (mg/dl) (median)	1.08 (0.46–6.00)	1.41 (0.56–5.48)	1.03 (0.46–6.00)	0.573
Respiratory leukostasis (%)	21 (55.3%)	2 (18.2%)	19 (70.4%)	0.010
Central Nervous System Leukostasis (%)	20 (52.6%)	5 (45.5%)	15 (55.6%)	0.836
Cardiovascular Leukostasis (%)	10 (26.3%)	3 (27.3%)	7 (25.9%)	1.000
Gastrointestinal Leukostasis (%)	22 (57.9%)	4 (36.4%)	18 (66.7%)	0.176
Tumor Lysis Syndrome (%)	15 (39.5%)	3 (27.3%)	12 (44.4%)	0.538
Disseminated Intravascular Coagulation (%)	13 (34.2%)	3 (27.3%)	10 (37%)	0.843
Chronic Comorbidities (%)	14 (36.8%)	2 (18.2%)	12 (44.4%)	0.250

Survival Outcomes

The median overall survival was 25 days (95% CI: 17.001–32.999 days) in the chemotherapy only group and 20 days (95% CI: 1.497–38.503) in the chemotherapy with leukapheresis group. At day 28, a total of 11 of the 27 patients receiving chemotherapy only (41.7%) and 5 of the 11 patients receiving chemotherapy with leukapheresis (45.5%) had died.

The 28-day survival was not statistically better in patients receiving chemotherapy with leukapheresis group than in chemotherapy only group (p Log Rank: 0.806; p Breslow: 0.827) (Figure 1). The hazard ratio for chemotherapy plus leukapheresis group when compared with chemotherapy only group for 28-day survival was 1.140 (95% CI: 0.396–3.283; p value: 0.809). Similarly, based on Kaplan–Meier curves, the survival rates of patients receiving chemotherapy plus leukapheresis were also not statistically

better at day-7 (p Log Rank: 0.528; p Breslow: 0.733), day-14 (p Log Rank: 0.825; p Breslow: 0.871), and day-21 (p Log Rank: 0.917; p Breslow: 0.949) when compared with their respective chemotherapy only groups (Figure 1). The hazard ratio for chemotherapy plus leukapheresis group when compared with chemotherapy only group for 7-day survival was 1.073 (95% CI: 0.277–4.152; p value: 0.919).

Univariate and Multivariate Analysis

Univariate and multivariate analyses were conducted for 28-day survival and 7-day survival. In 28-day survival, the use of chemotherapy plus leukapheresis did not statistically improve 28-day survival (HR: 1.140; 95% CI: 0.396–3.283; p: 0.809). Other factors such as age, blast percentage, creatinine level, presence of gastrointestinal leukostasis, TLS, DIC, and chronic comorbidities in univariate analysis
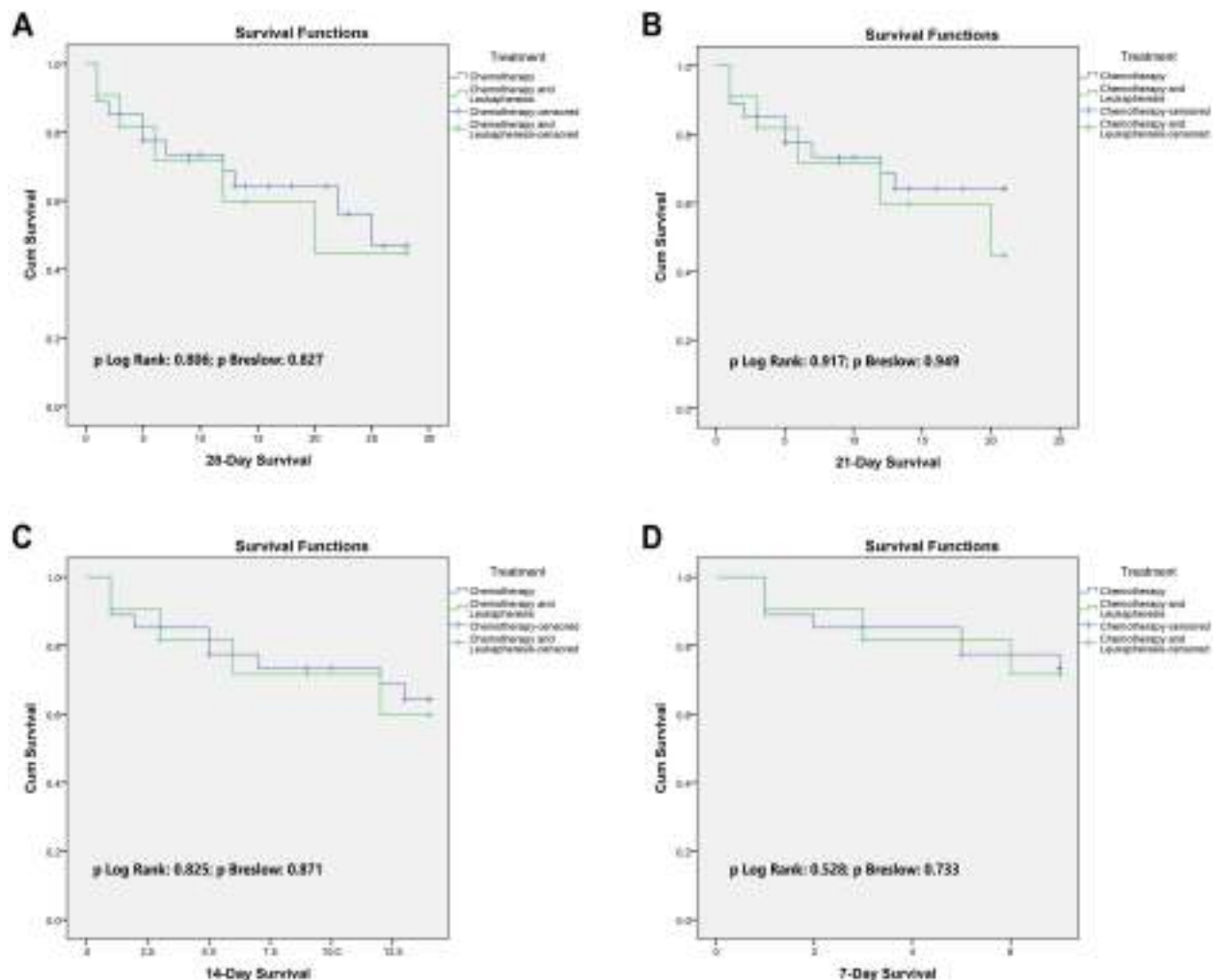
Figure 1 Survival of acute myeloid leukemia patients with leukostasis based on chemotherapy plus leukapheresis treatment and chemotherapy only treatment. (**A**) 28 Days; (**B**) 21 days; (**C**) 14 days; (**D**) 7 days.

also did not affect 28-day survival significantly (Table 2). However, patients with BMI of ≥25 were shown to have worse survival (HR: 3.292; 95% CI: 1.180–9.183; p: 0.023) in the univariate analysis. Variables from the univariate analysis which fulfilled proportional hazard assumption test and thus were selected for multivariate analysis can be seen in Supplementary Table S1. In the multivariate analysis, age ≥60 years, blast percentage ≥90%, creatinine ≥1.4, and presence of DIC were associated with worse 28-day survival (Table 2). Hence, BMI no longer remained statistically significant in the multivariate analysis.

Discussion
Acute Myeloid Leukemia and Leukostasis

In AML, there is an unregulated clonal proliferation of immature blast cells with concomitant deterioration of bone marrow function. AML is currently the most common leukemia in adults. Left untreated, up to 20% of de novo AML patients progress to hyperleukocytosis state defined as leukocyte count above >100 000/μL.[3,12,22] Although hyperleukocytosis is a laboratory abnormality, hyperleukocytosis is a very important clinical entity when encountered by

physicians due to numerous high-mortality-complications that can arise such as leukostasis, TLS, and DIC.

Leukostasis is a clinical manifestation of organ ischemia due to intravascular lumen obstruction by immature blast cells which often occurs in hyperleukocytosis and is associated with significant mortality.[2,23,24] The diagnosis of leukostasis is generally made clinically and empirically based on the clinical manifestations associated with organ ischemia in patients with hyperleukocytosis, after excluding other potential etiologies.[25] For example, if an AML patient with hyperleukocytosis suddenly develops dyspnea, the clinician must first be able to exclude other potential causes of dyspnea before making a diagnosis of leukostasis.[12,25] Hence, patients suspected of having leukostasis are often given a series of diagnostic tests such as chest X-ray, blood cultures, brain imaging, cerebrospinal fluid analysis, liver enzymes, lactic dehydrogenase, and peripheral blood morphology.

Organs most commonly affected by leukostasis are the CNS and lungs. The exact reasons why these organs have a tendency to be affected by leukostasis is currently unknown, however, it can be speculated that the rich vasculature and the physiological functions of CNS and

Table 2 Univariate Analysis and Multivariate Analysis for 28-Day Survival of Acute Myeloid Leukemia Patients

Variable	Category	Univariate Analysis			Multivariate Analysis		
		Hazard Ratio	95% CI	P value	Hazard Ratio	95% CI	P value
Therapy	Chemotherapy + leukapheresis Chemotherapy	1.140 1	0.396–3.283	0.809	–	–	–
Age	≥60 years <60 years	2.513 1	0.805–7.851	0.113	5.541 1	1.031–29.781	0.046
BMI	≥25 kg/m^2 <25 kg/m^2	3.292 1	1.180–9.183	0.023	3.465 1	0.934–12.959	0.063
Blast	≥90% <90%	2.322 1	0.747–7.224	0.146	6.058 1	1.464–25.075	0.013
Creatinine	≥1.4 mg/dL <1.4 md/dL	2.516 1	0.925–6.844	0.071	5.749 1	1.792–18.442	0.003
Gastrointestinal Leukostasis	Yes No	1.567 1	0.562–4.370	0.391	–	–	–
Tumor Lysis Syndrome	Yes No	0.656 1	0.226–1.902	0.437	–	–	–
Disseminated Intravascular Coagulation	Yes No	2.005 1	0.738–5.444	0.172	6.541 1	1.822–23.490	0.004
Chronic Comorbidities	Yes No	0.439 1	0.141–1.369	0.156	–	–	–

lungs themselves, predispose these organs to disruption from leukostasis. The rich vasculature and high surface area of the lung for example, may increase the chance of blast cells to bind toward endothelium through E-selectin and vascular cell adhesion molecule-1 (VCAM-1).[26,27] More studies are urgently needed to unravel the mechanisms in leukostasis initiation with the hope to discover a clinically relevant molecular target which can be used to prevent or remove leukostasis other than cytoreduction.

Leukapheresis for AML Patients with Hyperleukocytosis

Leukapheresis or leukocytapheresis is defined as removal of white blood cells from the blood by the apheresis machine through centrifugation. Other constituents of the blood are maintained and then infused back to the patient. Leukapheresis is one of the modalities available for management of hyperleukocytosis and leukostasis, not only in AML but also in ALL, chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML).[6]

Leukapheresis is widely accepted to be very effective in rapid reduction of white blood cell count with up to 70% of leukocytes removed in just a single session.[28,29] However, like all medical treatments and procedures, there are always limitations. The major limitation of leukapheresis is that the procedure does not remove blast cells from bone marrow.[30] These "leftover" blast cells in bone marrow may cause a short term rebound of hyperleukocytosis due to mobilization of blast cells from bone marrow into circulation which necessitates the use of chemotherapy to prevent rebound.[4,13,30] Additionally, leukapheresis also does not remove organs' infiltrating blast cells. These blast cells then remain in affected organs and may continuously produce inflammatory response.[30] Finally, already aggregated blast cells in intravascular lumen are also not removed.[30] Hence, there appears to be some limitations of leukapheresis.

Another important factor that should be taken into consideration is that insertion of a central venous catheter is required for leukapheresis, which may cause several complications associated with catheter placement such as infection, pneumothorax, and bleeding.[3,4] Furthermore, due to citrate being commonly used as anticoagulant during the procedure, hypocalcemia and its symptoms may occur during or after the procedure due to citrate's affinity to bind to calcium.[31,32] The most dangerous complication is QTc interval prolongation.[32] Hence, periodic monitoring of the patient during and after the procedure is very important.

Current Evidence on the Use of Leukapheresis for AML

Currently, there are no randomized studies that evaluated the survival benefit of leukapheresis in AML patients with leukostasis. Furthermore, guidelines from professional medical organizations are scarce. For example, there are no guidelines from American Society of Hematology (ASH) and European Hematology Association (EHA) regarding whether to give leukapheresis or not. One of the guidelines currently available is 2013 Guidelines on the Use of Therapeutic Apheresis in Clinical Practice from American Society for Apheresis (ASFA) which stated that leukapheresis is indicated for symptomatic patients (Grade IB evidence).[33] However, in the latest version of the guidelines in 2019, the grade of evidence was downgraded to IIB.[34] Meanwhile, the grade of evidence for prophylactic leukapheresis is still IIC.[33,34] Even now, all research on leukapheresis in AML come from observational studies.

In the literature, Ganzel et al stated that leukapheresis is recommended to be performed on all patients with hyperleukocytosis either with or without clinical manifestations to prevent imminent symptoms of leukostasis and to reduce the severity of tumor lysis syndrome.[2,35] However, the effect of leukapheresis in improving mortality outcome is still controversial with studies producing conflicting results.[13,15,16,19,36] For example, a retrospective study by Malkan et al with 28 AML subjects consisting of 10 subjects receiving leukapheresis and 18 subjects not receiving leukapheresis with outcome of early deaths in 15 days post-treatment, showed no difference in early death, although the study stated that leukapheresis can effectively lower plasma leukocytes.[17] Other retrospective studies with bigger sample size such as by Pastore et al, with 52 AML patients, on the use of prophylactic leukapheresis and by Choi et al on therapeutic leukapheresis with 44 matched AML patients, also showed no difference in early mortality.[10,36] Meanwhile, a study by Giles et al with a total of 146 AML patients showed that the use of leukapheresis reduced 2-week mortality.[15] The study by Bug et al also supported that leukapheresis can reduce early 21-day mortality.[16] It should be noted that although the study by Giles et al and Bug et al observed benefit in early mortality, no statistically significant difference was

observed in long-term survival.[15,16] A recent retrospective study by Göçer et al in 2021 found benefit in both early mortality and overall survival.[37] However, differences in methodology and measured endpoint in these studies make it relatively difficult to interpret together.

A well-written systematic review and meta-analysis by Bewersdorf et al compared early mortality rates of AML patients treated with leukapheresis versus AML patients not receiving leukapheresis in 13 retrospective studies.[38] The systematic review and meta-analysis found no short-term benefit form leukapheresis and the authors discourage the use of routine leukapheresis for AML patients.[38] However, due to all studies used being retrospective in design and including pediatric patients, there was mild heterogeneity in the pooled analysis.[38] Given the rarity of AML patients with hyperleukocytosis, ethical issues, and preferences of clinicians in deciding which patients receive leukapheresis or not, it is theoretically very difficult for a randomized controlled study to be conducted.[12]

In Indonesia itself, leukapheresis was only introduced around the year 2002 and was only implemented as a means of therapy much later on.[39] There were initially financial limitations, limited availability of blood components, as well as differences in knowledge of physicians and medical personnel regarding leukapheresis procedures in Indonesia that hindered the development of leukapheresis therapy.[39] However, leukapheresis is now a common procedure in many Indonesian hospitals. The uncertainty of mortality benefit from leukapheresis is therefore a major concern not just for clinicians in Indonesia but also for clinicians worldwide in providing treatment for acute leukemia patients with leukostasis due to widespread availability of leukapheresis. Hence, this retrospective cohort study was conducted to measure early survival since leukostasis is an acute manifestation with significant early mortality and any important differences in survival should be detected within one month or less instead of a longer period.

Baseline Characteristics

Our research was conducted at Cipto Mangunkusumo National General Hospital and Dharmais National Cancer Hospital which recruited 38 acute myeloid leukemia patients with leukostasis for survival analysis. The mean age in all subjects was 42.45 years (Table 1). The mean age in treatment group and comparator group was similar (p: 0.685). When compared with other studies, the mean age in our study is younger.[15–17,19] In terms of gender and

BMI, there were no significant differences between treatment and comparator group. However, the median baseline leukocyte count was considerably higher in chemotherapy plus leukapheresis group than chemotherapy only group (353,830/mm^3 versus 170,930/mm^3; p value: 0.003). This difference also occurred in studies by Nan et al and Shallis et al.[18,19] When compared with other studies, the overall median baseline leukocyte count of this study was higher.[10,16,17] Finally, there was also significant difference in proportion of patients with pulmonary leukostasis between the two groups.

Survival and Multivariate Analysis

The one-month survival was not different between two groups (Figure 1). According to univariate Kaplan–Meier survival statistical analysis, there was no significant difference in 28-day survival (HR: 1.140; 95% CI: 0.396–3.283; p value: 0.809) and 7-day survival (HR: 1.073; 95% CI: 0.277–4.152; p value: 0.919). Our results are in line with several studies.[10,19–21,36,38] Despite similar conclusion, there were major differences in those studies that should be noted. For example, the study by Stahl et al used inclusion criteria of WBC > 50 x 10^9/L and also included patients without leukostasis, while all patients in this study had leukostasis symptoms.[21] In contrast, the study by Ventura et al concluded that patients receiving leukapheresis had better mortality, however, it should be noted that this study was conducted more than several decades ago and in the study, non-leukostasis patients were more likely than leukostasis patients to receive leukapheresis.[2] A retrospective study by Jin et al attempted to seek factors that influence efficacy of therapeutic leukapheresis in patients with hyperleukocytosis leukemia.[29] In the study, the authors found that lymphocyte count, mean corpuscular hemoglobin (MCH), and hematocrit levels prior to apheresis procedures were independent factors affecting survival.[29] From the study by Jin et al, it can be speculated that there may have been a group of patients that received more benefit from leukapheresis based on lymphocyte count, MCH, and hematocrit levels. Hence, further studies to elucidate and confirm whether there is a group of patients that would benefit from leukapheresis are needed.

In this study, leukapheresis and chemotherapy were given to patients with higher median leukocytes than patients receiving chemotherapy alone. From previous studies, it was found that leukocyte count is a prognostic factor for survival in AML.[40–42] It is

therefore possible that the higher median leukocyte count in leukapheresis and chemotherapy group affected the survival outcome in this study. The higher number of leukostasis manifestations in chemotherapy only group may also have affected the result by causing lower survival in the chemotherapy group, however, the leukostasis manifestations were not found to be prognostic factors in this study. Finally, many studies including this study recruited patients who were receiving leukapheresis therapy along with chemotherapy such as hydroxyurea or low-dose chemotherapy, resulting in difficulty to evaluate the effect of leukapheresis itself.[4]

The multivariate analysis in this study showed several interesting findings. The first was that age ≥ 60 years old was associated independently with worse one-month survival (HR: 5.541; 95% CI: 1.031–29.781; p: 0.046). This result is in concordance with the study by Liu et al, Kuo et al, and Kantarjian et al where age was a risk factor for early mortality.[42–44] Older age is associated with worse physiological function and functional capacity, which may explain the finding of the multivariate analysis.[7] Multiple comorbidities were also more commonly observed in the elderly.[45] All of these factors may explain the lower survival in the elderly.

Our study also observed that creatinine of ≥1.4 was associated with worse 28-day and 7-day survival. Similarly, the study by Pastore et al revealed that creatinine was associated with early death, but the confidence interval in the study was extremely wide.[10] While the 95% CI in the multivariate analysis of this study was also wide, it was more precise than the study by Pastore et al.[10] Other studies also supported the detrimental role of impaired kidney function in survival in AML patients.[42,46] The exact reasons how kidney function impacts survival are currently unclear. We speculate that impaired kidney function is associated with poor performance status and other comorbidities which may indirectly decrease survival.

Another result from this study is that BMI ≥25 was observed as prognostic factor for worse survival in the univariate analysis for 28-day survival and 7-day survival. However, BMI did not achieve statistical significance in multivariate analysis. The association between BMI and survival is in concordance with the study by Dhakal et al.[47] However, the role of BMI in survival of acute leukemia patients is still unclear, as several studies show contradictory findings and the exact mechanisms of how BMI affects pharmacokinetics of treatments in acute leukemia are still under investigation. It is possible that BMI may affect the impact of pharmacokinetics of chemotherapy.[48,49] Finally, both blast percentage ≥90% and presence of DIC were associated with worse 28-day survival.

To Use or Not to Use Leukapheresis

The question remains whether acute leukemia patients with hyperleukocytosis and leukostasis should receive leukapheresis or not. It is undeniable that leukapheresis is very effective in reducing the number of WBC, however, leukapheresis does not remove aggregated blast cells in microcirculation or infiltrated tissues and hence may not improve survival, as there may still be tissue hypoxia and inflammation due to the leftover aggregated blast cells.[4,30] Leukapheresis also confers procedural risk to patients especially those with comorbidities.[12] Additionally, without removal of blast cells in bone marrow, patients with leukapheresis only treatment may have very high risk of relapse short-term.[4] Hence, we do not recommend the addition of leukapheresis for AML patients with leukostasis, as its use together with chemotherapy does not provide better survival when compared with chemotherapy only, and there are many disadvantages of leukapheresis.

Study Limitations

There were several limitations in this study that should be taken into consideration when interpreting the results. First, we only had a limited sample size of 38 patients which may not be adequate for analyzing all confounders. Secondly, we also did not analyze the impact of cytogenetic and genetic mutation on survival. Finally, this was a retrospective cohort study which had all limitations associated with a cohort study.

Conclusion

AML patients with leukostasis who received both chemotherapy and leukapheresis did not have better 28-day survival and 7-day survival when compared with patients receiving chemotherapy only. Old age, high blast percentage, high creatinine, and presence of disseminated intravascular coagulation were prognostic factors for worse 28-day survival.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

Author Contributions

Funding

Disclosure

References

1. Giammarco S, Chiusolo P, Piccirillo N, et al. Hyperleukocytosis and leukostasis: management of a medical emergency. *Expert Rev Hematol*. 2017;10(2):147–154. doi:10.1080/17474086.2017.1270754
2. Ganzel C, Becker J, Mintz PD, Lazarus HM, Rowe JM. Hyperleukocytosis, leukostasis and leukapheresis: practice management. *Blood Rev*. 2012;26(3):117–122. doi:10.1016/j.blre.2012.01.003
3. Ali A, Mirrakhimov A, Abboud C, Cashen A. Leukostasis in adult acute hyperleukocytic leukemia: a clinician's digest. *Hematol Oncol*. 2016;34(2):69–78. doi:10.1002/hon.2292
4. Korkmaz S. The management of hyperleukocytosis in 2017: do we still need leukapheresis? *Transfus Apher Sci*. 2018;57(1):4–7. doi:10.1016/j.transci.2018.02.006
5. Barrett CL, Louw VJ, Webb MJ. Exchange transfusion as a life-saving intervention in three patients with different haematological malignancies with severe hyperleukocytosis where leukapheresis was not available. *Transfus Apher Sci*. 2013;49(3):397–402.
6. Aqui N, O'Doherty U. Leukocytapheresis for the treatment of hyperleukocytosis secondary to acute leukemia. *Hematology Am Soc Hematol Educ Program*. 2014;2014(1):457–460. doi:10.1182/asheducation-2014.1.457
7. Porcu P, Cripe LD, Ng EW, et al. Hyperleukocytic leukemias and leukostasis: a review of pathophysiology, clinical presentation and management. *Leuk Lymphoma*. 2000;39(1–2):1–18. doi:10.3109/10428190009053534
8. Howard SC, Jones DP, Pui C-H. The tumor lysis syndrome. *N Engl J Med*. 2011;364(19):1844–1854. doi:10.1056/NEJMra0904569
9. Aslam HM, Zhi C, Wallach SL. Tumor lysis syndrome: a rare complication of chemotherapy for metastatic breast cancer. *Cureus*. 2019;11(2):e4024. doi:10.7759/cureus.4024.
10. Pastore F, Pastore A, Wittmann G, Hiddemann W, Spiekermann K, Winn RA. The role of therapeutic leukapheresis in hyperleukocytotic AML. *PLoS One*. 2014;9(4):7–10. doi:10.1371/journal.pone.0095062
11. Singh N, Lubana SS, Dabrowski L, Sidhu G. Leukostasis in chronic lymphocytic leukemia. *Am J Case Rep*. 2020;21:1–5. doi:10.12659/AJCR.924798
12. Röllig C, Ehninger G. How I treat hyperleukocytosis in acute myeloid leukemia. *Blood*. 2015;125(21):3246–3252. doi:10.1182/blood-2014-10-551507
13. Porcu P, Farag S, Marcucci G, Cataland SR, Kennedy MS, Bissell M. Leukocytoreduction for acute leukemia. *Ther Apher*. 2002;6(1):15–23. doi:10.1046/j.1526-0968.2002.00402.x
14. Ventura GJ, Hester JP, Smith TL, Keating MJ. Acute myeloblastic leukemia with hyperleukocytosis: risk factors for early mortality in induction. *Am J Hematol*. 1988;27(1):34–37. doi:10.1002/ajh.2830270109
15. Giles FJ, Shen Y, Kantarjian HM, et al. Leukapheresis reduces early mortality in patients with acute myeloid leukemia with high white cell counts but does not improve long-term survival. *Leuk Lymphoma*. 2001;42(1–2):67–73. doi:10.3109/10428190109097677
16. Bug G, Anargyrou K, Tonn T, et al. Impact of leukapheresis on early death rate in adult acute myeloid leukemia presenting with hyperleukocytosis. *Transfusion (Paris)*. 2007;47(10):1843–1850. doi:10.1111/j.1537-2995.2007.01406.x
17. Malkan UY, Ozcebe OI. Leukapheresis do not improve early death rates in acute myeloid leukemia patients with hyperleukocytosis. *Transfus Apher Sci*. 2017;56(6):880–882. doi:10.1016/j.transci.2017.11.002
18. Shallis RM, Stahl M, Wei W, et al. Patterns of care and clinical outcomes of patients with newly diagnosed acute myeloid leukemia presenting with hyperleukocytosis who do not receive intensive chemotherapy. *Leuk Lymphoma*. 2020;61(5):1220–1225. doi:10.1080/10428194.2020.1728753
19. Nan X, Qin Q, Gentille C, et al. Leukapheresis reduces 4-week mortality in acute myeloid leukemia patients with hyperleukocytosis–a retrospective study from a tertiary center. *Leuk Lymphoma*. 2017;58(9):2110–2117. doi:10.1080/10428194.2016.1277386
20. Malkan UY, Gunes G, Eliacik E, et al. The factors affecting early death after the initial therapy of acute myeloid leukemia. *Int J Clin Exp Med*. 2015;8(12):22564–22569.
21. Stahl M, Shallis RM, Wei W, et al. Management of hyperleukocytosis and impact of leukapheresis among patients with acute myeloid leukemia (AML) on short- and long-term clinical outcomes: a Large, Retrospective, Multicenter, International Study. *Leukemia*. 2020;34(12):3149–3160. doi:10.1038/s41375-020-0783-3
22. Bewersdorf JP, Zeidan AM. Hyperleukocytosis and leukostasis in acute myeloid leukemia: can a better understanding of the underlying molecular pathophysiology lead to novel treatments? *Cells*. 2020;9(10):2310. doi:10.3390/cells9102310
23. Stucki A, Rivier A-S, Gikic M, Monai N, Schapira M, Spertini O. Endothelial cell activation by myeloblasts: molecular mechanisms of leukostasis and leukemic cell dissemination. *Blood*. 2001;97(7):2121–2129. doi:10.1182/blood.V97.7.2121
24. McKee LC, Collins RD. Intravascular leukocyte thrombi and aggregates as a cause of morbidity and mortality in leukemia. *Medicine (Baltimore)*. 1974;53(6):463–478. doi:10.1097/00005792-197411000-00006
25. Zuckerman T, Ganzel C, Tallman MS, Rowe JM. How I treat hematologic emergencies in adults with acute leukemia. *Blood*. 2012;120(10):1993–2002. doi:10.1182/blood-2012-04-424440
26. Langleben D, Orfanos SE, Giovinazzo M, et al. Pulmonary capillary surface area in supine exercising humans: demonstration of vascular recruitment. *Am J Physiol Lung Cell Mol Physiol*. 2019;317(3):L361–8. doi:10.1152/ajplung.00098.2019
27. Cavenagh JD, Gordon-Smith EC, Gibson FM, Gordon MY. Acute myeloid leukaemia blast cells bind to human endothelium in vitro utilizing E-selectin and vascular cell adhesion molecule-1 (VCAM-1). *Br J Haematol*. 1993;85(2):285–291. doi:10.1111/j.1365-2141.1993.tb03168.x

28. Hölig K, Moog R. Leukocyte depletion by therapeutic leukocytapheresis in patients with leukemia. *Transfus Med Hemother.* 2012;39 (4):241–245. doi:10.1159/000341805

29. Jin Y, Guo S, Cui Q, et al. A hospital based retrospective study of factors influencing therapeutic leukapheresis in patients presenting with hyperleukocytic leukaemia. *Sci Rep.* 2018;8:294. doi:10.1038/s41598-017-17534-4

30. Hoffman R, Benz E, Silberstein L, et al. *Hematology: Basic Principles and Practice.* 7th ed. Elsevier; 2017:924–953.

31. Davenport A, Tolwani A. Citrate anticoagulation for continuous renal replacement therapy (CRRT) in patients with acute kidney injury admitted to the intensive care unit. *NDT Plus.* 2009;2(6):439–447. doi:10.1093/ndtplus/sfp136

32. Shelat SG. Practical considerations for planning a therapeutic apheresis procedure. *Am J Med.* 2010;123(9):777–784. doi:10.1016/j.amjmed.2010.01.022

33. Schwartz J, Winters JL, Padmanabhan A, et al. Guidelines on the use of therapeutic apheresis in clinical practice-evidence-based approach from the writing committee of the American Society for apheresis: the sixth special issue. *J Clin Apher.* 2013;28(3):145–284. doi:10.1002/jca.21276

34. Padmanabhan A, Connelly-Smith L, Aqui N, et al. Guidelines on the use of therapeutic apheresis in clinical practice – evidence-based approach from the writing committee of the American Society for apheresis: the eighth special issue. *J Clin Apher.* 2019;34 (3):171–354.

35. Makroo RN, Kakkar B, Chowdhry M, Agrawal S, Seth S, Thakur UK. Therapeutic leukapheresis in a tertiary care hospital: a case series. *Asian J Transfus Sci.* 2017;11(1):65–68. doi:10.4103/0973-6247.200772

36. Choi MH, Choe YH, Park Y, et al. The effect of therapeutic leukapheresis on early complications and outcomes in patients with acute leukemia and hyperleukocytosis: a Propensity Score-Matched Study. *Transfusion (Paris).* 2018;58(1):208–216. doi:10.1111/trf.14329

37. Göçer M, Kurtoğlu E. Effect of prophylactic leukapheresis on early mortality and overall survival in acute leukemia patients with hyperleukocytosis. *Ther Apher Dial.* 2021. doi:10.1111/1744-9987.13645

38. Bewersdorf JP, Giri S, Tallman MS, Zeidan AM, Stahl M. Leukapheresis for the management of hyperleukocytosis in acute myeloid leukemia—a systematic review and meta-analysis. *Transfusion (Paris).* 2020;60(10):2360–2369. doi:10.1111/trf.15994

39. Triyono T, Hans V. Therapeutic apheresis in Asia: an Indonesia single center experience. *J Clin Apher.* 2014;28(April 2013):349–355.

40. de Jonge HJM, Valk PJM, de Bont ESJM, et al. Prognostic impact of white blood cell count in intermediate risk acute myeloid leukemia: relevance of mutated NPM1 and FLT3-ITD. *Haematologica.* 2011;96 (9):1310–1317. doi:10.3324/haematol.2011.040592

41. Padilha SL, Matos MCC, Domino NR, Domino NR. Acute myeloid leukemia: survival analysis of patients at a university hospital of Paraná. *Rev Bras Hematol Hemoter.* 2015;37(1):21–27. doi:10.1016/j.bjhh.2014.11.008

42. Liu C, Hong Y, Kuan AS, et al. The risk of early mortality in elderly patients with newly diagnosed acute myeloid leukemia. *Cancer Med.* 2020;9(4):1572–1580. doi:10.1002/cam4.2740

43. Kuo KHM, Callum JL, Panzarella T, et al. A retrospective observational study of leucoreductive strategies to manage patients with acute myeloid leukaemia presenting with hyperleucocytosis. *Br J Haematol.* 2015;168(3):384–394. doi:10.1111/bjh.13146

44. Kantarjian H, Ravandi F, O'Brien S, et al. Intensive chemotherapy does not benefit most older patients (age 70 years or older) with acute myeloid leukemia. *Blood.* 2010;116(22):4422–4429. doi:10.1182/blood-2010-03-276485

45. Malfuson J-V, Etienne A, Turlure P, et al. Risk factors and decision criteria for intensive chemotherapy in older patients with acute myeloid leukemia. *Haematologica.* 2008;93(12):1806–1813. doi:10.3324/haematol.13309

46. Malkawi A, Anand A, Al-Ameri A, Abdelfatah M, Kanaan Z, Haller N. Outcome of patients with acute myeloid leukemia/high risk MDS according to the kidney function. *Blood.* 2013;122 (21):5007. doi:10.1182/blood.V122.21.5007.5007

47. Dhakal P, Lyden E, Lee A, et al. Effects of obesity on overall survival of adults with acute myeloid leukemia. *Clin Lymphoma Myeloma Leuk.* 2020;20(3):e131–6. doi:10.1016/j.clml.2019.11.001

48. Navarro WH. Impact of obesity in the setting of high-dose chemotherapy. *Bone Marrow Transplant.* 2003;31(11):961–966. doi:10.1038/sj.bmt.1704052

49. Kendrick J, Warkentin D, Ensom MHH. The impact of obesity on pharmacokinetics and dosing of leukemia chemotherapy. In: Mittelman SD, Berger NA, editors. *Energy Balance and Hematologic Malignancies [Internet].* Boston, MA: Springer US; 2012:97–127. Energy Balance and Cancer. doi:10.1007/978-1-4614-2403-1_5

Coagulopathy in SARS-CoV-2 Infected Patients: Implication for the Management of COVID-19

Sisay Getu, Tegenaw Tiruneh, Henok Andualem, Wasihun Hailemichael, Teklehayimanot Kiros, Demeke Mesfin Belay & Mulugeta Kiros

Submit your article to this journal

Article views: 221

View related articles

View Crossmark data

Citing articles: 5 View citing articles

REVIEW

Coagulopathy in SARS-CoV-2 Infected Patients: Implication for the Management of COVID-19

Sisay Getu [1]
Tegenaw Tiruneh [1]
Henok Andualem [2]
Wasihun Hailemichael [2]
Teklehayimanot Kiros [3]
Demeke Mesfin Belay [4]
Mulugeta Kiros [3]

[1]Hematology and Immuno-hematology, Medical Laboratory Science, College of Health Sciences, Debre Tabor University, Debre Tabor, Ethiopia; [2]Immunology and Molecular Biology, Medical Laboratory Science, College of Health Sciences, Debre Tabor University, Debre Tabor, Ethiopia; [3]Microbiology, Medical Laboratory Science, College of Health Sciences, Debre Tabor University, Debre Tabor, Ethiopia; [4]Pediatrics and Child Health Nursing, College of Health Sciences, Debre Tabor University, Debre Tabor, Ethiopia

Abstract: COVID-19 disease has led to an extraordinary inclusive health crisis globally. Elevation of D-dimer is the major remarkable abnormal coagulation test in seriously ill COVID-19 patients. In nearly 50% of COVID-19 patients, the value of D-dimer was significantly enhancing. Recent literature indicated that COVID-19 patients were at higher risk of developing disseminated intravascular coagulation. Pro-inflammatory cytokines and chemokines are some of the factors leading to these conditions. The majority of COVID-19 patients showed a higher profile of pro-inflammatory cytokines and chemokines in severe clinical conditions. Tumor necrosis factor-α (TNF-α) and interleukins (ILs) elevated in COVID-19 infected patients. TNF-α, IL-6, and IL-1 are major cytokines vital for the inhibition of intrinsic anticoagulant pathways. COVID-19 becomes a higher complication with a significant effect on blood cell production and hemostasis cascades. Deep vein thrombosis and arterial thrombosis are common complications. Changes in hematological parameters are also frequently observed in COVID-19 patients. Especially, thrombocytopenia is an indicator for poor prognosis of the disease and is highly expected and aggravates the likelihood of death of SARS-CoV-2 infected individuals. Thrombopoiesis reduction in COVID-19 patients might be due to viral abuse of the bone marrow/the viral load may affect thrombopoietin production and function. In other ways, immune-inflammation-mediated destruction and increased consumption of platelets are also the possible proposed mechanisms for thrombocytopenia. Therefore, the counting of platelet cells is an easily accessible biomarker for disease monitoring. All SARS-CoV-2 infected patients should be admitted and identifying potential higher-risk patients. It is also obligatory to provide appropriate treatments with intensive care and strict follow-up. In addition, considerations of chronic diseases are essential for better prognosis and recovery. The current review discusses coagulopathy among SARS-CoV-2 infected individuals and its complication for the management of the disease.

Keywords: COVID-19, SARS-CoV-2, coagulopathy, DIC

Introduction

There are various genera of coronaviruses. However, only seven viruses have been identified that could infect humans. Among these viruses, the human coronavirus (HCoV)-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 are the primary causes of common cold, while the other three (severe acute respiratory syndrome (SARS)-CoV, Middle East respiratory syndrome (MERS)-CoV and SARS-CoV-2) are known to cause atypical pneumonia.[1] Nowadays, we are in the era of the recently emerged coronavirus called Severe Acute Respiratory Syndrome corona virus-2 (SARS-CoV-2). SARS-CoV-2 is an enveloped coronavirus with a spherical

Correspondence: Sisay Getu
Hematology and Immuno-hematology, Medical Laboratory Science, College of Health Sciences, Debre Tabor University, Debre Tabor, Ethiopia
Email sis.gtu@gmail.com

shape that belongs to the genus similar to SARS-CoV and MERS-CoV known as β-coronavirus though there is a minuscule difference in genetic sequence.[1,2] It is the etiologic agent of coronavirus disease 2019 (COVID-19), which was initially appeared in China, Wuhan by December 2019. The disease is still continued as a global concern and greatly impacts humanity's culture and economy.[3,4]

Physiologic hemostasis is an intensive process of retaining blood fluid in the circulation either in normal or abnormal conditions.[5] When blood vessel damages or an injury occurs, a clot is produced to stop bleeding and then localized thrombus formation at the site of the break-in vascular integrity. This is followed by fibrinolysis that dissolves the clot and thereby healing the wound.[6] Whenever there are imbalance hemostasis cascades, excessive clot formation, or thrombosis might have occurred. The imbalance can also lead to bleeding as a result of excessive fibrinolysis. It is therefore essential to understand the hemostasis systems such as blood vessels, platelets, and plasma proteins to manage diseases related to hemostasis.[5]

In patients with severe COVID-19 disease, the involvement of fibrin deposition has been reported. The severe manifestation is usually characterized by acute respiratory distress syndrome (ARDS); a lung inflammation that leads to an insufficient oxygen supply that could involve multiorgan failure and consecutive death.[7] Fibrin formation is steadily seen in lung parenchyma cells of ARDS patients. Fibrin accumulation supports the thickening of the hyaline membrane that further promotes the accumulation of fibrin in the alveoli and thus provokes respiratory dysfunction.[8–12] In the majority of COVID-19 patients with severe coagulation abnormalities, such as disseminated intravascular coagulation (DIC) and thrombotic microangiopathy, unrecognized thromboembolic complications and a higher risk of death could be occurred in some conditions.[13–16]

Method

In this review, the data were retrieved from electronic databases, such as PubMed, Google, and Google Scholar. Articles conducted with the aim of coagulopathy in SARS-CoV-2 infected patients and its complications for the management of COVID-19 have been included through searching using key terms "coagulopathy, COVID-19, SARS-CoV-2 infected patients, SARS-CoV-2, and COVID-19 complication" separately and in combination. The full-text articles were first to read for eligibility

(relevance for the topic), and those which are relevant were included in this review. Articles from a systematic review and meta-analysis have been also used while writing this document. All references were cited using Endnote X8 referencing manager. Most of the articles used in this review were published within this year (2020). However, articles not open accessed and published in languages other than English were not included in this review.

Coagulation Abnormalities in COVID-19 Patients

Several studies indicated that COVID-19 patients had higher production of pro-inflammatory cytokines and chemokines. Tumor necrosis factor-α (TNF-α), interleukin-6 ((IL-6), and IL-1 are the key cytokines that vigorously inhibit the intrinsic anticoagulation pathways. IL-6 promotes tissue factor expression on lymphocytes, monocytes, and macrophages that consequently recruits coagulation activation and thrombin formation. On the other side, they are also related to the significant initiation of the fibrinolytic system. In addition to the cytokines, the inflammations associated with COVID-19 have been reported to induce the higher production of fibrinolytic factors, such as urokinase-type and plasminogen. This indicates there is active fibrinolysis and waning clotting. This is corroborated by a finding that showed a higher level of D-dimers, a fibrin degradation product; in seriously ill COVID-19 patients.[17–19] Further on, in some infected patients, the endothelial cell in the alveoli initiates the release of tissue factor (TF) on surfaces of leukocytes and endorses fibrin deposition. On top of that, there is an elevation of plasminogen activator inhibitor 1 (PAI-1), indicating the association of COVID-19 disease with the hypofibrinolytic state. Together, it seems shreds of evidence are equivocal on whether COVID-19 is related to hypo fibrinolysis or hyper-fibrinolysis conditions.[20,21]

Studies indicated that patients with COVID-19 showed leukocytosis, thrombocytopenia, a higher concentration of partial thromboplastin time (PTT), APTT, fibrinogen degradation products (FDPs), and lower levels of antithrombin activity.[22,23] In addition to this, a study from China revealed that the presence of markedly elevated values of von Willebrand factor (VWF) antigen, VWF activity, and factor VIII.[24] At the last stages of the disease,

patients are at higher risk of developing venous thromboembolism (VTE) and DIC.[25]

Complications of Coagulation Disorder Associated with COVID-19

As discussed above, it is reasonable to assume that SARS-CoV-2 infection has a considerable effect on hemostasis and blood cell production when the disease progression is becoming worse. Thrombocytopenia and DIC are common complications in seriously ill patients.

Thrombocytopenia in Patients with COVID-19

Changes in hematological parameters are frequently seen in COVID-19 patients, for example thrombocytopenia, which is usually related to poor prognosis of the disease and even death.[26] A recent study done in China showed that COVID-19 patients demonstrated a reduction in platelet cells. Thus is correlated with an increased risk of mortality.[27] The reduction of platelet cells was more prominent in patients with more severe cases than in mild cases.[28] Therefore, the counting of platelets cells could be a biomarker for monitoring the disease progression.[29–31] Platelet count reduction and thrombocytopenia may occur after sepsis, DIC, or drug-induced in most other cases. However, in COVID-19 patients might also occur due to an immune disorder, immune thrombocytopenia (ITP). ITP is an intermittent autoimmune disorder with the feature of platelet count lower than $100 \times 109/$ L, resulting in an imbalance of blood hemostasis and leading to the risk of bleeding.[32–35] The other study indicated that 36% of hospitalized COVID-19 patients could develop thrombocytopenia.[3]

Mechanisms of Thrombocytopenia in COVID-19 Patients

The mechanisms behind thrombocytopenia in COVID-19 patients can be explained in various means. Platelets recognized SARS-CoV-2 via the surface of toll-like receptors, integrins, and P-selectin.[36] SARS-CoV-2 may reduce platelet production by the direct impact of the virus on hematopoiesis, megakaryocyte maturation, the elevation of platelet adhesion, and activation. Platelet production could be also reduced because either viral abuse to the bone marrow or the viral load may affect thrombopoietin production and function. Finally, the large consumption of platelets to heal the damaged tissue leading to

thrombocytopenia.[37] The pooled results of nine studies conducted in China, Wuhan among 1779 COVID-19 patients showed low platelets in severe cases.[38,39] Thrombocytopenia could also have occurred after heparin therapy, known as heparin-induced thrombocytopenia (HIT).[28,40–42]

Heparin-induced thrombocytopenia appears when platelet factor 4 (PF4) that discharges from activated platelets binds to heparin. Subsequently, antibodies are produced against this complex (heparin-PF4). In the next step, the three complexes (antibody-PF4-heparin) bind to platelets via the FcγIIa receptor, this complex further stimulates platelet activation and aggregation. This induces coagulation pathways and subsequent reduction of circulating platelets which finally leading to thrombocytopenia.[43,44] A higher dose of heparin to treat COVID-19 patients could provoke thrombocytopenia and leads to death. Therefore, other optional anticoagulants should be used to treat critical COVID-19 patients.[41] Another study indicated that 71.4% of the patients infected with SARS-CoV-2 develop thrombosis and DIC leading to higher platelet consumption with fatal outcomes. In other mechanisms, SARS-CoV-2 infection could also lead to alveolar damage that captures megakaryocytes and delays the release of platelets from it to the circulation after maturation.[45,46]

Generally, thrombocytopenia in COVID-19 patients is primarily associated with either immune-mediation[47] or reduction of platelet production (thrombopoiesis)[48,49] because of the direct influence of the virus on hematopoietic tissue,[50–52] higher platelet consumption[53] due to increased adherence of platelets[54] on injured endothelial cells[55] as indicated in the Figure 1.

Disseminated Intravascular Coagulation

When endothelial cells are injured, the Hageman factor is activated and leading to the initiation of the intrinsic clotting system. Extrinsic clotting system activation is continued after tissue injury. In the next step, coagulant phospholipids are released from injured cells, such as platelets and red blood cells. The final shared product for these two processes is thrombin production that cleaves fibrinogen and activates factor XIII. Next to this, the fibrinolysis system is produced by the release and aggregation of platelets. In the final stages of the cascade, FDPs are formed through plasmin which contributes to the
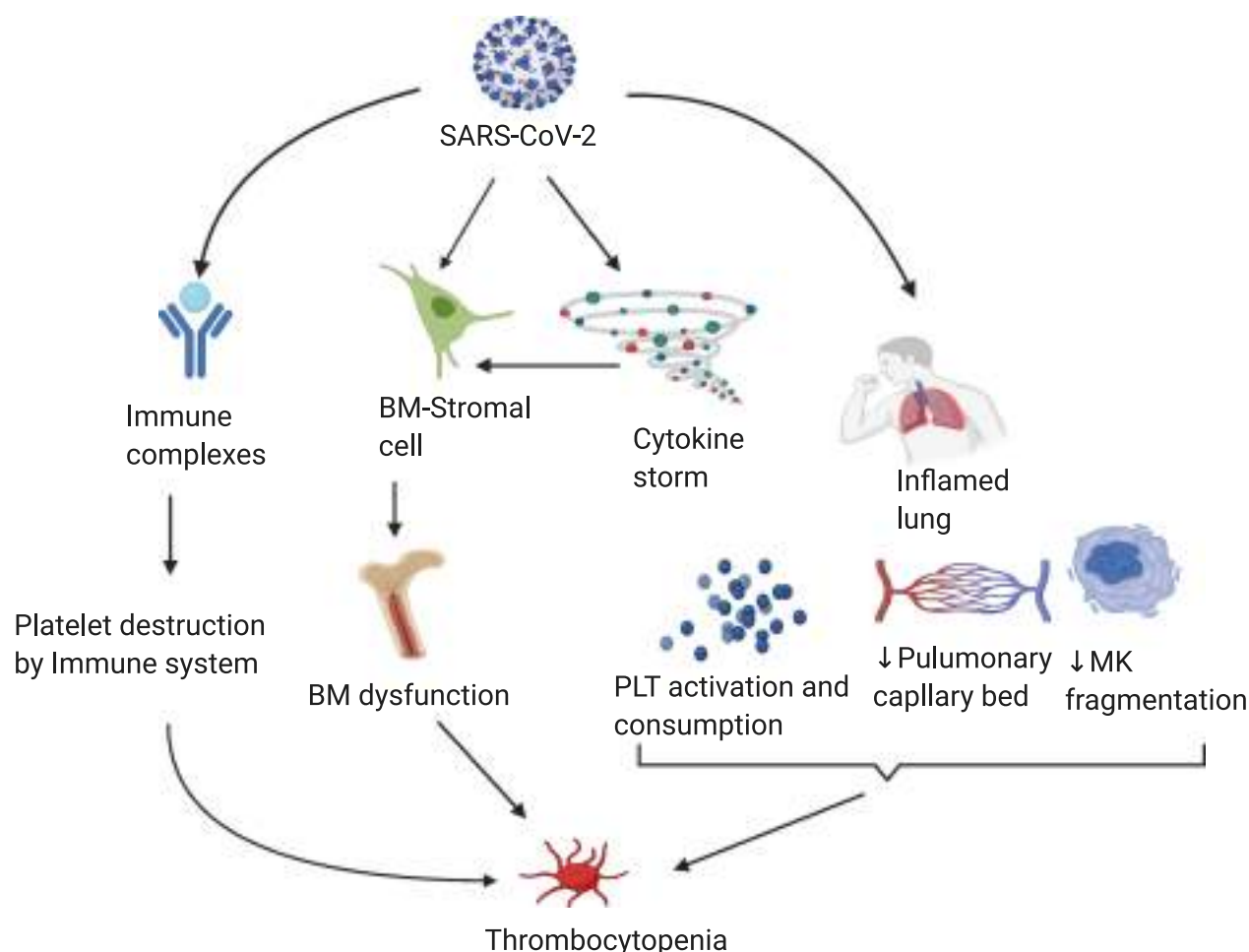
Figure 1 Mechanisms of thrombocytopenia in COVID-19 patients.
Notes: Bone marrow stromal cells may be infected either directly by the virus or higher cytokine storm because of infection may distract the cell. Thus, in turn leads to bone marrow dysfunction and finally thrombocytopenia can occur. In addition, thrombocytopenia in COVID-19 patients might be due to lung inflammation with SARS-CoV-2 infection which leads to platelets activation with higher consumption, reduction of pulmonary capillary bed and megakaryocytes fragmentation. Similarly, thrombocytopenia with SARS-CoV-2 infection could occur after antigen and antibody complexes formation to protect against the virus results to platelets distraction by the immune system. The figure is created with BioRender; https://app.biorender.com **Abbreviations**: PLT, platelets; MK, mega kayrocytes, BM; bone marrow.

hemorrhagic diathesis which is the hall marker in the diagnosis of DIC.[56]

In systemic infections, coagulation cascades are triggered and resulting in DIC when the disease is highly complicated. However, in subclinical conditions, DIC occurs as a result of increment in thrombin and fibrin generation.[57] DIC was firstly found among blood cancer patients though it is also seen in intensive care units (ICU) COVID-19 patients and results in severe bleeding and organ failure nowadays.[32,58–61] The study conducted by Lodigiani C et al, showed that development of VTE in ICU COVID-19 patients.[62] COVID-19 patients that developed DIC showed that thrombocytopenia, elevated D-dimer, decreased factors, such as II, V, VII, and X,

lower levels of plasma concentrations of antithrombin and protein C.[63–65] Similarly, endothelial and mononuclear cells are induced by proinflammatory cytokine. Thus, tissue factor stimulation and unregulated thrombin generation could have occurred after a while.[66]

Thrombotic Microangiopathy

Thrombotic microangiopathy (TMA) is one the hemolytic anemia that occurs after increment of platelets activation, aggregation, and adhesion to the veins, capillaries, and small arteries walls. TMA is the causes of thrombocytopenia due to higher consumption of platelets. It also causes organ damage, such as renal insufficiency, neurological disease and microangiopathic hemolysis as the results of

microscopic blood clot formation in vascular endothelium. A postmortem tissue examination from COVID-19 patients showed that accumulation and trapping of inflammatory cells particularly neutrophils and deposition of platelet-rich vascular thrombotic in vessels of lungs which is equivalent to pulmonary TMA.[67–69] However, thrombocytopenic thrombotic purpura (TTP) which is the cause of both thrombocytopenia and intravascular hemolysis is not a clinical manifestation of COVID-19.[4]

In TMA, higher platelet interaction to the vessel wall is observed as a consequence of extreme-large von Willebrand factor multimers (VWFM) stimulation and release from injured endothelial cells. In normal conditions, ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type I motif-13) cleaves the VWFM. In COVID-19 patients ADAMTS-13 concentration was reduced. Therefore, higher concentration of VWFM leads to increased deposition of platelet-rich-thrombi in the micro vascular area.[70,71]

Pathophysiology of Complement-Mediated TMA

The activation of complement pathways is a crucial mechanism in the occurrence of TMAs. When the microbes invade the human body, the complement system protects against invaders as part of innate immunity. After initiation of complement pathway activation, stimulation of platelets and endothelial cells, recruitments of immune cells, and blood coagulation are followed. The ultimate goal of the three complement pathways activation (alternative, classical, and lectin) is membrane attack complex constitution on the surface of target cells and vascular fragment deposition.[72,73] Recent studies suggest that complement-mediated TMAs were leading to thrombosis.

Angiotensin-converting enzyme 2 (ACE2) is highly expressed on podocytes and epithelial cells after SARS-CoV-2 infection causes the risk of cardiac damage and multiple organ dysfunction. Additionally, studies on coronaviruses showed that the de-activation of C3 notably mitigates the lung headed to pro-inflammatory cytokines elevation. Either absences of C3 or a blockage of its activation has a higher contribution to the spread of the virus through inhibition of immune cell activation and recruitment via lungs.[74–77] A recent study also revealed that SARS-CoV-2's protein binds to a protein of the lectin pathway resulting in complement-mediated inflammatory lung injury. A further study conducted by Magro et al has

also recently shown that a broad accumulation of C5b-9, C4d, and Mannan-binding lectin serine protease 2 in the micro-vessel of lung of severe COVID-19 patients due to extreme activation of alternative and lectin pathway.[78–80]

Coagulation Tests in COVID-19

An increment of D-dimer value is the main remarkable abnormal coagulation test investigated in seriously ill COVID-19 patients. In nearly 50% of COVID-19 patients, the value of D-dimer is significantly enhanced. Severe COVID-19 patients are at higher risks of death unless they are supplied oxygen with mechanical ventilation.[4,17,81,82] In addition, thrombocytopenia is the other abnormality in most severe patients. Most COVID-19 patients have a platelet count of (100 to 150) x10^9/L or lower than this value.[3,17,83–85] Slight prolongation of the PT, and aPTT were also found in ICU COVID-19 patients. Similarly, the average plasma fibrinogen levels are notably increased in the majority of severe COVID-19 patients because fibrinogen is an acute phase reactant protein. Nevertheless, a reduction of plasma fibrinogen concentration was shown in some patients. Besides, mild decrement of plasma antithrombin and protein C concentrations are revealed among survived ICU patients.[81,86]

Management of Coagulopathy

All hospital-admitted COVID-19 patients could develop VTE. Therefore, control measurements should be taken according to the international treatment guidelines for all high-risk COVID-19 patients. Likewise, respiratory distress, pulmonary thromboembolism (PTE), and blood pressure reduction should need strict follow-up. Vitamin K antagonist medications, such as warfarin causes stroke mostly in VTE patients. Consequently, patients who take this drug should be controlled.[87] All SARS-CoV-2 infected patients should be admitted for identifying potentially higher-risk patients and provide treatment with intensive care. In severe patients with tissue hypoxia; determinations of coagulation biomarkers levels, for example, PT, INR, platelet count, D-dimers, and plasma fibrinogen concentration are not sufficient for clinical decision. Therefore, complete clinical assessment and molecular test investigations should be also taken into account. However, D-dimer increments by three to four-fold have a guarantee for admission.[88,89]

Conclusion and Future Perspective

There are no effective therapies for COVID-19. Hence, strict follow-up and consideration of chronic diseases are essential

for better prognosis and recovery. COVID-19 leads to coagulation abnormalities with multiple complications, such as DIC and TMA. Thrombo-embolic is one of the complications and specific features of severe COVID-19. It is an indicator for severe COVID-19 and helps to create various management strategies to overcome the disease.

Vitamin K antagonist medications, such as warfarin, and lower molecular weight heparin could not be given to prevent stroke in COVID-19 patients. Anticoagulants other than heparin should use to treat severe cases. Prolonged PT, aPTT, and elevated D-dimer levels in serious COVID-19 patients lead to DIC combined with severe thrombocytopenia could be used as an alert for quick intervention. Hence, upon observation of these abnormalities, quick responses should be given to reduce death prevalence.

Both ambulatory and hospital-admitted COVID-19 patients are highly suspected of VTE and stroke. Therefore, earlier management of cases is recommended along with blood donation. Generally, health professionals should always be alerted for hemorrhagic conditions in critically ill COVID-19 patients, and laboratory investigations related to hemorrhagic conditions should be strictly controlled and interpreted.

Abbreviations

ACE2, Angiotensin-Converting Enzyme 2; ADAMTS-13, A Disintegrin and Metalloproteinase with a Thrombospondin Type I Motif-13; ARDS, Acute Respiratory Distress Syndrome; COVID-19, Coronavirus Disease 2019; CoVs, Coronaviruses; DIC, Disseminated Intravascular Coagulation; FDPs, Fibrinogen Degradation Products; HCoV, Human Coronavirus; HIT, Heparin-Induced Thrombocytopenia; ICU, Intensive Care Unit; ILs, Interleukins; ITP, Immune Thrombocytopenia; MAC, Membrane Attack Complex; MERS, Middle East Respiratory Syndrome; PAI-1, Plasminogen Activator Inhibitor 1; PF4, Platelet Factor 4; PT, Prothrombin Time; PTE, Pulmonary Thromboembolism; PTT, Partial Thromboplastin Time; SARS-CoV-2, Severe Acute Respiratory Syndrome 2; TF, Tissue Factor; TMA, Thrombotic Microangiopathy; TNF-α, Tumor Necrosis Factor-α; T-PA, Tissue-type Plasminogen Activator; TTP, Thrombocytopenic Thrombotic Purpura; VTE, Venous Thromboembolism; VWF, Von Willebrand Factor; VWFM, Von Willebrand Factor Multimers.

Author Contributions

All authors made a significant contribution to the work reported in the current review, whether in the conception, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

References

1. Chen Z, Zhang W, Lu Y, et al. From SARS-CoV to Wuhan 2019-CoV outbreak: similarity of early epidemic and prediction of future trends. *Cell-Host-Microbe*. 2020;D-20-00063.
2. Lancet T. Emerging understandings of 2019-nCoV. *Lancet*. 2020;395 (10221):311. doi:10.1016/S0140-6736(20)30186-0
3. Guan W-J, Ni Z-Y, Hu Y, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. 2020;382(18):1708–1720. doi:10.1056/NEJMoa2002032
4. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *The lancet*. 2020;395 (10229):1054–1062. doi:10.1016/S0140-6736(20)30566-3
5. Hokland P. *Hematology: Clinical Principles and Applications*. 2011.
6. Meduri GU, Annane D, Chrousos GP, Marik PE, Sinclair SE. Activation and regulation of systemic inflammation in ARDS: *r*ationale for prolonged glucocorticoid therap*y*. *Chest*. 2009;136 (6):1631–1643. doi:10.1378/chest.08-2408
7. Rocco P, Dos Santos C, Pelosi P. Lung parenchyma remodeling in acute respiratory distress syndrome. *Minerva Anestesiol*. 2009;75 (12):730–740.
8. Bertozzi P, Astedt B, Zenzius L, et al. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. *N Engl J Med*. 1990;322(13):890–897. doi:10.1056/ NEJM199003293221304
9. Ware LB, Matthay MA, Parsons PE, Thompson BT, Januzzi JL, Eisner MD. Pathogenetic and prognostic significance of altered coagulation and fibrinolysis in acute lung injury/acute respiratory distress syndrome. *Crit Care Med*. 2007;35(8):1821.
10. Ham W, Schoonhoven L, Schuurmans MJ, Leenen LP. Pressure ulcers from spinal immobilization in trauma patients: a systematic review. *J Trauma Acute Care Surg*. 2014;76(4):1131–1141. doi:10.1097/TA.0000000000000153
11. Laterre P, Wittebole X, Jf D. Anticoagulant therapy in acute lung injury. *Crit Care Med*. 2003;31:S329–S36.
12. Ware LB, Camerer E, Welty-Wolf K, Schultz MJ, Matthay MA. Bench to bedside: targeting coagulation and fibrinolysis in acute lung injury. *Am J Physio Lung Cellular Mol Physiol*. 2006;291(3): L307–L11. doi:10.1152/ajplung.00157.2006
13. Levi M, Scully M. How I treat disseminated intravascular coagulation. *Blood J Am Soc Hematol*. 2018;131(8):845–854.
14. Oudkerk M, Büller HR, Kuijpers D, et al. Diagnosis, prevention, and treatment of thromboembolic complications in COVID-19: report of the National Institute for Public Health of the Netherlands. *Radiology*. 2020;201629.
15. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thrombosis Haemostasis*. 2020;18(5):1094–1099. doi:10.1111/jth.14817

16. Thachil J, Tang N, Gando S, et al. ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thrombosis Haemostasis*. 2020;18(5):1023–1026. doi:10.1111/jth.14810

17. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet*. 2020;395 (10223):497–506. doi:10.1016/S0140-6736(20)30183-5

18. Mehta P, McAuley DF, Brown M, et al. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet*. 2020;395 (10229):1033. doi:10.1016/S0140-6736(20)30628-0

19. Klok F, Kruip M, Van der Meer N, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res*. 2020.

20. Al-Samkari H, Karp Leaf RS, Dzik WH, et al. COVID and Coagulation: bleeding and Thrombotic Manifestations of SARS-CoV2 Infection. *Blood*. 2020;136(4):489–500. doi:10.1182/blood.2020006520

21. Whyte CS, Morrow GB, Mitchell JL, Chowdary P, Mutch NJ. Fibrinolytic abnormalities in acute respiratory distress syndrome (ARDS) and versatility of thrombolytic drugs to treat COVID-19. *J Thrombosis Haemostasis*. 2020;18(7):1548–1555. doi:10.1111/jth.14872

22. Zhang Y, Xiao M, Zhang S, et al. Coagulopathy and antiphospholipid antibodies in patients with Covid-19. *N Engl J Med*. 2020;382(17):e38. doi:10.1056/NEJMc2007575

23. Pavoni V, Gianesello L, Pazzi M, Stera C, Meconi T, Frigieri FC. Evaluation of coagulation function by rotation thromboelastometry in critically ill patients with severe COVID-19 pneumonia. *J Thromb Thrombolysis*. 2020;1.

24. Leonard-Lorant I, Delabranche X, Severac F, et al. Acute pulmonary embolism in COVID-19 patients on CT angiography and relationship to D-dimer levels. *Radiology*. 2020;201561.

25. Pavoni V, Gianesello L, Pazzi M, Stera C, Meconi T, Frigieri FC. Evaluation of coagulation function by rotation thromboelastometry in critically ill patients with severe COVID-19 pneumonia. *J Thromb Thrombolysis*. 2020;50::281–6. doi:10.1007/s11239-020-02130-7

26. Chen N, Zhou M, Dong X, et al. Epidemiological and clinical characteristics of 99 cases of 2019 322 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet*. 2020;395::507–323. doi:10.1016/S0140-6736(20)30211-7

27. Yang X, Yang Q, Wang Y, et al. Thrombocytopenia and its association with mortality in patients with COVID-19. *J Thrombosis Haemostasis*. 2020;18(6):1469–1472. doi:10.1111/jth.14848

28. Lippi G, Plebani M, Henry BM. Thrombocytopenia is associated with severe coronavirus disease 2019 (COVID-19) infections: a meta-analysis. *Clinica Chimica Acta*. 2020.

29. Hui P, Cook DJ, Lim W, Fraser GA, Arnold DM. The frequency and clinical significance of thrombocytopenia complicating critical illness: a systematic review. *Chest*. 2011;139(2):271–278. doi:10.1378/chest.10-2243

30. Raju NA, Rao SV, Joel JC, et al. Predictive value of serum myoglobin and creatine phosphokinase for development of acute kidney injury in traumatic rhabdomyolysis. Indian journal of critical care medicine: peer-reviewed, official publication of. *Ind Soc Crit Care Med*. 2017;21(12):852. doi:10.4103/ijccm.IJCCM_186_17

31. Vanderschueren S, De Weerdt A, Malbrain M, et al. Thrombocytopenia and prognosis in intensive care. *Crit Care Med*. 2000;28(6):1871–1876. doi:10.1097/00003246-200006000-00031

32. Gralnick HR, Bagley J, Abrell E. Heparin treatment for the hemorrhagic diathesis of acute promyelocytic leukemia. *Am J Med*. 1972;52 (2):167–174. doi:10.1016/0002-9343(72)90066-6

33. Zulfiqar -A-A, Lorenzo-Villalba N, Hassler P, Andrès E. Immune thrombocytopenic purpura in a patient with Covid-19. *N Engl J Med*. 2020;382(18):e43. doi:10.1056/NEJMc2010472

34. Cooper N, Ghanima W. Immune thrombocytopenia. *N Engl J Med*. 2019;381(10):945–955. doi:10.1056/NEJMcp1810479

35. Swinkels M, Rijkers M, Voorberg J, Vidarsson G, Leebeek FW, Jansen A. Emerging concepts in immune thrombocytopenia. *Front Immunol*. 2018;9:880. doi:10.3389/fimmu.2018.00880

36. Page MJ, Pretorius E, editors. *A Champion of Host Defense: A Generic Large-Scale Cause for Platelet Dysfunction and Depletion in Infection. Seminars in Thrombosis and Hemostasis*. Thieme Medical Publishers; 2020.

37. Yang M, Ng MH, Li CK. Thrombocytopenia in patients with severe acute respiratory syndrome. *Hematology*. 2005;10(2):101–105. doi:10.1080/10245330400026170

38. Kolb-Mäurer A, Goebel W. Susceptibility of hematopoietic stem cells to pathogens: role in virus/bacteria tropism and pathogenesis. *FEMS Microbiol Lett*. 2003;226(2):203–207. doi:10.1016/S0378-1097(03)00643-8

39. Alexander T, Thomson VS, Malviya A, Mohan B, Wander GS, et al. Guidance for health care providers on management of cardiovascular complications in patients suspected or confirmed with COVID 19 virus infection. *J Assoc Phys India*. 2020;68:46.

40. Kravitz MS, Shoenfeld Y. Thrombocytopenic conditions—autoimmunity and hypercoagulability: commonalities and differences in ITP, TTP, HIT, and APS. *Am J Hematol*. 2005;80(3):232–242. doi:10.1002/ajh.20408

41. Liu X, Zhang X, Xiao Y, et al. Heparin-induced thrombocytopenia is associated with a high risk of mortality in critical COVID-19 patients receiving heparin-involved treatment. *medRxiv*. 2020.

42. Warkentin TE, Aird WC, Rand JH. Platelet-endothelial interactions: sepsis, HIT, and antiphospholipid syndrome. *ASH Educ Prog Book*. 2003;2003(1):497–519.

43. Rauova L, Zhai L, Kowalska MA, Arepally GM, Cines DB, Poncz M. Role of platelet surface PF4 antigenic complexes in heparin-induced thrombocytopenia pathogenesis: diagnostic and therapeutic implications. *Blood*. 2006;107(6):2346–2353. doi:10.1182/blood-2005-08-3122

44. Warkentin TE, Levine MN, Hirsh J, et al. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *N Engl J Med*. 1995;332(20):1330–1336. doi:10.1056/NEJM199505183322003

45. Arachchillage DR, Laffan M. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thrombosis Haemostasis*. 2020;18(5):1233–1234. doi:10.1111/jth.14820

46. Mandal RV, Mark EJ, Kradin RL. Megakaryocytes and platelet homeostasis in diffuse alveolar damage. *Exp Mol Pathol*. 2007;83 (3):327–331. doi:10.1016/j.yexmp.2007.08.005

47. Nicholson AC, Hajjar DP. Herpesviruses in atherosclerosis and thrombosis: etiologic agents or ubiquitous bystanders? *Arterioscler Thromb Vasc Biol*. 1998;18(3):339–348. doi:10.1161/01.ATV.18.3.339

48. Bierman H, Nelson E. Hematodepressive virus diseases of Thailand. *Ann Intern Med*. 1965;62(5):867–884. doi:10.7326/0003-4819-62-5-867

49. Nakao S, Lai C-J, Young NS Dengue virus, a flavivirus, propagates in human bone marrow progenitors and hematopoietic cell lines. 1989.

50. Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. *Rev Infect Dis*. 1989;11 (Supplement_4):S830–S9. doi:10.1093/clinids/11.Supplement_4.S830

51. Terada H, Baldini M, Ebbe S, Madoff M. Interaction of influenza virus with blood platelets. *Blood*. 1966;28(2):213–228. doi:10.1182/blood.V28.2.213.213

52. Kabra S, Jain Y, Singhal T, Ratageri V. Dengue hemorrhagic fever: clinical manifestations and management. *Indian J Pediatrics*. 1999;66(1):93. doi:10.1007/BF02752362

53. Kapdi M, Shah I. Dengue and haemophagocytic lymphohistiocytosis. *Scand J Infect Dis*. 2012;44(9):708–709. doi:10.3109/00365548.2011.652667

54. Curwen K, Gimbrone JM, Handin R. In vitro studies of thromboresistance: the role of prostacyclin (PGI2) in platelet adhesion to cultured normal and virally transformed human vascular endothelial cells. Laboratory investigation. *j Technical Methodspathol*. 1980;42 (3):366–374.

55. Stemerman MB. Vascular intimal components: precursors of thrombosis. *Prog Hemost Thromb*. 1974;2:1.

56. Colman RW, Robboy SJ, Minna JD. Disseminated intravascular coagulation (DIC): an approach. *Am J Med*. 1972;52(5):679–689. doi:10.1016/0002-9343(72)90058-7

57. Levi M, Levi M, Ten Cate H, van der Poll T, Sj VD. Pathogenesis of disseminated intravascular coagulation in sepsis. *JAMA*. 1993;270 (8):975–979. doi:10.1001/jama.1993.03510080079035

58. Müller-Berghaus G, Ten Cate H, Levi M. Disseminated intravascular coagulation: clinical spectrum and established as well as new diagnostic approaches. *Thromb Haemost*. 1999;82(08):706–712. doi:10.1055/s-0037-1615900

59. Ten Cate H, Timmerman JJ, Levi M. The pathophysiology of disseminated intravascular coagulation. *Thromb Haemost*. 1999;82 (08):713–717. doi:10.1055/s-0037-1615901

60. Levi M, Ten Cate H. Disseminated intravascular coagulation. *N Engl J Med*. 1999;341(8):586–592. doi:10.1056/NEJM199908193410807

61. Rosenthal RL. Acute promyelocytic leukemia associated with hypofibrinogenemia. *Blood*. 1963;21(4):495–508. doi:10.1182/blood. V21.4.495.495

62. Lodigiani C, Iapichino G, Carenzo L, et al. Venous and arterial thromboembolic complications in COVID-19 patients admitted to an academic hospital in Milan, Italy. *Thromb Res*. 2020;191:9–14. doi:10.1016/j.thromres.2020.04.024

63. Delabranche X, Stiel L, Severac F, et al. Evidence of netosis in septic shock-induced disseminated intravascular coagulation. Shock: injury, Inflammation, and Sepsis. *Lab Clin Approaches*. 2017;47 (3):313–317.

64. Levi M, editor. *Platelets in Critical Illness. Seminars in Thrombosis and Hemostasis*. Thieme Medical Publishers; 2016.

65. Valerio L, Head RN. Neck, and Abdominopelvic Septic Thrombophlebitis: current Evidence and Challenges in Diagnosis and Treatment. *Hämostaseologie*. 2020;40(03):301–310. doi:10.1055/a-1177-5127

66. Levi M, Van der Poll T, Büller HR. Bidirectional relation between inflammation and coagulation. *Circulation*. 2004;109(22):2698–2704. doi:10.1161/01.CIR.0000131660.51520.9A

67. Scully M, How GT. I treat thrombotic thrombocytopenic purpura and atypical haemolytic uraemic syndrome. *Br J Haematol*. 2014;164 (6):759–766. doi:10.1111/bjh.12718

68. Fox SE, Akmatbekov A, Harbert JL, Li G, Brown JQ, Vander Heide RS. Pulmonary and cardiac pathology in African American patients with COVID-19: an autopsy series from New Orleans. *Lancet Respir Med*. 2020;8(7):681–686. doi:10.1016/S2213-2600(20)30243-5

69. Ruggenenti P, Noris M, Remuzzi G. Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int*. 2001;60(3):831–846. doi:10.1046/j.1523-1755.2001.060003831.x

70. Levi M, Scully M, Singer M. The role of ADAMTS-13 in the coagulopathy of sepsis. *J Thrombosis Haemostasis*. 2018;16 (4):646–651. doi:10.1111/jth.13953

71. Schwameis M, Schörgenhofer C, Assinger A, Steiner MM, Jilma B. Von Willebrand factor excess and ADAMTS13 deficiency: a unifying pathomechanism linking inflammation to thrombosis in DIC, malaria, and TTP. *Thromb Haemost*. 2015;113(4):708. doi:10.1160/TH14-09-0731

72. Willan J, Aj K, Djebbari F, et al. Assessing the Impact of Lockdown: fresh Challenges for the Care of Haematology Patients in the COVID-19 Pandemic. *Br J Haematol*. 2020.

73. Peto J. Covid-19 mass testing facilities could end the epidemic rapidly. *BMJ*. 2020;368.

74. Chen L, Li X, Chen M, Feng Y, The XC. ACE2 expression in human heart indicates new potential mechanism of heart injury among patients infected with SARS-CoV-2. *Cardiovasc Res*. 2020;116 (6):1097–1100. doi:10.1093/cvr/cvaa078

75. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. *J Virol*. 2020;94:7. doi:10.1128/JVI.00127-20

76. Gralinski L, Sheahan T, Morrison T, et al. Complement activation contributes to severe acute respiratory syndrome coronavirus pathogenesis. *mBio*. 2018;9:e01753–18. doi:10.1128/mBio.01753-18

77. Jiang Y, Zhao G, Song N, et al. Blockade of the C5a–C5aR axis alleviates lung damage in hDPP4-transgenic mice infected with MERS-CoV. *Emerging Microbes Infect*. 2018;7(1):1–12.

78. Gao T, Hu M, Zhang X, et al. Highly pathogenic coronavirus N protein aggravates lung injury by MASP-2-mediated complement over-activation. *MedRxiv*. 2020.

79. Magro C, Mulvey JJ, Berlin D, et al. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. *Trans Res*. 2020;220:1–13. doi:10.1016/j.trsl.2020.04.007

80. Abbaszadeh-Goudarzi K, Radbakhsh S, Pourhanifeh MH, et al. Circular RNA and diabetes: epigenetic regulator with diagnostic role. *Curr Mol Med*. 2020;20(7):516–526. doi:10.2174/1566524020666200129142106

81. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thrombosis Haemostasis*. 2020;18(4):844–847. doi:10.1111/jth.14768

82. Henry BM, Benoit SW, Hoehn J, Lippi G, Favaloro EJ, Benoit JL, editors. Circulating plasminogen concentration at admission in patients with coronavirus disease 2019 (COVID-19). In: *Seminars in Thrombosis and Hemostasis*. Thieme Medical Publishers; 2020.

83. Christensen B, Favaloro EJ, Lippi G, Van Cott EM. Hematology laboratory abnormalities in patients with coronavirus disease 2019 (COVID-19).*Semin Thrombosis Hemost*. 2020;46(7):845–849.

84. Brogaard Larsen J, Pasalic L, Hvas A. Platelets in coronavirus disease. *Semin Thromb Hemost*. 2020;46(07):823–825. doi:10.1055/s-0040-1710006

85. Akca S, Haji-Michael P, De Mendonça A, Suter P, Levi M, Vincent J-L. Time course of platelet counts in critically ill patients. *Crit Care Med*. 2002;30(4):753–756. doi:10.1097/00003246-200204000-00005

86. Henry BM, De Oliveira MHS, Benoit S, Plebani M, Lippi G. Hematologic, biochemical and immune biomarker abnormalities associated with severe illness and mortality in coronavirus disease 2019 (COVID-19): a meta-analysis. *Clin Chem Lab Med*. 2020;58 (7):1021–1028. doi:10.1515/cclm-2020-0369

87. Obe BH, Retter A, McClintock C. Practical guidance for the prevention of thrombosis and management of coagulopathy and disseminated intravascular coagulation of patients infected with COVID-19. 2020.

88. Akima S, McLintock C, Hunt BJ. RE: ISTH interim guidance to recognition and management of coagulopathy in COVID-19. *J Thrombosis Haemostasis*. 2020;18(8):2057–2058. doi:10.1111/jth.14853

89. Barrett CD, Moore HB, Yaffe MB, Moore EE. ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thrombosis Haemostasis*. 2020;18(8):2060–2063. doi:10.1111/jth.14860

Primary Cauda Equina Lymphoma Treated with CNS-Centric Approach: A Case Report and Literature Review

Justin J Kuhlman, Muhamad Alhaj Moustafa, Vivek Gupta, Liuyan Jiang & Han W Tun

Published online: 21 Jul 2021.

Submit your article to this journal ⬚

Article views: 164

View related articles ⬚

View Crossmark data ⬚

Citing articles: 5 View citing articles ⬚

CASE REPORT

Primary Cauda Equina Lymphoma Treated with CNS-Centric Approach: A Case Report and Literature Review

Justin J Kuhlman [1]
Muhamad Alhaj Moustafa [2]
Vivek Gupta[3]
Liuyan Jiang [4]
Han W Tun [2]

[1]Department of Internal Medicine, Mayo Clinic Florida, Jacksonville, FL, USA; [2]Division of Hematology and Medical Oncology, Mayo Clinic Florida, Jacksonville, FL, USA; [3]Department of Radiology, Mayo Clinic Florida, Jacksonville, FL, USA; [4]Department of Pathology and Laboratory Medicine, Jacksonville, FL, USA

Abstract: Primary cauda equina lymphoma is an extremely rare entity previously documented in only 24 reported cases. Primary cauda equina lymphoma represents a subtype of neurolymphomatosis, which occurs when lymphoma cells with neurotropism infiltrate and destroy peripheral nerves, spinal nerve roots, nerve plexuses and cranial nerves. The cauda equina is an anatomic structure located in the lower part of the spinal canal consisting of multiple lumbar and sacral nerve roots. Herein, we report a unique case of primary cauda equina diffuse large B-cell lymphoma presenting as a tumor mass in the lower spinal canal, which was treated with a CNS-centric treatment approach followed by autologous hematopoietic stem cell transplantation.

Keywords: primary cauda equina lymphoma, neurolymphomatosis, diffuse large B cell cauda equina lymphoma, MATRIX chemoimmunotherapy, autologous stem cell transplant

Introduction

In rare instances, malignant lymphoma cells can be discovered as primary lesions originating within the spinal cord, and even more rarely, within the cauda equina itself. The cauda equina is an anatomic structure in a crowded lower spinal canal consisting of multiple lumbar and sacral nerve roots. Primary cauda equina lymphoma (PCEL) presents as a primary lesion within the cauda equina, and has only been reported in a limited number of case reports. PCEL represents an extremely rare and distinct subcategory of neurolymphomatosis. Neurolymphomatosis is a rare lymphomatous manifestation in which lymphoma cells with neurotropism infiltrate and destroy peripheral nerves, spinal nerve roots, nerve plexuses and cranial nerves. Histologically, the majority of neurolymphomatosis cases involve B cell lymphomas, with previous studies documenting frequencies of B-cell involvement as high as 82% and 97.5%.[1–3] Occurring less frequently, T-cell lymphomas have been found to range anywhere from 2.5% to 10% of cases.[1,3] Typically presenting due to secondary involvement from systemic lymphoma, primary neurolymphomatosis is extremely rare. PCEL appears to comprise less than 1% of neurolymphomatosis cases.[2] We report a unique case in which diffuse large B-cell lymphoma involved multiple nerve roots in the cauda equina, presenting as a large tumor occupying the lower spinal canal. Moreover, we describe and highlight the unique treatment approach incorporated in the case of our patient.

Case Presentation

A 55 year-old Asian female patient presented to an outside facility with complaints of tingling, numbness, and significant pain radiating into both lower extremities. Her pain was accompanied by loss of bowel and bladder function as well as significant weakness in both legs resulting
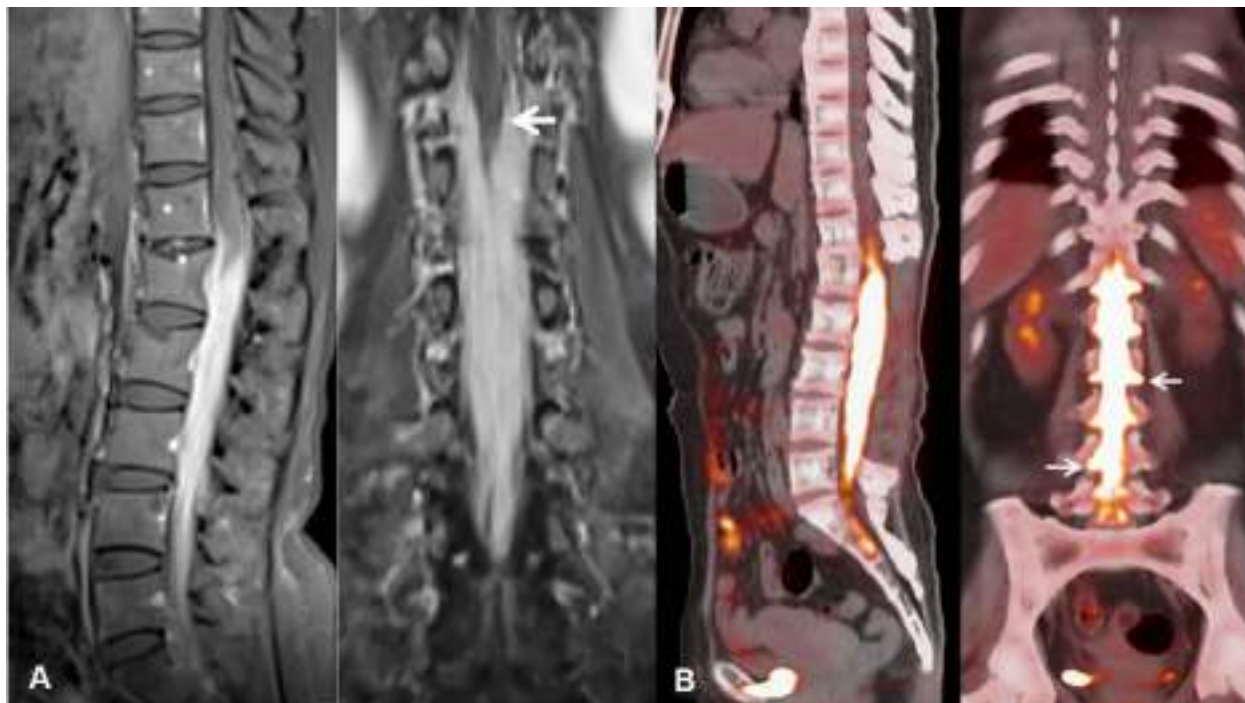
Figure 1 (**A**) Gadolinium enhanced sagittal and coronal T1 MRI showing diffuse involvement of cauda equina with encasement of conus medullaris (arrows). (**B**) Staging whole-body 18F-FDG PET-CT coronal and sagittal views showing hypermetabolic mass in the cauda equina and lumbar nerve root sleeves (arrows).
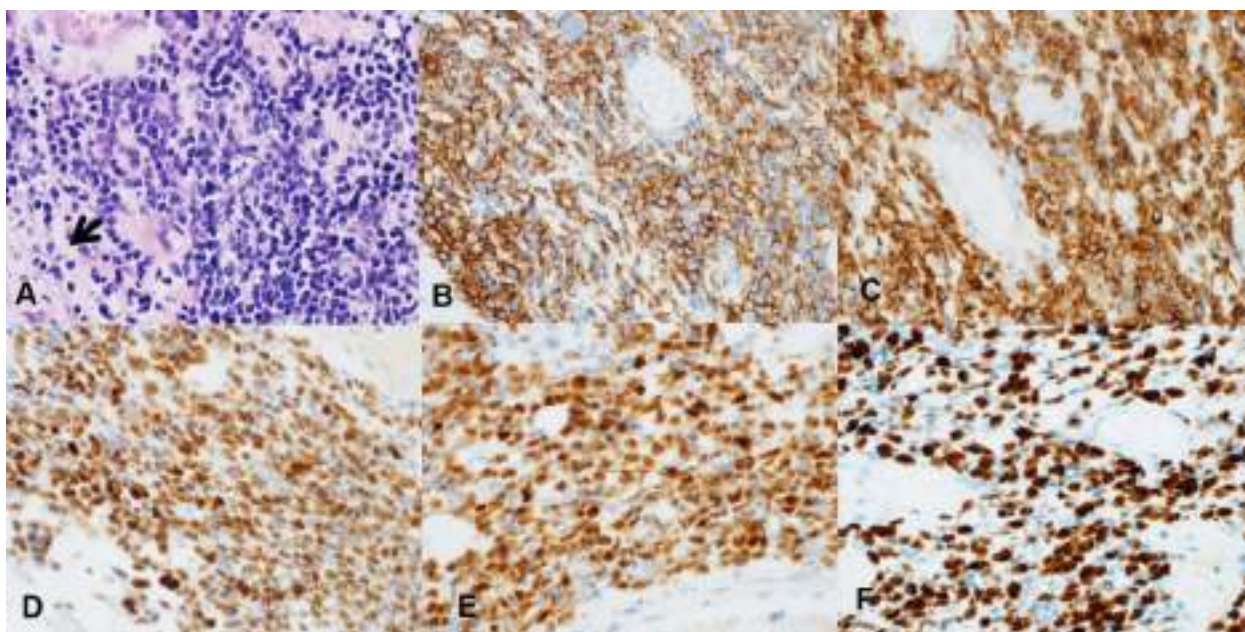
Figure 2 (**A**) Pathology examination by H&E staining revealing diffusely infiltrating large atypical lymphocytes dissecting through the nerve bundles (arrow indicating residual nerve). IHC studies showed that the neoplastic lymphocytes were positive for (**B**) CD20, (**C**) BCL2, (**D**) BCL6, (**E**) MUM1, (**F**) with a high proliferate rate (>90%) by Ki-67.

in multiple falls, inability to ambulate and subsequent wheel-chair dependency. She further complained of consistent radicular pain radiating around her abdomen. Initial magnetic resonance imaging (MRI) of the lumbar spine identified a homogeneously enhancing mass diffusely infiltrating the cauda equina and filum terminale, superiorly encasing the conus medullaris (Figure 1A). PET-CT scan of the whole body revealed a markedly hypermetabolic tumor occupying the spinal canal and nerve root sleeves from T12 to S1 with no evidence of the disease outside the spine (Figure 1B).

The patient underwent L1-L4 lumbar laminectomy with biopsy of the tumor. Hematoxylin and eosin (H&E) sections of the tumor demonstrated diffusely infiltrating large atypical lymphocytes encasing the lumbar nerve (Figure 2A). Immunohistochemical (IHC) studies showed that the neoplastic lymphocytes were positive for CD20, CD79a, BCL6, BCL2, and MUM1 (Figure 2B–E); negative for CD10, TdT, cyclin D1, and MYC. The proliferative rate by ki-67 was also high (>90%) (Figure 2F). Fluorescence in situ hybridization (FISH) analysis from resected tissue was negative for MYC and BCL2 rearrangement, but positive for BCL6 rearrangement in 80% of nuclei. Cerebrospinal fluid (CSF) cytology was positive for lymphoma cells. These findings were consistent with activated B cell subtype of diffuse large B-cell lymphoma (ABC-DLBCL). A bone marrow aspirate and biopsy were negative for lymphomatous involvement.

The patient was subsequently initiated on systemic therapy with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) with mid-cycle high-dose methotrexate (HD-MTX). After completing 5 cycles of treatment, restaging MR of the spine and PET-CT scans revealed some residual hypermetabolic abnormalities within the spine despite improved resolution of the spinal lesion. It was at this time that the patient presented to our department for further management and evaluation for potential autologous stem cell transplant. Although her neurological status seemed to be somewhat improved with increased strength in her lower extremities, the patient continued to endorse sensory symptoms in the lower extremities and abdomen as well as significant weakness in her right lower extremity. These clinical findings in combination with restaging PET scan indicated a partial response to previous treatment with R-CHOP and HD-MTX.

She was then initiated on consolidation treatment with 2 cycles of methotrexate, cytarabine, thiotepa, and rituximab (MATRIX) chemoimmunotherapy due to previously reported success of MATRIX therapy in patients with primary central nervous system lymphoma (PCNSL).[4] Following the first cycle of MATRIX, the patient reported significant improvement in symptoms as her legs felt much stronger and she was finally able to ambulate on her own with the assistance of a walker. Restaging following MATRIX therapy with PET-CT, MRI of the brain, and MRI of the spine revealed complete resolution of the original lesion with no evidence of disease elsewhere in the body (Figure 3). She then underwent high-dose Bis-chloroethyl nitrosourea (BCNU) plus thiotepa followed by autologous hematopoietic stem cell transplantation (auto-SCT). Restaging after auto-SCT with MRI of the thoracic and lumbar spine at day +100 continued to reveal no evidence of disease. She was then initiated on maintenance therapy with Ibrutinib 560 mg daily. At 18 months following the initial diagnosis, the patient has remained in complete remission and her neurological status has greatly improved with regaining of her right lower extremity strength with the help of intensive physical therapy.

Discussion and Literature Review

The clinical and pathologic findings for our patient are most consistent with PCEL with CSF involvement. Her presenting neurological findings fit the classical presentation of cauda equina syndrome. Radiologic review of her imaging scans from the time of initial presentation did not show any evidence of intramedullary involvement of the spinal cord, but they did reveal a mass encasing multiple nerve roots in the cauda equina. Unlike typical neurolymphomatosis which most commonly represents a challenging diagnostic entity due to patchy nerve involvement requiring multiple biopsies and investigations over time,[5,6] our patient presented with a solid tumor due to lymphomatous involvement of multiple nerve roots in the cauda equina region in a tight spinal canal. This unique presentation, in contrast to most cases of neurolymphomatosis, allowed for a more expedient diagnosis and prevented any delays in treatment. The pathologic findings in our case were consistent with activated B cell subtype of DLBCL associated with BCL6 translocation. The immunophenotype is similar to that of PCNSL; there was positive expression of CD20, BCL6, and MUM1.

Our patient was initially treated with R-CHOP with mid-cycle HD-MTX achieving a partial response. Under our care, she was further treated with two cycles of MATRIX resulting in complete remission. Although
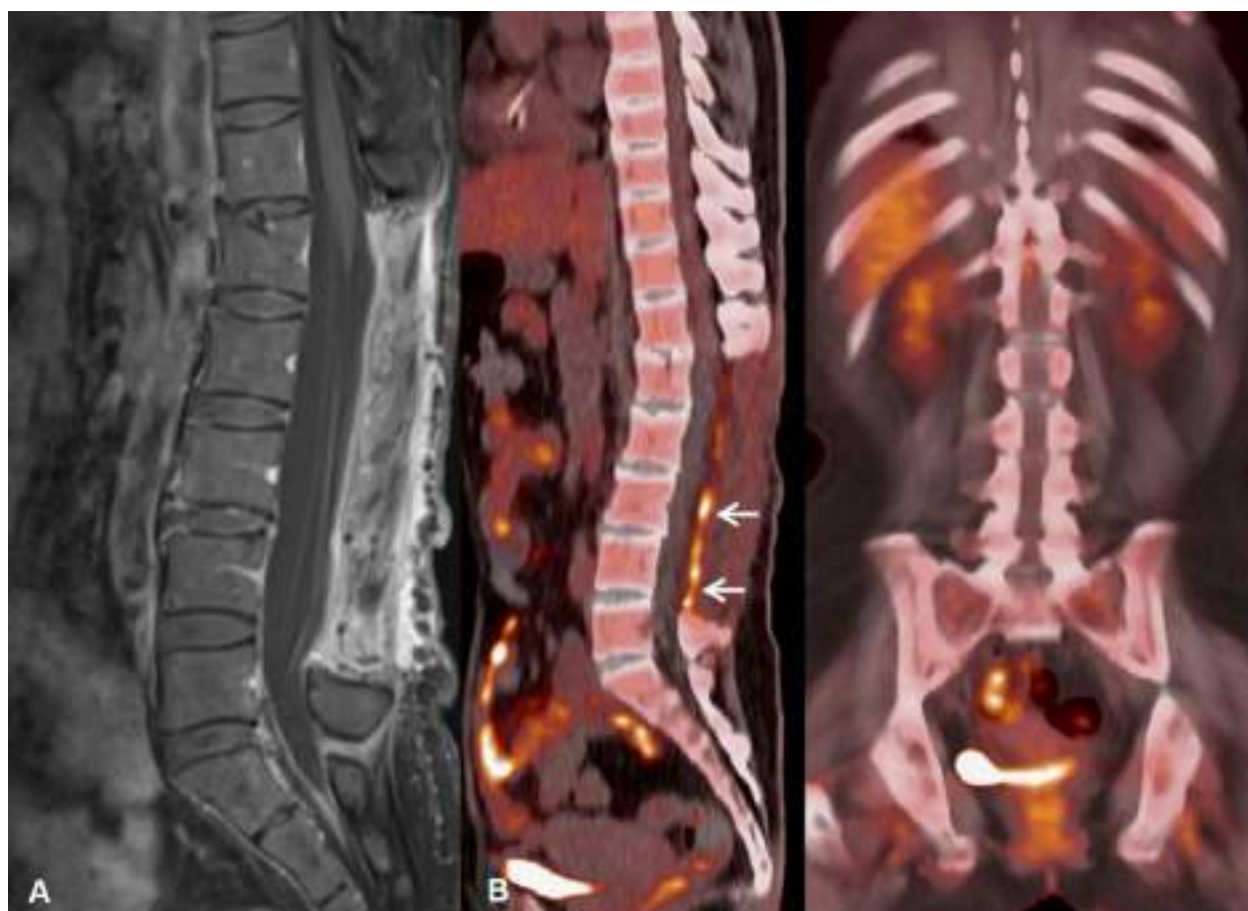
Figure 3 Follow up MRI (**A**) and PET-CT (**B**) after chemotherapy showing complete resolution of the hypermetabolic cauda equina lesion. Linear FDG uptake in the posterior lumbar dura (arrows) is postoperative in nature.

radiation therapy was originally offered to the patient as an alternative consolidation therapy, the patient declined any radiation therapy and elected to undergo high-dose chemotherapy followed by auto-SCT. Since it is well known that PCNSL is more amenable to cure at initial diagnosis that in the relapsed/refractory setting, we believed that it was critical for our patient to receive CNS penetrating agents to ensure that the entire CNS was permeated with therapeutic agents. Because we felt that she did not receive good CNS coverage during her initial induction treatment, it was decided that she receive consolidation therapy with two cycles of MATRIX therapy. She was further consolidated with high-dose BCNU and Thiotepa chemotherapy followed by auto-SCT. She has remained in complete remission following the transplant. We decided to put her on maintenance Ibrutinib as her lymphoma was aggressive with significant neurological impacts. Ibrutinib has previously shown significant therapeutic efficacy in ABC-DLBCL and PCNSL with excellent CNS penetration.[7–11]

It is also pertinent to emphasize the importance of the role of intensive physical therapy, which tremendously helped our patient during her recovery.

Our medical literature review identified 25 PCEL patients (Table 1) with a median age of 55.5 years (range 11–79) at the time of diagnosis and a 3:2 male to female predominance.[12–34] Of those cases that describe lymphoma type, 91% (22/24) classify as B-cell lymphoma whereas only 9% (2/24) describe lymphomas that are T-cell in origin. Diffuse large B-cell lymphoma clearly appears to be the most common subtype as it comprises 77% of those lymphomas that are B-cell in nature and 72% of all PCEL cases in general (17/24). It is also worth noting that over half of patients presented with a mass-like lesion in the cauda equina region on imaging of the spine (62.5%, 15/24) and CSF involvement by lymphoma was reflected on cytology in more than half of cases when lumbar puncture was performed (52.6%, 10/19). Of the 23 cases which included descriptions of treatment, the most common

Table 1 Traits of 25 PCEL Patients Reported in the Literature

Reference	Age/ Sex	Pathology	CSF Involvement (Cytology)	Mass- Like Lesion	Treatment	Outcome
Nakashima[2014]	59/M	DLBCL	Yes	Yes	Radiotherapy, intravenous methotrexate	Alive; 1 year
Mauney[1983]	68/F	DLBCL	Yes	Yes	Radiotherapy	Alive; 3 months
Toner[1989]	59/M	DLBCL	Yes	No	Radiotherapy, intrathecal methotrexate, intravenous cyclophosphamide, adriamycin, vincristine, etoposide, and prednisolone	Alive; 2 years
Klein[1990]	29/F	B-cell lymphoma	No	Yes	Tumor resection	Died; 5 weeks
Knopp[1994]	69/F	N/a	N/a	No	N/a	N/a
Ooi[1996]	16/M	T-lymphoblastic lymphoma	N/a	Yes	Radiotherapy, intrathecal methotrexate, intravenous methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and dexamethasone	Died; 8 months
Zagami[2003]	71/F	DLBCL	Yes	No	Intrathecal methotrexate, cyclophosphamide, doxorubicin, vincristine, prednisone	Died; 16 months
Kumar[2005]	60/M		No	No	IVIG, Rituximab	Alive; 6 months
Tajima[2007]	67/F	DLBCL	No	Yes	Radiotherapy and intrathecal methotrexate, IVIG, intravenous carboplatin	Alive; 3 years
Morita[2009]	67/M	NK/T-cell lymphoma	N/a	Yes	Radiotherapy, surgical resection, and etoposide	Died; 14 months
Teo[2012]	58/M	DLBCL	No	Yes	Radiotherapy, steroids, and intravenous chemotherapy	Alive; 2 years
Iwasaki[2012]	69/M	DLBCL	N/a	No	Radiotherapy and intravenous methotrexate	Died; 1.5 years
Nishida[2012]	47/M	DLBCL	Yes	Yes	Radiotherapy, intravenous methotrexate and cytarabine, and intrathecal methotrexate, cytarabine, prednisolone	Alive; 1.5 years
Broen[2014]	75/F	DLBCL	No	No	Oral dexamethasone	Died; 10 months
Broen[2014]	71/F	DLBCL	No	No	Intravenous doxorubicin, vincristine, cyclophosphamide, prednisone, and rituximab, and intrathecal methotrexate.	Alive; n/a
Shin[2016]	79/F	DLBCL	N/a	Yes	Radiotherapy and chemotherapy	Alive; N/A
Belcastro[2016]	47/M	DLBCL	No	Yes	Intravenous steroids, intrathecal methotrexate, cytarabine, and rituximab	Died; 2 months
Giobbia[1999]	30/F	DLBCL	Yes	No	Radiotherapy, intrathecal methotrexate, cytosine arabinoside, and hydrocortisone	Alive; 1 year
Khong[2008]	16/M	DLBCL	No	Yes	Radiotherapy, intravenous dexamethasone, cyclophosphamide, cytarabine, doxorubicin, leucovorin, methotrexate, vincristine, rituximab	Alive; 1 year

(*Continued*)

Table 1 (Continued).

Reference	Age/ Sex	Pathology	CSF Involvement (Cytology)	Mass- Like Lesion	Treatment	Outcome
Beitzke[2010]	69/M	DLBCL	Yes	No	Glucocorticoid and intravenous chemotherapy	Died; days after diagnosis
Cugati[2012]	11/M	B-cell NHL	N/a	Yes	Radiotherapy and intravenous cyclophosphamide, doxorubicin, vincristine, prednisone	Alive; 1 year
Wang[2016]	69/M	B-cell nerve	Yes	Yes	N/a	N/a
Sasaki[2019]	62/M	B-cell lymphoma	No	No	Intravenous methotrexate and rituximab, radiotherapy	Alive; 2 years
Suzuki[2018]	65/M	DLBCL	Yes	Yes	Intravenous cytarabine and methotrexate	Alive; 6 years
Current Case	55/F	DLBCL	Yes	Yes	Intravenous rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone, methotrexate, cytarabine, thiotepa, BCNU, auto-SCT	Alive; 1.5 years

Abbreviations: DLBCL, Diffuse large B-cell lymphoma; NHL, Non-Hodgkin lymphoma; LPL, Lymphoplasmacytic lymphoma.

method of treatment appears to be both chemotherapy and radiation (56.5%), followed by chemotherapy (30.4%), and lastly radiation (4.3%), tumor resection (4.3%), and oral steroids (4.3%). Similar to the standard treatment of lymphoma with CNS involvement, the majority of chemotherapy regimens in PCEL patients included CNS penetrating agents, including high dose methotrexate and cytarabine. The death rate at the time of publication in this review appears to be 34.8% (8/23). Of those patients that survived longer than a year, the large majority were treated with systemic chemotherapy incorporating CNS penetrating agents (85.7%, 12/14). Our case appears to be the first to incorporate MATRIX therapy for CNS penetration as well as the only report to include auto-SCT as part of the treatment for PCEL.

Conclusion

In conclusion, PCEL is a rare, yet frequently aggressive lymphoma that can manifest as a mass lesion in the lower spinal canal. The congregation and conglomeration of multiple nerve roots involved by neurolymphomatosis in a tight anatomical space helps facilitate a proper diagnosis and prevents delays in treatment. We propose that PCEL should be treated like PCNSL, with regimens consisting of CNS-penetrating agents. CNS-penetrating high-dose chemotherapy followed by auto-SCT should be considered in eligible patients.

Ethics and Consent

Written informed consent was obtained from the patient for the publication of this manuscript and any accompanying images. Institutional approval was not required for publication.

Disclosure

Dr Han W Tun reports grants, personal fees from Acrotech pharmaceutical, grants from Celgene, grants from Bristol Myers Squibb, grants from Mundipharma, grants from TG Therapeutic, grants from Curis, grants from Zhejiang DTRM Biopharma, outside the submitted work. In addition, Dr Han W Tun has a patent 9464093 (USA), a patent 9839632 (USA), and a patent 10301273 (USA). The authors declare that there are no other conflicts of interest regarding the publication of this manuscript.

References

1. Grisariu S, Avni B, Batchelor TT, et al. Neurolymphomatosis: an international primary CNS lymphoma collaborative group report. *Blood*. 2010;115(24):5005–5011.

2. Baehring JM, Damek D, Martin EC, Betensky RA, Hochberg FH. Neurolymphomatosis. *Neuro Oncol*. 2003;5(2):104–115. doi:10.1215/15228517-5-2-104

3. Novo M, Nowakowski G, Micallef I, Ristow K, Habermann T, Johnston P. Clinical features, treatment and outcome of neurolymphomatosis: single institution experience. *Age*. 2019;60:37–83.

4. Ferreri AJ, Cwynarski K, Pulczynski E, et al. Chemoimmunotherapy with methotrexate, cytarabine, thiotepa, and rituximab (MATRix regimen) in patients with primary CNS lymphoma: results of the first randomisation of the International Extranodal Lymphoma Study Group-32 (IELSG32) Phase 2 trial. *Lancet Haematol*. 2016;3(5): e217–e227. doi:10.1016/S2352-3026(16)00036-3

5. Keddie S, Nagendran A, Cox T, et al. Peripheral nerve neurolymphomatosis: clinical features, treatment, and outcomes. *Muscle Nerve*. 2020;62(5):617–625. doi:10.1002/mus.27045

6. Li V, Jaunmuktane Z, Cwynarski K, Carr A. Diagnostic delay in a case of T-cell neurolymphomatosis. *BMJ Case Rep*. 2019;12(12): e232538. doi:10.1136/bcr-2019-232538

7. Grommes C, Pastore A, Gavrilovic I, et al. *Single-Agent Ibrutinib in Recurrent/Refractory Central Nervous System Lymphoma*. Washington, DC: American Society of Hematology; 2016.

8. Grommes C, Younes A. Ibrutinib in PCNSL: the curious cases of clinical responses and aspergillosis. *Cancer Cell*. 2017;31 (6):731–733. doi:10.1016/j.ccell.2017.05.004

9. Lionakis MS, Dunleavy K, Roschewski M, et al. Inhibition of B cell receptor signaling by ibrutinib in primary CNS lymphoma. *Cancer Cell*. 2017;31(6):833–843. e835. doi:10.1016/j.ccell.2017.04.012

10. Soussain C, Choquet S, Blonski M, et al. Ibrutinib monotherapy for relapse or refractory primary CNS lymphoma and primary vitreoretinal lymphoma: final analysis of the Phase II 'proof-of-concept'iLOC study by the Lymphoma study association (LYSA) and the French oculo-cerebral lymphoma (LOC) network. *Eur J Cancer*. 2019;117:121–130.

11. Wilson WH, Young RM, Schmitz R, et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat Med*. 2015;21(8):922–926. doi:10.1038/nm.3884

12. Beitzke M, Enzinger C, Beitzke D, Neureiter D, Ladurner G, Fazekas F. Primary leptomeningeal lymphoma of the cauda equina: a rare cause of radiculopathy. *J Neurol*. 2010;257(10):1734–1737. doi:10.1007/s00415-010-5574-8

13. Belcastro V, Bellocchi S, Patriarca C, et al. Cauda equina syndrome due to large B-cell lymphoma: a case report. *Neurol Sci*. 2016;37 (5):825–827. doi:10.1007/s10072-015-2470-0

14. Broen M, Draak T, Riedl RG, Weber WE. Diffuse large B-cell lymphoma of the cauda equina. *Case Rep*. 2014;2014: bcr2014205950.

15. Cugati G, Singh M, Symss NP, Pande A, Vasudevan M, Ramamurthi R. Primary spinal intradural extramedullary lymphoma causing cauda equina syndrome. *J Craniovertebr Junction Spine*. 2012;3(2):58. doi:10.4103/0974-8237.116538

16. Giobbia M, Carniato A, Scotton P, Vaglia A, Marchiori G. Primary EBV-associated cauda equina lymphoma. *J Neurol*. 1999;246 (8):739–740. doi:10.1007/s004150050445

17. Iwasaki M, Hida K, Yano S, Aoyama T, Kaneko S, Iwasaki Y. Primary cauda equina lymphoma treated with high-dose methotrexate. *Neurol Med Chir (Tokyo)*. 2012;52(9):679–683. doi:10.2176/nmc.52.679

18. Khong P, Pitham T, Owler B. Isolated neurolymphomatosis of the cauda equina and filum terminale: case report. *Spine*. 2008;33(21): E807–E811. doi:10.1097/BRS.0b013e31818441be

19. Klein P, Zientek G, VandenBerg SR, Lothman LE. Primary CNS lymphoma: lymphomatous meningitis presenting as a cauda equina lesion in an AIDS patient. *Can J Neurol Sci*. 1990;17(3):329–331. doi:10.1017/S0317167100030687

20. Knopp EA, Chynn KY, Hughes J. Primary lymphoma of the cauda equina: myelographic, CT myelographic, and MR appearance. *Am J Neuroradiol*. 1994;15(6):1187–1189.

21. Kumar N, Dyck PJB. Hypertrophy of the nerve roots of the cauda equina as a paraneoplastic manifestation of lymphoma. *Arch Neurol*. 2005;62(11):1776. doi:10.1001/archneur.62.11.1776

22. Mauney M, Sciotto CG. Primary malignant lymphoma of the cauda equina. *Am J Surg Pathol*. 1983;7(2):185–190. doi:10.1097/ 00000478-198303000-00009

23. Morita M, Osawa M, Naruse H, Nakamura H. Primary NK/T-cell lymphoma of the cauda equina: a case report and literature review. *Spine*. 2009;34(24):E882–E885. doi:10.1097/ BRS.0b013e3181b29de6

24. Nakashima H, Imagama S, Ito Z, et al. Primary cauda equina lymphoma: case report and literature review. *Nagoya J Med Sci*. 2014;76 (3–4):349.

25. Nishida H, Hori M, Obara K. Primary B-cell lymphoma of the cauda equina, successfully treated with high-dose methotrexate plus high-dose cytarabine: a case report with MRI findings. *Neurol Sci*. 2012;33(2):403–407. doi:10.1007/s10072-011-0752-8

26. Ooi G, Peh W, Fung C. Magnetic resonance imaging of primary lymphoma of the cauda equina. *Br J Radiol*. 1996;69 (827):1057–1060. doi:10.1259/0007-1285-69-827-1057

27. Sasaki R, Ohta Y, Yamada Y, et al. A case of neurolymphomatosis in the cauda equina diagnosed by an open biopsy. *Intern Med*. 2019;1018–1049.

28. Shin HK, Oh SK, Woo CG, Huh JR, Suh CW, Jeon SR. Cauda equina lymphoma mimicking non-neoplastic hypertrophic neuropathy of the cauda equina: a case report. *Br J Neurosurg*. 2016;30(6):678–680. doi:10.3109/02688697.2015.1111295

29. Suzuki K, Yasuda T, Hiraiwa T, Kanamori M, Kimura T, Kawaguchi Y. Primary cauda equina lymphoma diagnosed by nerve biopsy: a case report and literature review. *Oncol Lett*. 2018;16 (1):623–631.

30. Tajima Y, Sudo K, Matumoto A. Malignant lymphoma originating in the cauda equina mimicking the inflammatory polyradiculoneuropathy. *Intern Med*. 2007;46(13):1029–1032. doi:10.2169/internalmedicine.46.0005

31. Teo MK, Mathieson C, Carruthers R, Stewart W, Alakandy L. Cauda equina lymphoma–a rare presentation of primary central nervous system lymphoma: case report and literature review. *Br J Neurosurg*. 2012;26(6):868–871. doi:10.3109/ 02688697.2012.697225

32. Toner GC, Holmes R, Sinclair RA, Tang S, Schwarz MA. Central nervous system lymphoma: primary lumbar nerve root infiltration. *Acta Haematol*. 1989;81(1):44–47. doi:10.1159/000205399

33. Wang G, Liu Y, He F. Primary lymphoma involving cranial nerves and cauda equina detected by 18F-FDG PET/CT and MRI. *Nuklearmedizin*. 2016;55(05):N46–N48. doi:10.1055/s-0037-1616497

34. Zagami A, Granot R. Non-Hodgkin's lymphoma involving the cauda equina and ocular cranial nerves: case reports and literature review. *J Clin Neurosci*. 2003;10(6):696–699. doi:10.1016/S0967-5868(03) 00089-4

Management of Adult Patients with Immune Thrombocytopenia (ITP): A Review on Current Guidance and Experience from Clinical Practice

Fei Song & Hanny Al-Samkari

Published online: 26 Jul 2021.

Submit your article to this journal ↗

View related articles ↗

View Crossmark data ↗

REVIEW

Management of Adult Patients with Immune Thrombocytopenia (ITP): A Review on Current Guidance and Experience from Clinical Practice

Fei Song[1,2]
Hanny Al-Samkari [2,3]

[1]Department of Medicine, Massachusetts General Hospital, Boston, MA, USA; [2]Harvard Medical School, Boston, MA, USA; [3]Division of Hematology, Massachusetts General Hospital, Boston, MA, USA

Abstract: Immune thrombocytopenia (ITP) is an autoimmune process resulting in increased destruction and inadequate production of platelets that can result in bleeding, fatigue, and reduced health-related quality of life. While treatment is not required for many patients with ITP, the occurrence of bleeding manifestations, severe thrombocytopenia, and requirement for invasive procedures are among the reasons necessitating initiation of therapy. Corticosteroids, intravenous immunoglobulin, and anti-RhD immune globulin are typical first-line and rescue treatments, but these agents typically do not result in a durable remission in adult patients. Most patients requiring treatment therefore require subsequent line therapies, such as thrombopoietin receptor agonists (TPO-RAs), rituximab, fostamatinib, splenectomy, or a number of other immunosuppressive agents. In this focused review, we discuss management of adult ITP in the acute and chronic settings.

Keywords: platelets, immune thrombocytopenia, ITP, treatment, corticosteroids, IVIG, splenectomy, thrombopoietin receptor agonist, rituximab, fostamatinib

Introduction

Immune thrombocytopenia (ITP) results from autoimmune destruction of platelets in the reticuloendothelial system due to platelet autoantibodies and other immune mechanisms, resulting in increased platelet turnover as well as inadequate platelet production.[1–5] Primary ITP is defined as an isolated thrombocytopenia $<100 \times 10^9$/L in the absence of other causes or disorders that may be associated with thrombocytopenia, as distinguished from secondary ITP, which is associated with other conditions such as infections, drug effects, rheumatological diseases, or lymphoproliferative disorders.[6,7] The incidence of ITP in the US population is approximately 6.1 per 100,000 persons per year, or 13.7 per 100,000 persons per year in those 65 years or greater, and results in significant economic burden.[8] Clinical presentation can vary between asymptomatic to severe bleeding complications, and prior to 2010, fatal bleeding rates were estimated at 1.62–3.89 cases per 100 patient-years and predicted 5-year mortality rates varied from 2.2% for persons <40 years up to 47.8% for those aged >60 years.[9] Although many laboratory studies can support diagnosis or guide treatment selection, ultimately diagnosis is clinical, after ruling out other etiologies of thrombocytopenia.[7,10,11]

Given the wide variation in presentation, not all patients require treatment immediately after diagnosis. The American Society of Hematology (ASH)

Correspondence: Hanny Al-Samkari
Division of Hematology, Massachusetts General Hospital, Suite 118, Room 112, Zero Emerson Place, Boston, MA, 02114, USA
Tel +617-643-6214
Fax +617-643-8840
Email hal-samkari@mgh.harvard.edu

Immune Thrombocytopenia Clinical Guideline provides a grading system for severity of ITP, defining severe ITP as clinical bleeding requiring treatment.[7] Of note, the degree of thrombocytopenia is not reliably indicative of bleeding risk when platelets are above 10×10^9/L.[12–15] Factors thought to increase risks of bleeding include reduced platelet count, female sex, and exposure to NSAIDs.[16] There is also concern of increased frequency of intracranial hemorrhage in elderly patients aged ≥70 years when platelets are less than $20–30 \times 10^9$/L, so clinicians should also consider age when deciding on a threshold for treatment.[17] The goal of treatment of ITP is to reduce bleeding risks by raising platelet counts. Treatment must be individualized, accounting for an individual patient's risks of bleeding given their history of bleeding, trauma risks, and the risk of adverse events from therapeutics.

In this focused review article, we will discuss the treatment of adult ITP, incorporating the most recent evidence as well as expert opinion.

Treatment of ITP
Response Criteria
The International Working Group (IWG) defines complete response (CR) to ITP treatment as a platelet count ≥100 x 10^9/L and absence of bleeding and response (R) as platelet count ≥ 30 x 10^9/L and >2 fold increase in platelet count from baseline and absence of bleeding, both measured on 2 occasions greater than 7 days apart.[6] No response (NR), per the IWG definition, is characterized by a platelet count <30 x 10^9/L or a less than 2-fold increase in platelet count from baseline, or the presence of bleeding.[6]

First-Line/Rescue Treatments
Corticosteroids
The standard first-line treatment and most common rescue therapy in newly diagnosed ITP are corticosteroids, often with prednisone (0.5–2 mg/kg daily for a 4–8 week tapering course) or high-dose dexamethasone (40 mg daily for 4 days for 1–4 cycles).[7,10,18] Corticosteroids have been shown to decrease capillary permeability, reduce platelet autoantibody production, increase platelet production, increase myeloid-derived suppressor cells, and change T cell subsets to decrease platelet destruction.[19–24]

Overall, the choice of corticosteroid agent should be made in consideration of adverse event risk and the need for a rapid response. In a meta-analysis comparing prednisone with dexamethasone in previously untreated adult primary ITP, one to three courses of high dose dexamethasone, compared with prednisone 1 mg/kg for 4 weeks with taper, showed a platelet count response (79% vs 58%) at 14 days, but there was no difference in overall platelet response at 6 months (54% vs 53%) or rates of sustained response.[25] High-dose dexamethasone may be more likely to precipitate acute psychotic complications in the elderly or those with a history of psychiatric disease, however, and this should be considered upon agent selection.

Intravenous Immunoglobulin (IVIG) and Intravenous Anti-RhD Immune Globulin
Intravenous immunoglobulin (IVIG) is another common first-line or rescue therapy often employed when a patient presents with significant bleeding. It can be administered when a more rapid increase in platelet count is required, and also be added to corticosteroid therapy or when corticosteroids are contraindicated.[7] IVIG is recommended to be given at a dose of 1 g/kg daily for 1–2 days (high-dose) or 0.4 g/kg daily for up to 5 days (low-dose).[10] IVIG has many complex mechanisms of action in decreasing inflammatory processes by multiple pathways, including inhibition of the IgG Fc receptor, which is crucial to link the adaptive and innate immune systems, inhibiting phagocytosis and suppression and/or elimination of platelet autoantibodies.[26–30]

In one meta-analysis, effect rate, time of cessation of bleeding, and rate of development of chronic ITP was not statistically different between high-dose and low-dose IVIG for acute ITP and low-dose IVIG was associated with decreased risk of side effects.[31] In a case-control study by Zhou et al., there was no difference in therapeutic response in groups receiving IVIG doses between 0.2–0.4 g/kg/day, which suggests that ITP patients could be treated more cost-effectively by lower conventional dosages of IVIG.[32]

Intravenous anti-RhD immune globulin (administered at a dose of 50 mcg/kg to 75 mcg/kg daily)[33,34] is an alternative to IVIG for non-splenectomized, Rh (+) patients. It is thought to saturate macrophage Fc receptors with anti-D coated RBCs to prevent destruction of autoantibody-coated platelets.[35] In one study, IVIG and IV anti-RhD immune globulin treatments of patients with ITP yielded no statistical difference in cumulative response and remission rates.[36] Anti-RhD immune globulin induces a controlled hemolysis, with a majority of patients experiencing a decrease in hemoglobin concentration of 0.5–2.0 g/dL 3–7 days after infusion, with recovery

to baseline within 3 weeks of administration.[37] Rarely, this hemolysis can degenerate into life-threatening disseminated intravascular coagulation.

Second/Subsequent Line Therapies

An estimated 68% of adult patients develop persistent ITP despite first-line treatments including corticosteroids and IVIG.[38] There are no randomized controlled trials directly comparing the different second-line therapy options, so the results of placebo-controlled trials of second-line therapy are examined. The choice of second-line therapy is primarily based on patient values and priorities as well as available resources.

Based on the available data, the most recent American Society of Hematology ITP clinical guidelines conditionally recommend TPO-RA rather than rituximab and rituximab is recommended over splenectomy, though choice of treatment should be individualized, e.g. depending on patient goals of avoidance of surgery, achieving durable response, or avoidance of long-term medications.[39]

Splenectomy

Splenectomy is an effective treatment as the spleen is a site of platelet destruction as well as a site of antibody production. Splenectomy has been shown to help 74% of patients achieve sustained CR lasting more than 6 months-[40] and 64% after a minimum of 5 years.[41] Previous studies have shown that mortality was 1.0% (48 of 4955 patients) with laparotomy and 0.2% (3 of 1301 patients) with laparoscopy.[42] Splenectomy also increases infection risk as well, with reports of sepsis in 2.1% of splenectomized patients[43] as well as a 2-4-fold risk of venous thromboembolism.[44,45] Given risks of the surgery and potential for spontaneous remission of ITP within the first year, splenectomy as a treatment option should be deferred until the patient is confirmed to have chronic ITP (ITP lasting for more than 12 months), when the rate of spontaneous remission is much lower.

Rituximab

Rituximab is a monoclonal anti-CD20 antibody that decreases anti-platelet antibody production by B cells and has been an off-label treatment for ITP for many years. Overall response rates of 40–70% were seen in patients given four weekly doses of 375 mg/m^2 of rituximab, though remission is rarely sustained, decreasing to only 21% at 5 years.[46–49] Despite the relapse rate, patients treated with rituximab had a longer duration of response

compared with placebo (median 8.2 months vs 1.8 months).[50] Studies examining different dosages of rituximab, including a lower dose of 100 mg/week for four weeks[51] and a dose of 1000 mg on days 1 and 15 did not show significant differences in response rate or infection risks.[52,53] Rituximab has also been studied in combination with other therapies such as high-dose dexamethasone in newly diagnosed ITP, with an initial strong overall response though sustained response after 12 months of follow up decreased to 61.5%, with an 11.1% incidence of adverse effects.[54] In a meta-analysis of randomized controlled trials, rituximab plus standard of care was shown to have a higher complete response rate (46.8% vs 32.5%) by 6 months than standard of care alone.[48] Infection is an important safety concern of rituximab: in a large ITP patient registry, the incidence of infection was 23.8% after 34 months, with 8.5% being severe (grade III to IV) infections.[46] Some clinicians use anti-infective prophylaxis for patients undergoing rituximab treatment, though evidence to support its use is lacking.[55] Given the ongoing COVID-19 pandemic, clinicians also must take into consideration the B-cell depletion effect of rituximab, which may impair vaccine response for at least 6 months after administration.[56]

Thrombopoietin Receptor Agonists

Thrombopoietin receptor agonists (TPO-RA) mimic endogenous TPO function to increase megakaryocyte maturation and platelet production.[57] In a large systematic review, treatment failure was seen in 21% of TPO-RA treated patients compared with 47% of control patients, with a lower risk of significant bleeding and all-cause mortality.[58] There are currently three TPO-RAs approved for treatment of ITP: romiplostim, eltrombopag, avatrombopag, described below and summarized in Table 1.[59] Thrombosis is the major potential adverse event of concern with TPO-RA use and though clinical trials have not found an increased thrombotic risk of TPO-RA agents compared with placebo, uncontrolled observational data suggest an increase in thrombotic risk on the order of 2-3-fold.[60]

Romiplostim is a peptide TPO-RA approved by the US FDA for ITP following the failure of a first-line treatment and is administered subcutaneously on a weekly schedule, starting at 1 mcg/kg, increased weekly to a maximum dose of 10 mcg/kg until platelet count is consistently 50–200 × 10^9/L.[61] In two parallel phase III trials, durable platelet response was achieved in 38–56% of patients with overall

Table 1 Phase III Trials of TPO-RAs in ITP

Study	Patient Number (n)	Location	Study Population	Major Results (Compared with Placebo)
Bussel 2009[108]	Eltrombopag n=76 Placebo n=38	Worldwide (63 sites)	Adults with ITP for ≥6 months and a pretreatment Plt <30 × 10^9/L 39% splenectomized	Significantly higher rate of platelet response[a] Significantly less bleeding
Cheng 2011[68]	Eltrombopag n=135 Placebo n=62	Worldwide (75 sites)	Adults with ITP for ≥6 months and a pretreatment Plt <30 × 10^9/L 36% splenectomized	Significantly higher rate of platelet response[a] Reduced use of concomitant ITP medications Reduced need for rescue therapy
Tomiyama 2012[69]	Eltrombopag n=15 Placebo n=8	Japan	Adults ≥20 years old with ITP for ≥6 months and a pretreatment Plt <30 × 10^9/L 70% splenectomized	Significantly higher rate of platelet response[a] Significantly less bleeding Lower doses of eltrombopag were effective in Japanese patients
Yang 2014[70]	Eltrombopag n=104 Placebo n=51	China	Adults with ITP for ≥12 months and a pretreatment Plt <30 × 10^9/L 16% splenectomized	Significantly higher rate of platelet response[a]
Kuter 2008[62]	Romiplostim n=83 Placebo n=42 (patients from two parallel studies)	United States and Europe	Adults with ITP for ≥12 months and a screening mean Plt <30 × 10^9/L 50% splenectomized	Significantly higher rate of platelet response[a] Reduced use of concomitant ITP medications
Kuter 2010[63]	Romiplostim n=157 Standard of care n=77	North America, Europe, and Australia	Adults with ITP for ≥12 months and a pretreatment Plt <50 × 10^9/L 0% splenectomized	Significantly higher rate of platelet response[a] Reduced use of concomitant ITP medications Lower rate of treatment failure Lower rate of splenectomy Significantly less bleeding and transfusions Significantly improved quality of life
Shirasugi 2011[109]	Romiplostim n=22 Placebo n=12	Japan	Adults ≥20 years old with ITP for ≥6 months and a screening Plt ≤30 × 10^9/L 44% splenectomized	Significantly higher rate of platelet response[a] Reduced need for rescue therapy
Jurczak 2018[79]	Avatrombopag n=32 Placebo n=17	Europe, Asia, and Australia	Adults with ITP for ≥12 months and a screening mean Plt <30 × 10^9/L 33% splenectomized	Significantly higher rate of platelet response[a] Reduced use of concomitant ITP medications

Notes: Each trial was a prospective, multicenter, randomized, placebo-controlled, double-blind study except Kuter et al. (2010) which was open label. Reproduced with permission from Al-Samkari and Kuter.[59] . [a]Platelet response defined as a platelet count ≥50 × 10^9/L at a given assessment on treatment with TPO-RA or placebo.
Abbreviations: ITP, immune thrombocytopenia; Plt, platelet count.

platelet response rate of 79–88% in patients given romiplostim, with platelet counts to $\geq 50 \times 10^9$/L for 13.8 weeks, compared with 0.8 weeks in the placebo group.[62] In another open-label study, comparing romiplostim to the medical standard of care in patients without history of splenectomy, patients who received romiplostim were 2.3 times as likely to have a platelet response as those who received the standard of care (71–92% patients who received romiplostim had a platelet response, compared with 51% in the standard of care group).[63] Though romiplostim does not have the ease of administration as oral TPO-RAs, a recent study has found that self-administration of romiplostim by patients did not increase adverse events compared with administration by healthcare professionals.[64,65]

Eltrombopag is a small molecule TPO-RA approved by the US FDA for ITP following the failure of a first-line treatment and is initiated orally at a dose of 50 mg daily in adults (or 25 mg daily in patients of East Asian descent), titrated to a maximum dose of 75 mg daily to reach a goal platelet count of 50–200 x 10^9/L.[66] Multiple studies have found eltrombopag significantly increases platelet response (59–79%) with less bleeding (statistically significant OR 0.49) and reduced use of concomitant ITP treatment.[67,68] Ethnic differences in eltrombopag were noted in patients of East Asian descent, with about 60% responding to a 12.5 mg or 25 mg daily dose.[69,70] Commonly reported adverse effects of eltrombopag include hepatotoxicity (11%), headache (2.9%), diarrhea, and upper respiratory tract infection.[68–71] A disadvantage of eltrombopag is the dietary restrictions (avoidance of dietary fat and divalent cations, such as calcium and magnesium in food) for a 4–6 hour window around taking the medication to prevent dietary and medication interference with adequate absorption.[72,73] Given the half-life of 26–35 hours,[74] one method proposed to address this is alternative intermittent dosing of eltrombopag less frequently than once daily, which has been shown to be effective in observational data.[75]

Avatrombopag is small molecule oral TPO receptor agonist approved for chronic ITP in adults as well as patients with liver disease scheduled to undergo a procedure. In ITP, it is initiated at a dose of 20 mg daily[76] titrated to a maximum dose of 40 mg daily to achieve a goal platelet count of 50–200 x 10^9/L. Unlike eltrombopag, avatrombopag does not require strict dietary restrictions for a 4–6 hour window around when it is taken. Also, it does not have a known signal for hepatotoxicity, nor

does it require dose adjustment for the race of the patient. In a phase II double-blind randomized controlled trial in patients with persistent and chronic ITP who failed or relapsed after prior therapy, 75% of patients receiving avatrombopag had an overall response, with the drug overall well-tolerated (common adverse events included fatigue and headache).[77] In a phase III study, avatrombopag was superior to placebo in mean cumulative number of weeks with platelet count $\geq$50 x 10^9/L during a 6-month treatment period with higher rates of reduced concomitant ITP medication use and durable response compared with placebo. In a post hoc analysis of the 2018 phase III study, avatrombopag was shown to have higher rates of platelet response and complete response in the first 6 months and a reduction in chronic corticosteroid use.[78,79] In addition to treatment of ITP, avatrombopag has also been studied extensively in patients with liver disease and has shown to be efficacious in the peri-procedural setting in patients with thrombocytopenia of chronic liver disease.[80–83]

A fourth TPO-RA, lusutrombopag, is also a small molecule oral TPO-RA, approved for thrombocytopenia due to chronic liver disease prior to an invasive procedure.[84] Its effect in ITP has not yet been well studied. A phase II study of lusutrombopag in ITP was recently terminated early due to results suggesting a higher dose was necessary to elicit an efficacy effect.

Thus far, there have not been any head-to-head randomized controlled trials completed comparing TPO-RAs. The relative potency of these agents is a topic of interest.[85] In a single center retrospective comparison of romiplostim and eltrombopag, there was no significant difference in platelet responses or tolerability.[86] One meta-analysis of nine randomized placebo-controlled trials showed no significant difference in overall response rate, bleeding incidence, incidence of adverse events, or durable response.[87] In a systematic review of 18 retrospective studies, the response rate after switching from one TPO-RA agent to another due to lack of efficacy, adverse events, or patient preference was 77.5%.[74]

There have been no formal guidelines regarding the discontinuation or tapering of TPO-RAs.[88] In a single-center observational study of patients who discontinued TPO-RAs, the 2-year treatment-free remission rate was 66.4% with 46% cumulative incidence of loss of complete response, but there was no clear predictive factor for sustained response.[89] In a meta-analysis, the incidence of remission after TPO-RA discontinuation ranged from 5–36%.[58] Tapering of TPO-RAs is dependent on multiple

factors, including platelet count at or above the lower limit of normal, lack of a major bleeding history, low trauma risk, and taking into account antiplatelet or anticoagulant agents the patient is taking.[90] In an expert consensus panel, the duration of ITP, duration on TPO-RA, and timing of platelet response did not affect the panel's recommendations regarding discontinuation.[90] A recent phase II study of sustained remission off treatment after discontinuation from TPO-RAs found that 25% of responders were able to maintain the response during 6 months after tapering from eltrombopag.[91] This study also reviewed biomarkers including TPO levels, which did not have a significant association with response or with sustained remission, and IL-10, IL-4, and TNF-α, each of which had negative predictive response. Other studies have found that TPO levels can predict response to TPO-RAs.[92] More research is needed to identify predictive factors that might be able to guide the tapering of TPO-RAs.

Fostamatinib

Fostamatinib is a spleen tyrosine kinase inhibitor which inhibits the inflammatory response and clearance of auto-antibody coated platelets by the reticuloendothelial system.[93] It was approved for ITP after the failure of other therapies at an initial dose of 100 mg twice daily, with uptitration to 150 mg twice daily for an inadequate response. Two phase III randomized placebo-controlled trials of fostamatinib in patients with persistent/chronic ITP showed an overall response in 43% of patients on fostamatinib with a median time to response of 15 days.[94] In the follow-up, open-label extension study, responses appeared durable, with 44% of patients achieving an over-all response for a median of >28 months.[95] In this study, most adverse events were mild to moderate, with the most common events including diarrhea, hypertension, nausea, epistaxis, and transaminase elevation.[95]

Therapies Currently Under Investigation

There is ongoing research involving Bruton's tyrosine kinase inhibitors in the potential treatment of immune-related diseases.[96] Rilzabrutinib is an oral, reversible small molecule selective BTK inhibitor that has shown preclinical efficacy in rapidly inhibiting antibody mediated innate immune response as well as antibody production, exhibiting potential for ITP treatment.[96] Rilzabrutinib was also shown to be safe and well tolerated in a phase I trial, with favorable pharmacokinetics that could result in fast onset of effect.[97]

Bortezomib is a proteasome inhibitor that was shown in a preclinical study to improve thrombocytopenia in ITP by inducing apoptosis in long-lived plasma cells which were thought to play a role in corticosteroid resistant ITP.[98] Bortezomib has been shown to have success in treatment of relapsing ITP in a case report,[99] though further clinical trials are needed.

Salvage Therapies

Other treatments of ITP include immunosuppressants (azathioprine, cyclophosphamide, cyclosporine, mycophenolate mofetil, vinca alkaloids), dapsone and danazol. These agents are typically used after failure of multiple standard second/subsequent-line treatment options. A brief summary of these therapies can be found in Table 2.[11] The management of patients with refractory ITP is discussed in more detail elsewhere.[100–102]

Special Considerations in ITP Management
Bleeding Emergencies in ITP

Management of bleeding emergencies in ITP is an important topic which requires further study. In the Updated International Consensus Report, Provan et al. provides a review of recommendations for treatment of life-threatening hemorrhage due to ITP.[88] Recommendations incorporate general supportive care with a combination of treatments, including IV corticosteroids, IVIG, and platelet transfusions in order to increase platelet count rapidly, and in the absence of significant response, the early addition of a TPO-RA should also be considered.[88] Ultimately, aggressive management to provide for a rise in the platelet count which often incorporates the use of multiple agents simultaneously without waiting for a single agent to be effective is appropriate. When TPO-RAs are used, they may be dosed more aggressively (e.g. romiplostim 5–10 mcg/kg to start). This is done with recognition of a potential thrombocytosis risk but with the understanding that the risk of ongoing severe thrombocytopenia and worsened bleeding is greater and requires urgent mitigation.

ITP in Pregnancy

Pregnancy complications in the setting of ITP include maternal hemorrhage, fetal loss, low birth weight, and may be treated to maintain a platelet level in the mother ($\geq 30 \times 10^9$/L until close to term), with the goal then adjusted based on delivery procedures.[10,103,104]

Table 2 Other Agents for Use in the Subsequent Treatment Setting in ITP

Agent	Mechanism	Time to Response	Response Rate	Response Durability	Major Adverse Effects	Comments
Azathioprine[110]	Prodrug of antimetabolite 6-mercaptopurine; steroid-sparing immunosuppressant	Delayed (weeks to months)	30%	Good	Bone marrow suppression Infection Hepatotoxicity	Thiopurine S-methyltransferase activity should be measured prior to initiation Accepted as safe in pregnancy
Cyclophosphamide[111,112]	Prodrug of phosphoramide mustard metabolite; immunosuppressant	Delayed (weeks to months)	30–40%	Good	Bone marrow suppression Hemorrhagic cystitis Infection	Low-dose oral cyclophosphamide typically used
Cyclosporine[113,114]	Calcineurin inhibitor immunosuppressant	Early (1–2 weeks)	30–40%	Moderate	Nephrotoxicity Hypertension, Metabolic side-effects	Trough levels should be monitored
Danazol[115–118]	Attenuated androgenic steroid hormone with glucocorticoid receptor activity	Delayed (weeks to months)	30–40%	Good	Virilization Hepatotoxicity Weight gain	May be combined with azathioprine but evidence for this is poor
Dapsone[119–121]	Antibiotic with immunomodulatory and anti-inflammatory properties	Delayed (weeks)	40–50%	Poor	Methemoglobinemia Hemolysis	Glucose-6-phosphate dehydrogenase activity should be measured prior to initiation
Mycophenolate mofetil[122–124]	Prodrug of mycophenolic acid, a purine synthesis inhibitor causing immunosuppression	Delayed (weeks)	40–50%	Good	Diarrhea Bone marrow suppression Infection	
Vinca alkaloids (vincristine, vinblastine)[125–128]	Microtubule toxin chemotherapeutic agents causing potent immunosuppression	Rapid (within 1 week)	70%	Poor	Vesication at infusion site Neuropathy Constipation SIADH	Administered as multiple weekly intravenous infusions; can be used as a rescue therapy of last resort

Notes: These agents are commonly labeled "third-line" treatments, although they may be used earlier or later in the treatment of ITP depending on clinical circumstances (i.e. pregnancy) or availability of more expensive agents (such as TPO-RAs or rituximab). Reproduced with permission from Al-Samkari and Kuter.[11]
Abbreviation: SIADH, syndrome of inappropriate antidiuretic hormone secretion.

Recommended treatments include corticosteroids or IVIG, or a combination.[105] In the event that both modalities fail, TPO-RAs can be considered as a salvage therapy in severe cases, on the basis of limited observational data. One multicenter observational study of 15 pregnant women showed a response rate of 77% to TPO-RA (romiplostim or eltrombopag) though mostly in combination with concomitant ITP therapy.[106] And while this study showed no thromboembolic events and aside from one case of neonatal thrombosis, no other fetal complications,[106] more information is needed about other therapies in pregnancy. The management of pregnant patients is discussed in more detail in a review by Gernsheimer et al.[107]

Conclusions and Future Directions

Modern treatment of ITP in adults involves a number of tried and true first-line therapies, primarily corticosteroids and IVIG, as well as newer therapies in the TPO-RAs and fostamatinib. Though there are many more options for the management of ITP at present than in even the relatively recent past, there remains unmet need in this disease. Thankfully, additional therapies are under development for ITP, including inhibitors of the Bruton tyrosine kinase (e.g. rilzabrutinib), complement inhibitory therapies (e.g. sutimlimab), neonatal Fc receptor antagonists (e.g. rozanolixizumab, efgartigimod), and others. Management of chronic ITP may involve cycling through multiple drug therapies, consideration of splenectomy, and in some patients, reaching for salvage therapies or clinical trials of novel agents. Lastly, bleeding emergencies in ITP require prompt, aggressive management, typically with multiple agents.

Acknowledgments

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

References

1. Harrington WJ, Minnich V, Hollingsworth JW, et al. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med*. 1951;38(1):1–10.
2. Shulman NR, Marder VJ, Weinrach RS. Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia in idiopathic purpura. Physiologic, serologic and isotopic studies. *Ann N Y Acad Sci*. 2006;124(2):499–542. doi:10.1111/j.1749-6632.1965.tb18984.x
3. Ballem PJ, Segal GM, Stratton JR, et al. Mechanisms of thrombocytopenia in chronic autoimmune thrombocytopenic purpura. Evidence of both impaired platelet production and increased platelet clearance. *J Clin Invest*. 1987;80(1):33–40. doi:10.1172/JCI113060
4. Al-Samkari H, Rosovsky RP, Karp Leaf RS, et al. A modern reassessment of glycoprotein-specific direct platelet autoantibody testing in immune thrombocytopenia. *Blood Adv*. 2020;4(1):9–18. doi:10.1182/bloodadvances.2019000868
5. Cheloff AZ, Kuter DJ, Al-Samkari H. Serum complement levels in immune thrombocytopenia: characterization and relation to clinical features. *Res Pract Thromb Haemost*. 2020;4(5):807–812. doi:10.1002/rth2.12388
6. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113(11):2386–2393. doi:10.1182/blood-2008-07-162503
7. Neunert C, Lim W, Crowther M, et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. *Blood*. 2011;117(16):4190–4207. doi:10.1182/blood-2010-08-302984
8. Weycker D, Hanau A, Hatfield M, et al. Primary immune thrombocytopenia in US clinical practice: incidence and healthcare burden in first 12 months following diagnosis. *J Med Econ*. 2020;23(2):184–192. doi:10.1080/13696998.2019.1669329
9. Cohen YC, Djulbegovic B, Shamai-Lubovitz O, et al. The bleeding risk and natural history of idiopathic thrombocytopenic purpura in patients with persistent low platelet counts. *Arch Intern Med*. 2000;160(11):1630–1638. doi:10.1001/archinte.160.11.1630
10. Provan D, Stasi R, Newland AC, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood*. 2010;115(2):168–186. doi:10.1182/blood-2009-06-225565
11. Al-Samkari H, Kuter DJ. Immune thrombocytopenia in adults: modern approaches to diagnosis and treatment. *Semin Thromb Hemost*. 2020;46(3):275–288. doi:10.1055/s-0039-1700512
12. Slichter SJ, Harker LA. Thrombocytopenia: mechanisms and management of defects in platelet production. *Clin Haematol*. 1978;7(3):523–539. doi:10.1016/S0308-2261(21)00047-3
13. Slichter SJ, Kaufman RM, Assmann SF, et al. Dose of prophylactic platelet transfusions and prevention of hemorrhage. *N Engl J Med*. 2010;362(7):600–613. doi:10.1056/NEJMoa0904084
14. Lacey JV, Penner JA. Management of idiopathic thrombocytopenic purpura in the adult. *Semin Thromb Hemost*. 2008;3(3):160–174. doi:10.1055/s-0028-1086135

15. Harker LA, Slichter SJ. The bleeding time as a screening test for evaluation of platelet function. *N Engl J Med*. 1972;287(4):155–159. doi:10.1056/NEJM197207272870401

16. Piel-Julian ML, Mahevas M, Germain J, et al. Risk factors for bleeding, including platelet count threshold, in newly diagnosed immune thrombocytopenia adults. *J Thromb Haemost*. 2018;16(9):1830–1842. doi:10.1111/jth.14227

17. Hato T, Shimada N, Kurata Y, et al. Risk factors for skin, mucosal, and organ bleeding in adults with primary ITP: a nationwide study in Japan. *Blood Adv*. 2020;4(8):1648–1655. doi:10.1182/bloodadvances.2020001446

18. McVerry BA. Management of idiopathic thrombocytopenic purpura in adults. *Br J Haematol*. 1985;59(2):203–208. doi:10.1111/j.1365-2141.1985.tb02985.x

19. Shulman NR, Weinrach RS, Libre EP, et al. The role of the reticuloendothelial system in the pathogenesis of idiopathic thrombocytopenic purpura. *Trans Assoc Am Physicians*. 1965;78:374–390.

20. Gernsheimer T, Stratton J, Ballem PJ, et al. Mechanisms of response to treatment in autoimmune thrombocytopenic purpura. *N Engl J Med*. 1989;320(15):974–980. doi:10.1056/NEJM198904133201505

21. Pang SJ, Lazarus AH. Mechanisms of platelet recovery in ITP associated with therapy. *Ann Hematol*. 2010;89(Suppl 1):31–35. doi:10.1007/s00277-010-0916-2

22. Li J, Wang Z, Hu S, et al. Correction of abnormal T cell subsets by high-dose dexamethasone in patients with chronic idiopathic thrombocytopenic purpura. *Immunol Lett*. 2013;154(1–2):42–48. doi:10.1016/j.imlet.2013.08.006

23. Hou Y, Feng Q, Xu M, et al. High-dose dexamethasone corrects impaired myeloid-derived suppressor cell function via Ets1 in immune thrombocytopenia. *Blood*. 2016;127(12):1587–1597. doi:10.1182/blood-2015-10-674531

24. Kitchens CS. Amelioration of endothelial abnormalities by prednisone in experimental thrombocytopenia in the rabbit. *J Clin Invest*. 1977;60(5):1129–1134. doi:10.1172/JCI108864

25. Mithoowani S, Gregory-Miller K, Goy J, et al. High-dose dexamethasone compared with prednisone for previously untreated primary immune thrombocytopenia: a systematic review and meta-analysis. *Lancet Haematol*. 2016;3(10):e489–e96. doi:10.1016/S2352-3026(16)30109-0

26. Hansen RJ, Balthasar JP. Mechanisms of IVIG action in immune thrombocytopenic purpura. *Clin Lab*. 2004;50(3–4):133–140.

27. Crow AR, Lazarus AH. The mechanisms of action of intravenous immunoglobulin and polyclonal anti-d immunoglobulin in the amelioration of immune thrombocytopenic purpura: what do we really know? *Transfus Med Rev*. 2008;22(2):103–116. doi:10.1016/j.tmrv.2007.12.001

28. Nimmerjahn F, Ravetch JV. Anti-inflammatory actions of intravenous immunoglobulin. *Annu Rev Immunol*. 2008;26:513–533. doi:10.1146/annurev.immunol.26.021607.090232

29. Brückner C, Lehmann C, Dudziak D, et al. Sweet SIGNs: IgG glycosylation leads the way in IVIG-mediated resolution of inflammation. *Int Immunol*. 2017;29(11):499–509. doi:10.1093/intimm/dxx053

30. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nat Rev Immunol*. 2013;13(3):176–189. doi:10.1038/nri3401

31. Qin YH, Zhou TB, Su LN, et al. The efficacy of different dose intravenous immunoglobulin in treating acute idiopathic thrombocytopenic purpura: a meta-analysis of 13 randomized controlled trials. *Blood Coagul Fibrinolysis*. 2010;21(8):713–721. doi:10.1097/MBC.0b013e3283401490

32. Zhou Z, Qiao Z, Li H, et al. Different dosages of intravenous immunoglobulin (IVIg) in treating immune thrombocytopenia with long-term follow-up of three years: results of a prospective study including 167 cases. *Autoimmunity*. 2016;49(1):50–57. doi:10.3109/08916934.2015.1104671

33. Newman GC, Novoa MV, Fodero EM, et al. A dose of 75 microg/kg/d of i.v. anti-D increases the platelet count more rapidly and for a longer period of time than 50 microg/kg/d in adults with immune thrombocytopenic purpura. *Br J Haematol*. 2001;112(4):1076–1078. doi:10.1046/j.1365-2141.2001.02627.x

34. Tarantino MD, Young G, Bertolone SJ, et al. Single dose of anti-D immune globulin at 75 microg/kg is as effective as intravenous immune globulin at rapidly raising the platelet count in newly diagnosed immune thrombocytopenic purpura in children. *J Pediatr*. 2006;148(4):489–494. doi:10.1016/j.jpeds.2005.11.019

35. Ware RE, Zimmerman SA. Anti-D: mechanisms of action. *Semin Hematol*. 1998;35(1Suppl 1):14–22.

36. Rodeghiero F, Schiavotto C, Castaman G, et al. A follow-up study of 49 adult patients with idiopathic thrombocytopenic purpura treated with high-dose immunoglobulins and anti-D immunoglobulins. *Haematologica*. 1992;77(3):248–252.

37. Scaradavou A, Woo B, Woloski BM, et al. Intravenous anti-D treatment of immune thrombocytopenic purpura: experience in 272 patients. *Blood*. 1997;89(8):2689–2700. doi:10.1182/blood.V89.8.2689

38. Moulis G, Germain J, Comont T, et al. Newly diagnosed immune thrombocytopenia adults: clinical epidemiology, exposure to treatments, and evolution. Results of the CARMEN multicenter prospective cohort. *Am J Hematol*. 2017;92(6):493–500. doi:10.1002/ajh.24702

39. Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. *Blood Adv*. 2019;3(23):3829–3866. doi:10.1182/bloodadvances.2019000966

40. Stasi R, Stipa E, Masi M, et al. Long-term observation of 208 adults with chronic idiopathic thrombocytopenic purpura. *Am J Med*. 1995;98(5):436–442. doi:10.1016/s0002-9343(99)80342-8

41. Rodeghiero F. A critical appraisal of the evidence for the role of splenectomy in adults and children with ITP. *Br J Haematol*. 2018;181(2):183–195. doi:10.1111/bjh.15090

42. Kojouri K, Vesely SK, Terrell DR, et al. Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, prediction of response, and surgical complications. *Blood*. 2004;104(9):2623–2634. doi:10.1182/blood-2004-03-1168

43. Bisharat N, Omari H, Lavi I, et al. Risk of infection and death among post-splenectomy patients. *J Infect*. 2001;43(3):182–186. doi:10.1053/jinf.2001.0904

44. Boyle S, White RH, Brunson A, et al. Splenectomy and the incidence of venous thromboembolism and sepsis in patients with immune thrombocytopenia. *Blood*. 2013;121(23):4782–4790. doi:10.1182/blood-2012-12-467068

45. Ruggeri M, Tosetto A, Palandri F, et al. Thrombotic risk in patients with primary immune thrombocytopenia is only mildly increased and explained by personal and treatment-related risk factors. *J Thromb Haemost*. 2014;12(8):1266–1273. doi:10.1111/jth.12636

46. Deshayes S, Khellaf M, Zarour A, et al. Long-term safety and efficacy of rituximab in 248 adults with immune thrombocytopenia: results at 5 years from the French prospective registry ITP-ritux. *Am J Hematol*. 2019;94(12):1314–1324. doi:10.1002/ajh.25632

47. Ghanima W, Khelif A, Waage A, et al. Rituximab as second-line treatment for adult immune thrombocytopenia (the RITP trial): a multicentre, randomised, double-blind, placebo-controlled trial. *Lancet*. 2015;385(9978):1653–1661. doi:10.1016/S0140-6736(14)61495-1

48. Chugh S, Darvish-Kazem S, Lim W, et al. Rituximab plus standard of care for treatment of primary immune thrombocytopenia: a systematic review and meta-analysis. *Lancet Haematol*. 2015;2(2):e75–e81. doi:10.1016/S2352-3026(15)00003-4

49. Arnold DM, Dentali F, Crowther MA, et al. Systematic review: efficacy and safety of rituximab for adults with idiopathic thrombocytopenic purpura. *Ann Intern Med*. 2007;146(1):25–33. doi:10.7326/0003-4819-146-1-200701020-00006

50. Tjønnfjord E, Holme PA, Darne B, et al. Long-term outcomes of patients treated with rituximab as second-line treatment for adult immune thrombocytopenia - follow-up of the RITP study. *Br J Haematol*. 2020;191(3):460–465. doi:10.1111/bjh.16672

51. Zaja F, Vianelli N, Volpetti S, et al. Low-dose rituximab in adult patients with primary immune thrombocytopenia. *Eur J Haematol*. 2010;85(4):329–334. doi:10.1111/j.1600-0609.2010.01486.x

52. Mahevas M, Ebbo M, Audia S, et al. Efficacy and safety of rituximab given at 1000 mg on days 1 and 15 compared to the standard regimen to treat adult immune thrombocytopenia. *Am J Hematol*. 2013;88(10):858–861. doi:10.1002/ajh.23518

53. Tran H, Brighton T, Grigg A, et al. A multi-centre, single-arm, open-label study evaluating the safety and efficacy of fixed dose rituximab in patients with refractory, relapsed or chronic idiopathic thrombocytopenic purpura (R-ITP1000 study). *Br J Haematol*. 2014;167(2):243–251. doi:10.1111/bjh.13029

54. Zhou H, Liu L, Shu X, et al. Clinical efficacy and safety of high-dose dexamethasone plus low-dose rituximab as first-line therapy in newly diagnosed primary immune thrombocytopenia. *Indian J Hematol Blood Transfus*. 2019;35(3):507–512. doi:10.1007/s12288-018-1061-7

55. Raso S, Napolitano M, Arrigo G, et al. Antimicrobial prophylaxis in patients with immune thrombocytopenia treated with rituximab: a retrospective multicenter analysis. *Ann Hematol*. 2021;100:653–659. doi:10.1007/s00277-021-04438-7

56. Nazi I, Kelton JG, Larché M, et al. The effect of rituximab on vaccine responses in patients with immune thrombocytopenia. *Blood*. 2013;122(11):1946–1953. doi:10.1182/blood-2013-04-494096

57. Kuter DJ. The biology of thrombopoietin and thrombopoietin receptor agonists. *Int J Hematol*. 2013;98(1):10–23. doi:10.1007/s12185-013-1382-0

58. Birocchi S, Podda GM, Manzoni M, et al. Thrombopoietin receptor agonists for the treatment of primary immune thrombocytopenia: a meta-analysis and systematic review. *Platelets*. 2020;32:1–11. doi:10.1080/09537104.2020.1745168.

59. Al-Samkari H, Kuter DJ. Optimal use of thrombopoietin receptor agonists in immune thrombocytopenia. *Ther Adv Hematol*. 2019;10:2040620719841735. doi:10.1177/2040620719841735

60. Al-Samkari H, Van Cott EM, Kuter DJ. Platelet aggregation response in immune thrombocytopenia patients treated with romiplostim. *Ann Hematol*. 2019;98(3):581–588. doi:10.1007/s00277-018-3556-6

61. Amgen Inc. *Nplate (Romiplostim) [Prescribing Information]*. Thousand Oaks, CA: Amgen; 2017.

62. Kuter DJ, Bussel JB, Lyons RM, et al. Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. *Lancet*. 2008;371 (9610):395–403. doi:10.1016/S0140-6736(08)60203-2

63. Kuter DJ, Rummel M, Boccia R, et al. Romiplostim or standard of care in patients with immune thrombocytopenia. *N Engl J Med*. 2010;363(20):1889–1899. doi:10.1056/NEJMoa1002625

64. Kuter DJ, Arnold DM, Rodeghiero F, et al. Safety and efficacy of self-administered romiplostim in patients with immune thrombocytopenia: results of an integrated database of five clinical trials. *Am J Hematol*. 2020;95(6):643–651. doi:10.1002/ajh.25776

65. Al-Samkari H, Grace RF, Kuter DJ. The role of romiplostim for pediatric patients with immune thrombocytopenia. *Ther Adv Hematol*. 2020;11:2040620720912992. doi:10.1177/2040620720912992

66. GlaxoSmithKline. *Promacta (Eltrombopag) [Prescribing Information]*. Research Triangle Park, NC.: GlaxoSmithKline; 2017.

67. Bussel JB, Kuter DJ, Pullarkat V, et al. Safety and efficacy of long-term treatment with romiplostim in thrombocytopenic patients with chronic ITP. *Blood*. 2009;113(10):2161–2171. doi:10.1182/blood-2008-04-150078

68. Cheng G, Saleh MN, Marcher C, et al. Eltrombopag for management of chronic immune thrombocytopenia (RAISE): a 6-month, randomised, phase 3 study. *Lancet*. 2011;377(9763):393–402. doi:10.1016/S0140-6736(10)60959-2

69. Tomiyama Y, Miyakawa Y, Okamoto S, et al. A lower starting dose of eltrombopag is efficacious in Japanese patients with previously treated chronic immune thrombocytopenia. *J Thromb Haemost*. 2012;10(5):799–806. doi:10.1111/j.1538-7836.2012.04695.x

70. Yang R, Hou M, Li J, et al. Effect of eltrombopag on platelet response and safety results in Chinese adults with chronic ITP-primary result of a phase III study. *Blood*. 2014;124(21):1464. doi:10.1182/blood.V124.21.1464.1464

71. Elgebaly AS, Ashal GE, Elfil M, et al. Tolerability and efficacy of eltrombopag in chronic immune thrombocytopenia: meta-analysis of randomized controlled trials. *Clin Appl Thromb Hemost*. 2017;23(8):928–937. doi:10.1177/1076029616663849

72. Williams DD, Peng B, Bailey CK, et al. Effects of food and antacids on the pharmacokinetics of eltrombopag in healthy adult subjects: two single-dose, open-label, randomized-sequence, crossover studies. *Clin Ther*. 2009;31(4):764–776. doi:10.1016/j.clinthera.2009.04.010

73. Wire MB, Bruce J, Gauvin J, et al. A randomized, open-label, 5-period, balanced crossover study to evaluate the relative bioavailability of eltrombopag powder for oral suspension (PfOS) and tablet formulations and the effect of a high-calcium meal on eltrombopag pharmacokinetics when administered with or 2 hours before or after PfOS. *Clin Ther*. 2012;34(3):699–709. doi:10.1016/j.clinthera.2012.01.011

74. González-Porras JR, Godeau B, Carpenedo M. Switching thrombopoietin receptor agonist treatments in patients with primary immune thrombocytopenia. *Ther Adv Hematol*. 2019;10:2040620719837906. doi:10.1177/2040620719837906

75. Al-Samkari H, Kuter DJ. An alternative intermittent eltrombopag dosing protocol for the treatment of chronic immune thrombocytopenia. *Br J Clin Pharmacol*. 2018;84(11):2673–2677. doi:10.1111/bcp.13717

76. AkaRx. *DOPTELET (Avatrombopag) [Prescribing Information]*. Durham, NC: AkaRx; 2020.

77. Bussel JB, Kuter DJ, Aledort LM, et al. A randomized trial of avatrombopag, an investigational thrombopoietin-receptor agonist, in persistent and chronic immune thrombocytopenia. *Blood*. 2014;123(25):3887–3894. doi:10.1182/blood-2013-07-514398

78. Al-Samkari H, Nagalla S. Efficacy and safety evaluation of avatrombopag in immune thrombocytopenia: analyses of a phase III study and long-term extension. *Platelets*. 2021;1–8. doi:10.1080/09537104.2021.1881952

79. Jurczak W, Chojnowski K, Mayer J, et al. Phase 3 randomised study of avatrombopag, a novel thrombopoietin receptor agonist for the treatment of chronic immune thrombocytopenia. *Br J Haematol*. 2018;183(3):479–490. doi:10.1111/bjh.15573

80. Cheloff AZ, Al-Samkari H. Avatrombopag for the treatment of immune thrombocytopenia and thrombocytopenia of chronic liver disease. *J Blood Med*. 2019;10:313–321. doi:10.2147/jbm.S191790

81. Terrault N, Chen YC, Izumi N, et al. Avatrombopag before procedures reduces need for platelet transfusion in patients with chronic liver disease and thrombocytopenia. *Gastroenterology*. 2018;155(3):705–718. doi:10.1053/j.gastro.2018.05.025

82. Virk ZM, Kuter DJ, Al-Samkari H. An evaluation of avatrombopag for the treatment of thrombocytopenia. *Expert Opin Pharmacother*. 2021;22(3):273–280. doi:10.1080/14656566.2020.1841748

83. Al-Samkari H. Avatrombopag maleate for the treatment of peri-procedural thrombocytopenia in patients with chronic liver disease. *Drugs Today*. 2018;54(11):647–655. doi:10.1358/dot.2018.54.11.2899441

84. Thrombopoietin Receptor Agonists. *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012.

85. Al-Samkari H, Kuter DJ. Relative potency of the thrombopoietin receptor agonists eltrombopag, avatrombopag and romiplostim in a patient with chronic immune thrombocytopenia. *Br J Haematol*. 2018;183(2):168. doi:10.1111/bjh.15432

86. Arnall JR, DiSogra KY, Downing L, et al. Comparative utilization and efficacy of thrombopoietin receptor agonists in relapsed/refractory immune thrombocytopenia. *Am J Ther*. 2021;Publish Ahead of Print. doi:10.1097/mjt.0000000000001335

87. Zhang J, Liang Y, Ai Y, et al. Eltrombopag versus romiplostim in treatment of adult patients with immune thrombocytopenia: a systematic review incorporating an indirect-comparison meta-analysis. *PLoS One*. 2018;13(6):e0198504. doi:10.1371/journal.pone.0198504

88. Provan D, Arnold DM, Bussel JB, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv*. 2019;3(22):3780–3817. doi:10.1182/bloodadvances.2019000812

89. Iino M, Sakamoto Y, Sato T. Treatment-free remission after thrombopoietin receptor agonist discontinuation in patients with newly diagnosed immune thrombocytopenia: an observational retrospective analysis in real-world clinical practice. *Int J Hematol*. 2020;112 (2):159–168. doi:10.1007/s12185-020-02893-y

90. Cuker A, Despotovic JM, Grace RF, et al. Tapering thrombopoietin receptor agonists in primary immune thrombocytopenia: expert consensus based on the RAND/UCLA modified delphi panel method. *Res Pract Thromb Haemost*. 2021;5(1):69–80. doi:10.1002/rth2.12457

91. Lucchini E, Palandri F, Volpetti S, et al. Eltrombopag second-line therapy in adult patients with primary immune thrombocytopenia in an attempt to achieve sustained remission off-treatment: results of a phase II, multicentre, prospective study. *Br J Haematol*. 2021;193:386–396. doi:10.1111/bjh.17334

92. Al-Samkari H, Kuter DJ. Thrombopoietin level predicts response to treatment with eltrombopag and romiplostim in immune thrombocytopenia. *Am J Hematol*. 2018;93(12):1501–1508. doi:10.1002/ajh.25275

93. Markham A. Fostamatinib: first global approval. *Drugs*. 2018;78 (9):959–963. doi:10.1007/s40265-018-0927-1

94. Bussel J, Arnold DM, Grossbard E, et al. Fostamatinib for the treatment of adult persistent and chronic immune thrombocytopenia: results of two phase 3, randomized, placebo-controlled trials. *Am J Hematol*. 2018;93(7):921–930. doi:10.1002/ajh.25125

95. Bussel JB, Arnold DM, Boxer MA, et al. Long-term fostamatinib treatment of adults with immune thrombocytopenia during the phase 3 clinical trial program. *Am J Hematol*. 2019;94(5):546–553. doi:10.1002/ajh.25444

96. Langrish CL, Bradshaw JM, Francesco MR, et al. Preclinical efficacy and anti-inflammatory mechanisms of action of the bruton tyrosine kinase inhibitor rilzabrutinib for immune-mediated disease. *J Immunol*. 2021;206(7):1454–1468. doi:10.4049/jimmunol.2001130

97. Smith PF, Krishnarajah J, Nunn PA, et al. A phase I trial of PRN1008, a novel reversible covalent inhibitor of Bruton's tyrosine kinase, in healthy volunteers. *Br J Clin Pharmacol*. 2017;83 (11):2367–2376. doi:10.1111/bcp.13351

98. Li G, Wang S, Li N, et al. Proteasome inhibition with bortezomib induces apoptosis of long-lived plasma cells in steroid-resistant or relapsed immune thrombocytopaenia. *Thromb Haemost*. 2018;118 (10):1752–1764. doi:10.1055/s-0038-1669921

99. Beckman JD, Rollins-Raval MA, Raval JS, et al. Bortezomib for refractory immune-mediated thrombocytopenia purpura. *Am J Ther*. 2018;25(2):e270–e272. doi:10.1097/mjt.0000000000000517

100. Cuker A, Neunert CE. How I treat refractory immune thrombocytopenia. *Blood*. 2016;128(12):1547–1554. doi:10.1182/blood-2016-03-603365

101. Miltiadous O, Hou M, Bussel JB. Identifying and treating refractory ITP: difficulty in diagnosis and role of combination treatment. *Blood*. 2020;135(7):472–490. doi:10.1182/blood.2019003599

102. Mahévas M, Gerfaud-Valentin M, Moulis G, et al. Characteristics, outcome, and response to therapy of multirefractory chronic immune thrombocytopenia. *Blood*. 2016;128(12):1625–1630. doi:10.1182/blood-2016-03-704734

103. Webert KE, Mittal R, Sigouin C, et al. A retrospective 11-year analysis of obstetric patients with idiopathic thrombocytopenic purpura. *Blood*. 2003;102(13):4306–4311. doi:10.1182/blood-2002-10-3317

104. Al-Jama FE, Rahman J, Al-Suleiman SA, et al. Outcome of pregnancy in women with idiopathic thrombocytopenic purpura. *Aust N Z J Obstet Gynaecol*. 1998;38(4):410–413. doi:10.1111/j.1479-828X.1998.tb03099.x

105. Nicolescu A, Vladareanu AM, Voican I, et al. Therapeutic options for Immune Thrombocytopenia (ITP) during pregnancy. *Maedica*. 2013;8(2):182–188.

106. Michel M, Ruggeri M, Gonzalez-Lopez TJ, et al. Use of thrombopoietin receptor agonists for immune thrombocytopenia in pregnancy: results from a multicenter study. *Blood*. 2020;136 (26):3056–3061. doi:10.1182/blood.2020007594

107. Gernsheimer T, James AH, Stasi R. How I treat thrombocytopenia in pregnancy. *Blood*. 2013;121(1):38–47. doi:10.1182/blood-2012-08-448944

108. Bussel JB, Provan D, Shamsi T, et al. Effect of eltrombopag on platelet counts and bleeding during treatment of chronic idiopathic thrombocytopenic purpura: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2009;373(9664):641–648. doi:10.1016/S0140-6736(09)60402-5

109. Shirasugi Y, Ando K, Miyazaki K, et al. Romiplostim for the treatment of chronic immune thrombocytopenia in adult Japanese patients: a double-blind, randomized Phase III clinical trial. *Int J Hematol*. 2011;94(1):71–80. doi:10.1007/s12185-011-0886-8

110. Quiquandon I, Fenaux P, Caulier MT, et al. Re-evaluation of the role of azathioprine in the treatment of adult chronic idiopathic thrombocytopenic purpura: a report on 53 cases. *Br J Haematol*. 1990;74(2):223–228. doi:10.1111/j.1365-2141.1990.tb02569.x

111. Verlin M, Laros RK, Penner JA. Treatment of refractory thrombocytopenic purpura with cyclophosphamine. *Am J Hematol*. 1976;1(1):97–104. doi:10.1002/ajh.2830010111

112. Pizzuto J, Ambriz R. Therapeutic experience on 934 adults with idiopathic thrombocytopenic purpura: multicentric trial of the cooperative Latin American group on hemostasis and thrombosis. *Blood*. 1984;64(6):1179–1183. doi:10.1182/blood.V64.6.1179.1179

113. Choudhary DR, Naithani R, Mahapatra M, et al. Efficacy of cyclosporine as a single agent therapy in chronic idiopathic thrombocytopenic purpura. *Haematologica*. 2008;93(10):e61–e62;discussion e63. doi:10.3324/haematol.13481

114. Kappers-Klunne MC, Van't Veer MB. Cyclosporin A for the treatment of patients with chronic idiopathic thrombocytopenic purpura refractory to corticosteroids or splenectomy. *Br J Haematol*. 2001;114(1):121–125. doi:10.1046/j.1365-2141.2001.02893.x

115. Fenaux P, Quiquandon I, Huart JJ, et al. The role of danazol in the treatment of refractory idiopathic thrombocytopenic purpura. A report of 22 cases. *Nouv Rev Fr Hematol.* 1990;32(2):143–146.

116. Li HQ, Zhang L, Zhao H, et al. Chronic idiopathic thrombocytopenic purpura in adult Chinese patients: a retrospective single-centered analysis of 1791 cases. *Chin Med J (Engl).* 2005;118(1):34–37.

117. Liu W, Gu X, Fu R, et al. The effect of danazol in primary immune thrombocytopenia: an analysis of a large cohort from a single center in China. *Clin Appl Thromb Hemost.* 2016;22 (8):727–733. doi:10.1177/1076029615622002

118. Maloisel F, Andres E, Zimmer J, et al. Danazol therapy in patients with chronic idiopathic thrombocytopenic purpura: long-term results. *Am J Med.* 2004;116(9):590–594. doi:10.1016/j. amjmed.2003.12.024

119. Damodar S, Viswabandya A, George B, et al. Dapsone for chronic idiopathic thrombocytopenic purpura in children and adults–a report on 90 patients. *Eur J Haematol.* 2005;75 (4):328–331. doi:10.1111/j.1600-0609.2005.00545.x

120. Godeau B, Durand JM, Roudot-Thoraval F, et al. Dapsone for chronic autoimmune thrombocytopenic purpura: a report of 66 cases. *Br J Haematol.* 1997;97(2):336–339. doi:10.1046/j.1365-2141.1997.412687.x

121. Zaja F, Marin L, Chiozzotto M, et al. Dapsone salvage therapy for adult patients with immune thrombocytopenia relapsed or refractory to steroid and rituximab. *Am J Hematol.* 2012;87(3):321–323. doi:10.1002/ajh.22266

122. Hou M, Peng J, Shi Y, et al. Mycophenolate mofetil (MMF) for the treatment of steroid-resistant idiopathic thrombocytopenic purpura. *Eur J Haematol.* 2003;70(6):353–357. doi:10.1034/j.1600-0609.2003.00076.x

123. Taylor A, Neave L, Solanki S, et al. Mycophenolate mofetil therapy for severe immune thrombocytopenia. *Br J Haematol.* 2015;171(4):625–630. doi:10.1111/bjh.13622

124. Zhang WG, Ji L, Cao XM, et al. Mycophenolate mofetil as a treatment for refractory idiopathic thrombocytopenic purpura. *Acta Pharmacol Sin.* 2005;26(5):598–602. doi:10.1111/j.1745-7254.2005.00088.x

125. Ahn YS, Harrington WJ, Mylvaganam R, et al. Slow infusion of vinca alkaloids in the treatment of idiopathic thrombocytopenic purpura. *Ann Intern Med.* 1984;100(2):192–196. doi:10.7326/0003-4819-100-2-192

126. Facon T, Caulier MT, Wattel E, et al. A randomized trial comparing vinblastine in slow infusion and by bolus i.v. injection in idiopathic thrombocytopenic purpura: a report on 42 patients. *Br J Haematol.* 1994;86(3):678–680. doi:10.1111/j.1365-2141.1994.tb04810.x

127. Fenaux P, Quiquandon I, Caulier MT, et al. Slow infusions of vinblastine in the treatment of adult idiopathic thrombocytopenic purpura: a report on 43 cases. *Blut.* 1990;60(4):238–241. doi:10.1007/BF01728791

128. Park YH, Yi HG, Lee MH, et al. Clinical efficacy and tolerability of vincristine in splenectomized patients with refractory or relapsed immune thrombocytopenia: a retrospective single-center study. *Int J Hematol.* 2016;103(2):180–188. doi:10.1007/s12185-015-1903-0

The Superiority of T2*MRI Over Serum Ferritin in the Evaluation of Secondary Iron Overload in a Chronic Kidney Disease Patient: A Case Report

Abdulrahman Al-Mashdali, Tahiya Alyafei & Mohamed Yassin

Published online: 26 Jul 2021.

Submit your article to this journal 🗗

Article views: 119

View related articles 🗗

View Crossmark data 🗗

CASE REPORT

The Superiority of T2*MRI Over Serum Ferritin in the Evaluation of Secondary Iron Overload in a Chronic Kidney Disease Patient: A Case Report

Abdulrahman
Al-Mashdali [1]
Tahiya Alyafei[2]
Mohamed Yassin [3]

[1]Department of Internal Medicine, Hamad Medical Corporation, Doha, Qatar; [2]Department of Clinical Imaging, Hamad Medical Corporation, Doha, Qatar; [3]National Center for Cancer Care and Research, Department of Oncology, Hematology and BMT Section, Hamad Medical Corporation, Doha, Qatar

Abstract: Secondary iron overload is increasingly encountered in chronic kidney disease (CKD) patients because of the frequent use of parenteral iron products, especially in hemodialysis patients. Serum ferritin has been commonly used to monitor iron overload in these patients; however, other conditions can be associated with the high serum ferritin, like infections and inflammatory conditions. Currently, T2*MRI of the heart and liver is the preferred investigation for evaluating liver iron concentration (LIC) and cardiac iron concentration, which reflect the state of iron overload. Few studies observe a positive correlation between serum iron and LIC in CKD patients and postulate that serum ferritin exceeding 290 mcg/L should indicate significant iron overload and necessitates further MRI evaluation. However, here, we present a patient with a history of ESRD for which she underwent renal transplantation twice referred to our clinic due to persistent elevation in serum ferritin level (>1000 mcg/L) for several years. T2*MRI of the heart and liver revealed the absence of iron overload. Our objective of this case is to demonstrate the accuracy of T2*MRI over serum ferritin in evaluating iron overload and questioning the positive correlation between serum ferritin and LIC in CKD patients.

Keywords: secondary iron overload, chronic kidney disease, serum ferritin, T2*MRI, liver iron concentration

Introduction

Our body normally contains up to 4 grams of iron, mainly in hemoglobin.[1] The liver is considered the main storage site for iron in the body. Total body iron content is established by balancing iron intake from diet or other sources like blood transfusion and iron loss, such as from menstrual bleeding or epithelial cells shedding.[2] However, no physiological mechanism for the excretion of excess iron has been found in the human body.[3] Iron overload occurs either due to the excess of iron intake, which exceeds its loss from the body, like thalassemia and sickle cell disease patients requiring chronic blood transfusion[4,5] or due to the increase in iron absorption from the gut despite normal intake, such in hereditary hemochromatosis.[6] Less frequently, iron overload can happen due to iatrogenic intoxication.[7]

In general, secondary iron overload in ESRD patients is uncommon; however, iron overload has been increasingly diagnosed in the ESRD population over the last decade for multiple reasons. First, given the risk of iron deficiency with erythropoiesis-stimulating agents (ESA) therapy, KDIGO 2012 guideline set 500 µg/L as the upper limit of serum ferritin in ESRD patients on hemodialysis, which encouraged

Correspondence: Abdulrahman Al-Mashdali
Department of Internal Medicine, Hamad Medical Corporation (HMC), P.O. Box 3050, Al Rayyan Road, Doha, Qatar
Tel +967 507 008 16
Email AAlmashdali@hamad.qa

the clinicians to give more intravenous iron to prevent iron deficiency in such patients. Moreover, the recent advance in the diagnostic test for iron overload (particularly MRI) plays a significant role in the early detection of more iron overload cases.[8,9]

Because of their availability and lower cost, serum ferritin and transferrin saturation (TSAT) are frequently used to monitor iron accumulation in the body. Though, these parameters can be affected by different conditions, especially infections and variable inflammatory conditions. However, the evidence for the correlation between serum ferritin with liver iron concentration (LIC) and heart iron concentration is still not well established in patients with chronic kidney disease suspected to have iron overload.[10]

Here, we present a patient with a history of end-stage renal disease (ESRD) and renal transplants referred to our clinic because of persistently elevated serum ferritin for several years. T2* MRI of the heart and liver revealed no evidence of iron overload. In this case, we highlight the superiority of T2* MRI of the heart and liver over serum ferritin in evaluating iron overload in CKD patients.

Case Presentation

A 57-year-old female who referred to our hematology clinic due to persistent elevation in her serum ferritin. Her past medical history was significant for end-stage renal disease (ESRD) due to diabetic nephropathy, for which she underwent kidney transplantation on two occasions. The first kidney transplant was done in 2002 (then, her kidney functions were stable from 2002 until 2016, when they started to deteriorate due to recurrent diabetic nephropathy in the transplanted kidney leading to chronic allograft dysfunction, which required hemodialysis thrice weekly for six months before the second transplant), and the second one in 2017 (Both kidneys transplants were donated voluntarily, the first one from patient's relative and the second from family's friend, with written informed consent, and the organ donation was conducted in accordance with the declaration of Istanbul). The patient also had a past medical history of hypertension and breast cancer diagnosed in 2006 treated with a combination of lumpectomy, chemotherapy, and radiotherapy.

At her initial visit to our clinic, the patient denied any symptoms related to iron overload complications. Her vital signs were within the normal limit. Her body mass index (BMI) was 27. Laboratory findings were significant for serum ferritin of 1219.0 μg/L (normal level < 300), serum iron of 14 μmol/L (normal 9–30), TIBC of 50

μmol/L (normal 40–80), iron saturation of 38% (normal 15–45%). Her liver function tests are normal. Also, Her HbA1C levels were between 7% and 8% for the last five years. Of note, her serum ferritin was repeatedly exceeding 1000 μg/L for the last five years (her renal functions, serum ferritin, TSAT were near normal from 2002 until 2016). Our patient did not have any chronic infection, inflammatory condition, liver disease, or active malignancy (she was asymptomatic with normal inflammatory markers) that could lead to elevated ferritin levels over the last five years. The patient denied smoking or alcohol drinking. We summarized the relevant data from 2016 to 2021 in Table 1.

Given her past medical history of ESRD requiring hemodialysis, secondary iron overload was suspected. Accordingly, an T2*MRI of the heart and liver was suggested to rule out iron deposition in body organs. MRI heart revealed iron deposition of less than 1.2 mg/g of dry heart weight. The MRI liver showed an iron deposition of less than 5 mg/g of dry liver weight, consistent with the absence of myocardial and hepatic iron overload (Figures 1 and 2). Table 2 shows the severity classification of iron overload (both for liver and heart) based on T2*MRI findings. Accordingly, the patient was reassured and treated conservatively. In most recent follow-up in our clinic, the patient was doing fine and asymptomatic, and her latest serum ferritin was 940 μg/L.

Discussion

Iron overload is classified into primary and secondary iron overload. The most common cause of the primary iron overload is hereditary hemochromatosis. The secondary iron overload might occur in the context of different hematological disorders, mainly hemoglobinopathies and CKD. Different mechanisms can lead to iron accumulation associated with hematological diseases, including long-term blood transfusion, chronic hemolysis, increased intestinal absorption of iron due to ineffective hematopoiesis, or genetic mutation in hepcidin.[11,12] Iron overload with CKD may happen due to frequent intravenous iron use, especially in patients on hemodialysis. However, the risk of iron toxicity in ESRD patients is usually insignificant because other factors minimize iron accumulation, including the concurrent use of erythropoiesis-stimulating agents (ESA) and the distribution of hepatic iron by the reticuloendothelial cells.[13] However, as mentioned in the introduction, iron overload is increasingly discovered in hemodialysis patients since that the majority of ESRD

Table 1 Summary of Relevant Data from 2016 to 2021 (Since the Detection of the Elevated Serum Ferritin)

Data	Reference Value	2016 (HD Initiated by the End of This Year)	2017(Before Renal Tx)	2018 (After Renal Tx)	2019	2020	2021 (When T2*MRI Done)
Hb level (range)	12–15 gm/dL	10.2–11	8.8–10.4	11.6–12.2	11.3–11.8	10.9–11.4	12.2–12.9
WBC	4–10 x10^3/uL	7.3	6.5	3.9	4.6	6.2	5.5
Serum ferritin	< 300 mcg/L	282	1150	1830	1650	1933	1219
TSAT	15–45%	39	49	52	46	48	38
Creatinine(range)	50–95 umol/L	168–205	320–380 (required HD 3 times /week)	75–118	105–115	100–120	105–123
Iron received	Total	None	Intravenous ferrous carboxymaltose (> 3 gm over 6 months)	None	None	None	None
Blood transfusion	Units/year	None	Two units of PRBC before renal Tx	None	None	None	None
ESA	Weekly	None	Received darbepoetin for six months before renal Tx	None	None	None	None
CRP level	< 6 mg/L	4	11.4	6.4	4.2	3.8	2.1

Abbreviations: CRP, C-reactive protein; Hb, hemoglobin; HD, hemodialysis; TSAT, transferrin saturation; TX, transplantation; WBC, white blood cells.

patients treated with ESA receive parenteral iron to replenish iron storage and ensure iron availability for hematopoiesis.[8]

Iron overload mainly affects the parenchymal cells of the liver, heart, and endocrine organs. Iron accumulation leads to tissue inflammation and damage through the formation of reactive oxygen species.[14] As the main store for iron in the body, the liver is the most affected organ by iron overload. Different manifestations may result from excessive iron deposition in the liver, ranging from elevated liver enzymes to liver cirrhosis. Indeed, liver cirrhosis is rarely encountered in hemodialysis patients who developed iron overload.[15,16] Iron deposition in the myocardial tissue can occasionally lead to cardiomyopathy.[17] Also, iron overload might increase atherosclerosis risk in ESRD patients.[18] In addition to its effects on different
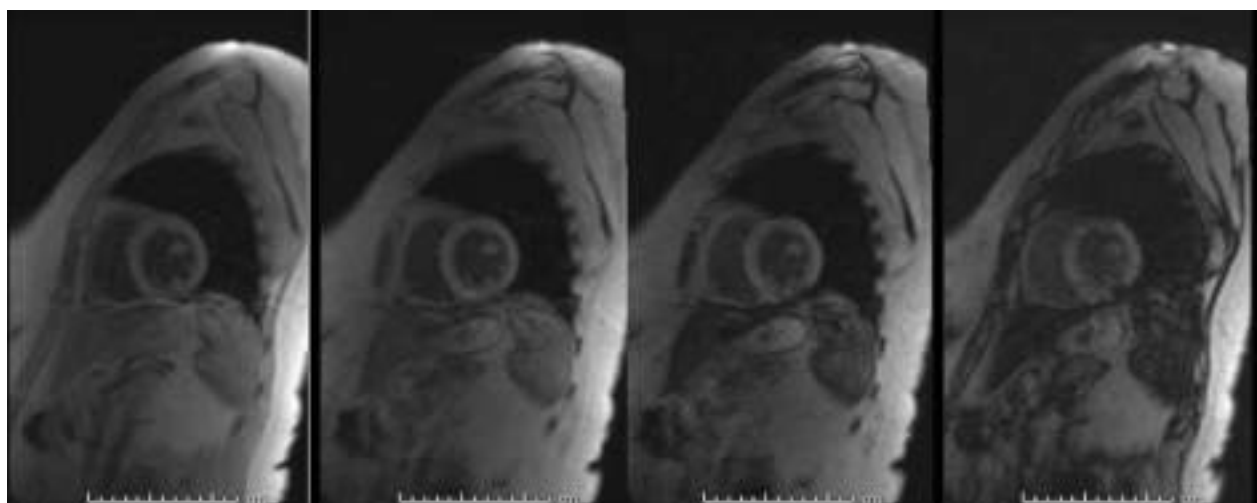
Figure 1 MRI 1.5 T (Siemens Avanto), using multi-TE gradient echo T2* MRI technique. Heart intensity is normal seen with the longest TE (14.68 msec). T2* =29.4 ms corresponding to <1.2 mg Iron/ g heart dry weight.
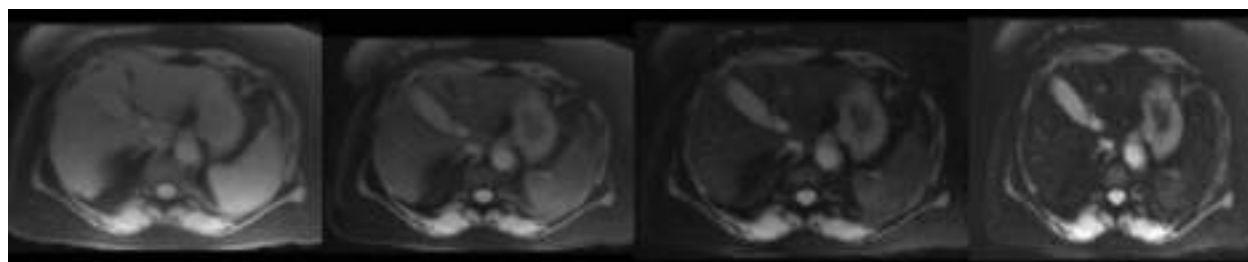
Figure 2 MRI 1.5 T (Siemens Avanto), using multi-TE gradient echo T2* MRI technique (using Garbowski method). Liver intensity is normal seen with the longest TE (14.68 msec). T2* =7.4 ms, corresponding to < 5 mg Iron/ g liver dry weight.

organs, iron overload may increase the risk of infection by disturbing the functions of different immune cells and, as an essential element, by enhancing bacterial growth and multiplication.[16]

Liver iron concentration (LIC) provides a precise representation of body iron storage in patients with iron overload.[19] At present, MRI becomes the modality of choice for the assessment of iron overload.[20] The sensitivity and specificity of standardized MRI protocol in determining LIC are estimated to be 94% and 100%, respectively.[21] The role of MRI methods for evaluating iron overload is well recognized in transfusion-dependent anemia patients; however, this role is still not well established in ESRD patients.[12,16] Given the very high sensitivity (resulting in the low false-negative result) of T2*MRI in the diagnosis of iron overload, we excluded secondary iron overload in our patient, especially in the absence of any clinical or laboratory evidence for that. Few studies were evaluated the correlation between serum ferritin and LIC in CKD patients on hemodialysis by using various MRI standardized protocols. A study done by Canavese et al assumed that the risk of iron overload is ten times more in CKD patients with serum ferritin value surpasses 500 mcg/L.[22] Another study concluded that there is no correlation between serum iron markers and LIC in CKD patients.[23] The latest studies of the correlation between serum ferritin and LIC in hemodialysis patients using T2*MRI found a positive correlation between these two variables. Therefore, it was suggested

that serum ferritin value above 290 mcg/L should justify further investigation with MRI to rule out iron overload in such cases.[24,25] Though different observation was reported in one EDRD patient with a serum ferritin level of >1000 mcg/L, his LIC measured by T2*MRI was suggestive of only mild iron accumulation liver.[12] In our case, we also observed that despite the serum ferritin was >1000 mcg/L for several years, the T2*MRI of the heart and liver showed no evidence of iron overload. Our finding could support the idea that serum ferritin might not correlate with LIC and cardiac iron concentration in ESRD patients.

Conclusion

Despite that, the existing guidelines recommend serum ferritin and transferrin saturation for monitoring secondary iron overload in CKD patients; the evidence for the correlation between serum ferritin and LIC measured by MRI is still scarce in CKD patients. Few studies observe a positive correlation between serum iron and LIC in CKD patients and postulate that serum ferritin above 290 mcg/L should indicate significant iron overload and necessitates MRI evaluation. However, our patient had a serum ferritin level of >1000 mcg/L for several years, but a T2*MRI of the heart and liver revealed the absence of iron overload. We think that further studies are necessary to prove this correlation and determine the cutoff level of serum ferritin that requires further evaluation of iron overload by MRI in CKD patients.

Table 2 Severity of Iron Overload Based on Hepatic and Myocardial T2*MRI and Our Patient Findings

Iron Load Severity	Normal	Mild	Moderate	Severe	Our Patient
Hepatic T2* by millisecond(ms), mg iron/ g of liver dry weight	>7.2ms,< 5 mg/g	3.3–7.2 ms, 5–10 mg/g	2.2–3.3 ms, 10–15 mg/g	< 2.2 ms, > 15 mg/g	T2*=7.4 ms, corresponding to < 5 mg iron/ g of liver dry weight (Normal)
Myocardial T2*by millisecond(ms), mg iron/ g of heart dry weight	> 20 ms, <1.2 mg/g	14–20 ms, 1.2–1.8 mg/g	10–14 ms, 1.8–2.7mg/g	< 10 ms, >2.7mg/g	T2* =29.4 ms, corresponding to <1.2 mg iron/ g of heart dry weight (Normal)

668

Journal of Blood Medicine 2021:12

Consent

Written informed consent was obtained from the patient for the publication of this case report. This case report was approved by the Hamad Medical Corporation's Medical Research Center (Protocol number: MRC-04-21-419).

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

References

1. Cook JD, Flowers CH, Skikne BS. The quantitative assessment of body iron. *Blood*. 2003;101(9):3359–3364. Epub 2003 Jan 9. PMID: 12521995. doi:10.1182/blood-2002-10-3071
2. McLaren GD, Muir WA, Kellermeyer RW. Iron overload disorders: natural history, pathogenesis, diagnosis, and therapy. *Crit Rev Clin Lab Sci*. 1983;19(3):205–266. PMID: 6373141. doi:10.3109/10408368309165764
3. Siddique A, Kowdley KV. Review article: the iron overload syndromes. *Aliment Pharmacol Ther*. 2012;35(8):876–893. Epub 2012 Mar 4. PMID: 22385471. doi:10.1111/j.1365-2036.2012.05051.x
4. Khan AM, Al-Sulaiti AM, Younes S, Yassin M, Zayed H. The spectrum of beta-thalassemia mutations in the 22 Arab countries: a systematic review. *Expert Rev Hematol*. 2021;14(1):109–122. Epub 2020 Dec 29. PMID: 33317346. doi:10.1080/17474086.2021.1860003
5. Yassin M, Soliman A, De Sanctis V, et al. Liver iron content (LIC) in adults with sickle cell disease (SCD): correlation with serum ferritin and liver enzymes concentrations in trasfusion dependent (TD-SCD) and non-transfusion dependent (NT-SCD) patients. *Mediterr J Hematol Infect Dis*. 2017;9(1):e2017037. PMID: 28698780; PMCID: PMC5499497. doi:10.4084/MJHID.2017.037
6. Cullis JO, Fitzsimons EJ, Griffiths WJ, Tsochatzis E, Thomas DW; British Society for Haematology. Investigation and management of a raised serum ferritin. *Br J Haematol*. 2018;181(3):331–340. Epub 2018 Apr 19. PMID: 29672840. doi:10.1111/bjh.15166
7. Yassin M, Soliman AT, De Sanctis V, Moustafa A, Samaan SA, Nashwan A. A young adult with unintended acute intravenous iron intoxication treated with oral chelation: the use of liver ferriscan for diagnosing and monitoring tissue iron load. *Mediterr J Hematol Infect Dis*. 2017;9(1):e2017008. PMID: 28101313; PMCID: PMC5224804. doi:10.4084/MJHID.2017.008
8. Rostoker G, Vaziri ND, Fishbane S. Iatrogenic iron overload in dialysis patients at the beginning of the 21st century. *Drugs*. 2016;76(7):741–757. PMID: 27091216; PMCID: PMC4848337. doi:10.1007/s40265-016-0569-0
9. Aldwairi MM, Yassin MA. Iatrogenic iron overload in an end stage renal disease patient. *Case Rep Oncol*. 2020;13(2):760–763. PMID: 32774272; PMCID: PMC7383200. doi:10.1159/000507979
10. Kanbour I, Chandra P, Soliman A, et al. Severe liver iron concentrations (LIC) in 24 patients with β-thalassemia major: correlations with serum ferritin, liver enzymes and endocrine complications. *Mediterr J Hematol Infect Dis*. 2018;10(1):e2018062. PMID: 30416694; PMCID: PMC6223579. doi:10.4084/MJHID.2018.062
11. Yassin MA, Soliman A, De Sanctis V, et al. The impact of iron overload in patients with acute leukemia and myelodysplastic syndrome on hepatic and endocrine functions. *Acta Biomed*. 2018;89(3–S):18–22. PMID: 29633728; PMCID: PMC6179097. doi:10.23750/abm.v89i3-S.7213
12. Ali M, Okar L, Iqbal P, Yassin MA. Iatrogenic iron overload in a patient with chronic kidney disease: is there a correlation between serum ferritin and liver iron concentration determined by MRI T2*? *Cureus*. 2020;12(6):e8914. PMID: 32742880; PMCID: PMC7389957. doi:10.7759/cureus.8914
13. Wish JB, Aronoff GR, Bacon BR, et al. Positive iron balance in chronic kidney disease: how much is too much and how to tell? *Am J Nephrol*. 2018;47(2):72–83. Epub 2018 Feb 13. PMID: 29439253. doi:10.1159/000486968
14. Le Lan C, Loréal O, Cohen T, et al. Redox active plasma iron in C282Y/C282Y hemochromatosis. *Blood*. 2005;105(11):4527–4531. Epub 2005 Jan 25. PMID: 15671444. doi:10.1182/blood-2004-09-3468
15. Soliman A, Yassin M, Al Yafei F, et al. Longitudinal study on liver functions in patients with thalassemia major before and after deferasirox (DFX) therapy. *Mediterr J Hematol Infect Dis*. 2014;6(1):e2014025. PMID: 24803998; PMCID: PMC4010606. doi:10.4084/MJHID.2014.025
16. Rostoker G, Vaziri ND. Iatrogenic iron overload and its potential consequences in patients on hemodialysis. *Presse Med*. 2017;46(12 Pt 2):e312–e328. Epub 2017 Nov 16. PMID: 29153377. doi:10.1016/j.lpm.2017.10.014
17. Gaenzer H, Marschang P, Sturm W, et al. Association between increased iron stores and impaired endothelial function in patients with hereditary hemochromatosis. *J Am Coll Cardiol*. 2002;40(12):2189–2194. PMID: 12505233. doi:10.1016/s0735-1097(02)02611-6
18. Reis KA, Guz G, Ozdemir H, et al. Intravenous iron therapy as a possible risk factor for atherosclerosis in end-stage renal disease. *Int Heart J*. 2005;46(2):255–264. PMID: 15876809. doi:10.1536/ihj.46.255
19. Angelucci E, Brittenham GM, McLaren CE, et al. Hepatic iron concentration and total body iron stores in thalassemia major. *N Engl J Med*. 2000;343(5):327–331. Erratum in: N Engl J Med 2000 Dec 7;343(23):1740.PMID: 10922422. doi:10.1056/NEJM200008033430503
20. Fischer R, Harmatz PR. Non-invasive assessment of tissue iron overload. *Hematology Am Soc Hematol Educ Program*. 2009;2009:215–221. PMID: 20008201. doi:10.1182/asheducation-2009.1.215
21. Lyle L, Hirose A. Iron overload in myelodysplastic syndromes: pathophysiology, consequences, diagnosis, and treatment. *J Adv Pract Oncol*. 2018;9(4):392–405. Epub 2018 May 1. PMID: 30719392; PMCID: PMC6347085.
22. Canavese C, Bergamo D, Ciccone G, et al. Validation of serum ferritin values by magnetic susceptometry in predicting iron overload in dialysis patients. *Kidney Int*. 2004;65(3):1091–1098. PMID: 14871430. doi:10.1111/j.1523-1755.2004.00480.x
23. Ferrari P, Kulkarni H, Dheda S, et al. Serum iron markers are inadequate for guiding iron repletion in chronic kidney disease. *Clin J Am Soc Nephrol*. 2011;6(1):77–83. Epub 2010 Sep 28. PMID: 20876673; PMCID: PMC3022252. doi:10.2215/CJN.04190510
24. Ghoti H, Rachmilewitz EA, Simon-Lopez R, et al. Evidence for tissue iron overload in long-term hemodialysis patients and the impact of withdrawing parenteral iron. *Eur J Haematol*. 2012;89(1):87–93. Epub 2012 May 18. PMID: 22435497. doi:10.1111/j.1600-0609.2012.01783.x
25. Rostoker G, Griuncelli M, Loridon C, et al. Reassessment of iron biomarkers for prediction of dialysis iron overload: an MRI Study. *PLoS One*. 2015;10(7):e0132006. PMID: 26182077; PMCID: PMC4504469. doi:10.1371/journal.pone.0132006

Human Immunodeficiency Virus and Syphilis Among Blood Donors at Western Oromia, Ethiopia

Milkias Abebe & Nagasa Marga

Dovepress

ORIGINAL RESEARCH

Human Immunodeficiency Virus and Syphilis Among Blood Donors at Western Oromia, Ethiopia

Milkias Abebe [1]
Nagasa Marga[2]

[1]Department of Medical Laboratory Sciences, Institute of Health Sciences, Wollega University, Nekemte, Oromia, Ethiopia; [2]Department Medical Laboratory, Bako Hospital, Bako, Oromia, Ethiopia

Background: Blood transfusion saves lives and improves health, but many patients requiring transfusion do not have timely access to safe blood. Human immunodeficiency virus and syphilis are the commonest transfused transmitted infections and threats to blood recipients. Proper donor selection and screening of the donated blood for major transfusion-transmitted infections evidently reduced challenge in blood safety. Therefore, the objective of this study was to determine the sero-prevalence HIV and syphilis infections among blood donors at East Wollega, West Ethiopia.

Patients and Methods: A five-year (from January 2015 to December 2019) retrospective study was conducted by reviewing blood donor laboratory test results from Nekemte blood bank which is serving hospitals in Western Oromia. Blood donor data were analyzed by Statistical Package for Social Sciences version 20 software.

Results: The total of 17,810 individual's blood was screened during the study period. The overall prevalence of HIV, syphilis, and their co-infection was 222 (1.25%), 142 (0.80%), and 5 (0.03%), respectively. The prevalence of HIV was associated with unmarried (AOR: 2.4; 95% CI: 1.5, 5.2), male (AOR: 2.1; 95% CI: 1.5, 2.9), and blood donors resident in a rural area (AOR: 1.5; 95% CI: 1.5, 5.9). Besides, the prevalence of syphilis was associated with education, age, marital status and residence of study participant.

Conclusion: In the current study, the sero-prevalence of HIV and syphilis among blood donors was low, when it was compared to other sub-Saharan Africa country. However, to ensure the health of all recipients screening blood using standard methods is highly recommended.

Keywords: epidemiology, predictor, transfusion-transmitted infection, blood bank

Introduction

Blood transfusion is a life-saving therapeutic intervention in which globally more than 81 million units of blood are donated every year. Among these 18 million are not screened for an infection that is potentially capable of being transmitted by blood transfusion due to the blood is a vector for harmful transfusion transmissible infections.[1] Human immunodeficiency virus and *Treponema palladium* are the common microorganisms for post-transfusion transmitted infection.[2]

The World Health Organization, to assure quality and safety, recommends screening of donated blood for a minimum of the major transfusion-transmitted infections (TTIs). Accordingly, screening for HIV, hepatitis B, hepatitis C, and syphilis should be mandatory.[3,4] Annually, the worldwide infection rate of HIV

Correspondence: Milkias Abebe
Tel +251912863004
Fax +251 57 661-7980
Email mickyelfe21@gmail.com

through blood transfusion alone ranges from 80,000 to 160,000.[5] Up to 500 people acquire TTIs due to contaminated blood transfusion daily in Africa[5] and in sub-Saharan Africa, blood transfusion accounts for 5–10% of HIV transmission.[6] The estimated prevalence of HIV infection among blood donors in Ethiopia ranged from 0.1% - 11.7%.[7–9]

There is a scarcity of comprehensive data on the prevalence and trends of major TTI among blood donors in the study area. Thus, the current study aimed to determine the prevalence and predictors of HIV and syphilis infections among blood donors in the Western Oromia, East Wollega, Ethiopia.

Methods
Study Area
The study was conducted in Nekemte Blood Bank, which is serving about 17 hospitals in Western Oromia. This blood bank is placed in the Nekemte town which is the capital city of the East Wollega Zone, Western Oromia, Ethiopia. Nekemte town is located 331 km west of the capital city, Addis Ababa.

Study Design
A five-year (January 2015 to December 2019) retrospective study was conducted by retrieving data from the Nekemte Blood Bank laboratory registration book.

Study Population
Blood donors who were registered on the laboratory registration of Nekemte Blood bank and screened for HIV and syphilis infection during the study period.

Data Collection, Laboratory Examination, and Statistical Analysis
The data extraction sheet was used to collect data regarding socio-demographic variables and blood donors laboratory test results of HIV and syphilis from Nekemte Blood Bank registration book. To identify HIV and syphilis of blood donors ELISA serological method was applied during the study period. In case of HIV, the WANTAI HIV 1 + 2 Ag/Ab ELISA test kit (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. China) was used. Besides, the blood donor sample was tested for anti-syphilis Ab: using DIALAB ELISA (Nora Kampitsch, MSc, India). Finally, the collected data were entered and analyzed by

using a statistical package for the social science version 20 software.

Results
From a total of 17,810 blood donors, 70.1% (12,480) of the study participants were males. Of the total, a majority of 74.4% (13,245) blood donors were single, 59.4% (10,572) were students, and 70% (12,456) donors were urban residents (Table 1).

The sero-prevalence of HIV was 1.25% (222/17,810). Of all study participants five (0.03%) of them had co-infection of HIV and syphilis. Furthermore, of the total infected participants, the majority (53.85%) of unmarried, 42.87% of males, 40.38% of age group within 18–30, 34.89% of students, 47.53% of resident in urban, and 41.48% of participants had above the secondary level of education were infected by HIV (Table 1). Besides, the sero-prevalence of HIV was 0.06% (10/17,810) in 2015 and increased to 0.29% (52) in 2016 and 0.48% (86/17,810) in 2017, then decreased to 0.26% (46/17,810) and 0.16% (28/17,810) in 2018, and 2019, respectively (Table 2).

The sero-prevalence of Syphilis was 0.8% (142/17,810). Of all study participants five (0.03%) of them had co-infection of HIV and syphilis. Moreover, of the total infected blood donors 29% of males, 34.89% of unmarried, 19.78% of age group within 35–65, 15.38% of students, and 24.45% resident in rural were infected with syphilis (Table 1). In addition, the seroprevalence of syphilis increased steadily from 0.06% (10/17,810) in 2015 to 0.02% (35/17,810) in 2019 (Table 2).

In the current study, the risk factors of HIV and syphillis were identified by multivariate logistic regression analysis. According to this study, the male blood donors were two times (AOR: 2.1; 95% CI: 1.5–2.9) more likely to be infected by HIV compared to the counterpart. Besides, the unmarried study participant was also two times (AOR: 2.4; 95% CI: 1.5–5.2) more likely to be infected by HIV when it was compared with that of married blood donor (Table 3).

Age, marital status, educational status, and residence were risk factors of syphilis in the present study. Those study participant at the age of 31–35 years (AOR = 2.8; 95% CI: 1.4, 3.9) and 45–65 years (AOR = 3.9; 95% CI: 1.5–3.7), unmarried blood donors (AOR = 2.5; 95% CI: 1.4,3.2), donors with no formal education (AOR = 3.5; 95% CI: 1.9, 4.5) and donor who were resident in rural

Table 1 Socio-Demographics Characteristics, Prevalence of HIV and Syphilis Among Blood Donor, Western Oromia, Ethiopia from January 2015 to December 2019

Variable	HIV Positive N (%)	Syphilis Positive N (%)	Total Infected Participants N (%)
Sex			
Male	156 (42.85)	107 (29.40)	263 (72.25)
Female	66 (18.13)	35 (9.62)	101 (27.75)
Age			
18–30	147 (40.38)	27 (7.42)	174 (47.80)
31–45	52 (14.29)	43 (11.81)	95 (26.10)
46–65	23 (6.32)	72 (19.78)	95 (26.10)
Marital Status			
Unmarried	196 (53.85)	127 (34.89)	323 (88.74)
Married	26 (7.14)	15 (4.12)	41 (11.26)
Occupational status			
Student	127 (34.89)	56 (15.38)	183 (50.27)
Private worker	22 (6.04)	15 (4.12)	37 (10.16)
Government Employs	63 (17.30)	52 (14.29)	115 (31.59)
Farmer	10 (2.75)	19 (5.22)	29 (7.97)
Educational status			
No formal	47 (12.91)	61 (16.76)	108 (29.67)
Primary school	24 (6.59)	9 (2.47)	33 (9.06)
Secondary and college	151 (41.48)	72 (19.78)	223 (61.26)
Residence			
Urban	173 (47.53)	53 (14.56)	226 (62.09)
Rural	49 (13.46)	89 (24.45)	138 (37.91)

Abbreviations: N,number; %, percent.

Table 2 Sero-Prevalence of HIV and Syphilis Infections with Respect to Donation Year Among Blood Donors, Western Oromia, Ethiopia from January 2015 to December 2019

Year of Donation	No of Donor	HIV Positive N (%)	Syphilis Positive N (%)
2015	2693	10 (0.06%)	10 (0.06%)
2016	2197	52 (0.29%)	30 (0.16%)
2017	4178	86 (0.48%)	25 (0.14%)
2018	4406	46 (0.26%)	42 (0.24%)
2019	4336	28 (0.16%)	35 (0.20%)
Total	17,810	222 (1.25%)	142 (0.80%)

Abbreviations: N,number; %,percent.

(AOR = 3.7; 95% CI: 1.9,4.6) were more likely to be infected by syphilis compared their counterpart (Table 4).

Discussion

In the current study, the majority of the donors (70.1%) were males, which were similar to the study in Gondar,[7] and Nigeria.[10] The numbers of female blood donors were few in the current study, and this may be attributed to behavioral and sociocultural drivers in Ethiopian society that the male is donated blood than female.[11] Regarding trend, seroprevalence of HIV and syphilis with respect to year has shown a trend. However, a study from Gondar, Ethiopia has shown that consistent increment in the seroprevalence from 2010 to 2012.[12] This might be a result of variation in the sero-prevalence of HIV and syphilis in the community since it is assumed that blood donors are a representative of the community. In addition, it might be as a result of the

Table 3 Logistic Regression of HIV with Socio-Demographic Characteristics of Blood, Western Oromia, Ethiopia from January 2015 to December 2019

Variable	HIV Status		COR (95% CI)	AOR (95% CI)
	Negative	**Positive**		
Sex				
Female	5264	36	1.00	
Male	12,324	186	2.2 (1.6–3.2)	2.1 (1.5–2.9)*
Age				
18–30	11,313	147	1.00	
31–45	5840	52	0.7 (0.4–2.9)	
46–65	435	23	4.1(0.3–4.6)	
Marital status				
Married	4539	26	1.00	
Unmarried	13,049	196	2.6 (1.6–5.5)	2.4 (1.5–5.2)*
Occupation				
Student	10,445	127	1.00	
Private worker	1208	22	1.5 (0.5–3.4)	
Government Employs	5413	63	0.9 (0.3–1.8)	
Farmer	522	10	1.5 (0.8, 2.4)	
Educational Status				
Primary school	5419	24	1.00	
Secondary and college	8367	151	4.1 (0.4–4.1)	
No formal education	3802	47	2.8 (0.3–3.4)	
Residence				
Rural	5305	49	1.00	
Urban	12,283	173	1.5 (1.6–6.6)	1.5(1.5–5.9)*

Note: *Statistically significance.
Abbreviations: COR, crude odd ratio; AOR, adjusted odd ratio; CI, confidence interval.

implementation of sensitive diagnostic test methods for screening of this infection.

The majority (64.5%) study participants' age in the present study were ranging from 18 to 30. Our result is similar to research conducted in North Gondar,[12] Southwest Ethiopia,[13] and Northwest Ethiopia.[7] The highest number of donations in this age group (18–30 years) is due to the fact that active age groups (18–30) of the population are actively participating in blood donations. Besides, a good awareness was created on these age groups in the current study area.

The seroprevalence of HIV in the present study was 1.25%. This result was similar to the study reported from Sudan, which was 1%[14] and 1.13% in South Africa.[15] However, it was lower than a study conducted by different scholars in Ethiopia which was 4.5% of Diro et al[16] and 3.8% of Tessema et al,[7] and 3.8% in another country.[17] The reason for the low prevalence of HIV seropositivity in

the present study is due to the fact that there is good voluntary counseling test coverage in the study area. On the other hand, the prevalence of HIV among blood donors in the present study was higher than a similar study conducted in Jigjiga which was 0.1%,[9] 0.18% in Eretria[18] and 0.00% in Egypt in which there were no cases reported.[19] The differences might be due to, differences in geographical locations, the burden of the disease in the society and difference in awareness of study participant on transmission and prevention of HIV.

In the current study, HIV infection was highest among blood donors who were male, college students, and 18–30 years of age which were similar to a report from another study area.[20] Besides, in the present study sex, marital status, and residence of blood donors were risk factors for HIV seroprevalence. Male blood donors were more likely to be infected with HIV compared to female which was agreed with other research reported by different

Table 4 Logistic Regression of Syphilis with Socio-Demographic Characteristics of Blood Donor, Western Oromia, Ethiopia from January 2015 to December 2020

Variable	Syphilis Status		COR (95% CI)	AOR (95% CI)
	Negative	**Positive**		
Sex				
Female	5295	35	1.00	
Male	12,373	107	1.3(0.8, 2.9)	
Age				
18–30	7633	27	1.00	
31–45	5849	43	3.1(1.5, 4.5)	2.8(1.4–3.9)*
46–65	4186	72	4.8(1.8–4.7)	3.9(1.5–3.7)*
Marital status				
Married	4550	15	1.00	
Unmarried	13,118	127	3(1.7–3.6)	2.5(1.4–3.2)*
Occupation				
Student	10,516	56	1.00	
Private worker	1215	15	2.3(0.5–2.9)	
Government Employs	5424	52	1.8 (0.1–4.5)	
Farmer	513	19	6.9(0.6–5.2)	
Educational Status				
Primary school	5424	19	1.00	
Secondary and college	8446	72	2.4(0.8,3.4)	
No formal	37,898	51	3.8 (1.9–4.7)	3.5(1.9–4.5)*
Residence				
Urban	12,403	53	1.00	
Rural	5265	89	3.9(1.8–4.9)	3.7(1.9–4.6)*

Note: *Statistically significance.
Abbreviations: COR, crude odd ratio; AOR, adjusted odd ratio; CI, confidence interval.

scholars.[7,21] This might be due to most females may know their HIV status at prenatal care which is mandatory for all pregnant women in our country. The present study has also shown that unmarried study participants were higher risk for HIV compared to married blood donors. The plausible explanation for this result is, married study participant might have stable sexual partners which decrease their risk of HIV infection.[22] Moreover, rural blood donors were more likely to be infected with HIV than the urban donors. Similar result had been reported from another study area.[23] The prevalence of HIV, which is high in rural study participant, is might be due to lack of awareness regarding the transmission and prevention of this virus from different electronic media.

The present study has shown that the prevalence of syphilis among study participant was 0.8%, which was similar to the research done in Hawassa, Ethiopia.[24] However, it was slightly higher than a similar study

conducted in Eritrea (0.49%)[18] and lower than a study reported by scholars in Gondar which was 1.7%, and Tanzania which was 4.7%.[17] These differences between different countries might be due to the quality of laboratory tests used to screen blood samples, the difference in prevention measures taken, the effectiveness of the program to choose blood donors. In this study, the prevalence of syphilis significantly associated with older blood donors within the age group 31–35 years and 45–65 years compared to the reference group. This result was similar with study conducted in different countries.[1,11,17,25,26] However, our result was not inconsistent with some study conducted in our country like in Gondar and Jigjiga.[9] Similar to study conducted in Eastern Ethiopia,[27] in the current study unmarried blood donors, donors with no formal education and donors who were living in rural were highly vulnerable to syphilis compared to their counterparts.

The current study has provided a good data on the prevalence and some associated predictors of HIV and syphilis due to the study tried to use long year blood donor's history with large sample size. However, some variables were missed since the study was retrospective, which was reviewed from laboratory registration book of the blood bank.

Conclusion

The result of the current study has shown that the seroprevalence of HIV and syphilis was low compared to study from countries in sub-Saharan Africa. Though, a substantial percentage of study participants had HIV and syphilis infections. Therefore, strict selection of blood donors using standard methods is necessary to make sure the health of the recipients is protected. Besides, increasing the awareness of rural people regarding the transmission and prevention of infection required to minimize the burden of HIV and syphilis should be encouraged.

Abbreviations

ELISA, enzyme linked immunosorbent assay; HIV, human immunodeficiency virus; SPSS, Statistical Package for Social Sciences; TTIs, transfused transmitted infections; WHO, World Health Organization.

Data Sharing Statement

All data generated or analyzed during this study were included in this article.

Ethics Approval and Consent to Participate

This research was conducted after obtaining ethical clearance from an ethical review committee of the Institute of Health Science, Wollega University with reference number DMLS/127/11. All data and samples obtained from them were kept confidential by using codes instead of any personal identifiers and were meant only for the purpose of the study. Besides, the study was conducted in accordance with the Declaration of Helsinki.

Acknowledgments

We would like to express our special thanks to all staff of the Nekemte blood bank for their support during data collection.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests in this work.

References

1. Adu-poku F, Agboli E, Tarkang EE, Region HV, Region HV. Seroprevalence of transfusion-transmissible infections among blood donors in the Hohoe Municipal Hospital, Ghana: 2015–2016: a retrospective hospital-based cross-sectional study. *PAMJ Clin Med*. 2020;2:12.
2. Song Y, Bian Y, Petzold M, Ung COL. Prevalence and trend of major transfusion-transmissible infections among blood donors in Western China, 2005 through 2010. *PLoS One*. 2014;9(4):e94528. doi:10.1371/journal.pone.0094528
3. Deshpande RH, Bhosale S, Gadgil PA, Sonawane M. Blood donor's status of HIV, HBV, HCV and syphilis in this region of Marathwada, India. *J Krishna Inst Med Sci Univ*. 2012;1(2):111–116.
4. Negash M, Ayalew M, Geremew D, Workineh M. Seroprevalence and associated risk factors for HIV, hepatitis B and C among blood donors in South Gondar District blood Bank, Northwest Ethiopia. *BMC Infect Dis*. 2019;19:430. doi:10.1186/s12879-019-4051-y
5. World Health Organization. 2020. Available from: https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability. Accessed July 17, 2021.
6. World Health Organization. Blood safety and availability fact sheet. 2019. Available from: https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability. Accessed July 17, 2021.
7. Tessema B, Yismaw G, Kassu A, et al. Seroprevalence of HIV, HBV, HCV and syphilis infections among blood donors at Gondar University Teaching Hospital, Northwest Ethiopia: declining trends over a period of five years. *BMC Infect Dis*. 2010;10:111. doi:10.1186/1471-2334-10-111
8. Bisetegen FS, Bekele FB, Ageru TA, Wada FW. Transfusion-transmissible infections among voluntary blood donors at Wolaita Sodo University Teaching Referral Hospital, South Ethiopia. *Can J Infect Dis Med Microbiol*. 2016;2016:6. doi:10.1155/2016/8254343
9. Mohammed Y, Bekele A. Seroprevalence of transfusion transmitted infection among blood donors at Jijiga blood bank, Eastern Ethiopia: retrospective 4 years study infectious diseases. *BMC Res Notes*. 2016;9:129. doi:10.1186/s13104-016-1925-6
10. Nwankwo E, Momodu I, Umar I, Musca B, Adeleke S. Seroprevalence of major blood-borne infections among blood donors in Kano, Nigeria. *Turk J Med Sci*. 2012;42(2):337–341.
11. Siraj N, Achila OO, Issac J, et al. Seroprevalence of transfusion-transmissible infections among blood donors at National Blood Transfusion Service, Eritrea: a seven-year retrospective study. *BMC Infect Dis*. 2018;18:264. doi:10.1186/s12879-018-3174-x

12. Biadgo B, Shiferaw E, Woldu B, Alene KA, Melku M. Transfusion-transmissible viral infections among blood donors at the North Gondar district blood bank, northwest Ethiopia: a three year retrospective study. *PLoS One.* 2017;12(7):e0180416. doi:10.1371/journal.pone.0180416

13. Kebede W, Mekonnen Z, Gerbi A, Abebe G. Transfusion-transmissible infection surveillance among blood donors in Southwest Ethiopia: a six years retrospective study. *Asian Pac J Trop Dis.* 2017;7(3):156–161. doi:10.12980/apjtd.7.2017D6-384

14. Abou MA, Abdalla M, Eltahir YM, Ali AS. Seroprevalence of hepatitis B virus and hepatitis C virus among blood donors in Nyala, South Dar Fur, Sudan. *Virol J.* 2009;6:1–4. doi:10.1186/1743-422X-6-146

15. Vermeulen M, Swaneveder R, Chowdhury D, et al. Use of blood donor screening to monitor prevalence of HIV and hepatitis B and C viruses, South Africa. *Emerg Infect Dis.* 2017;23(9):1560–1563. doi:10.3201/eid2309.161594

16. Diro E, Alemu S, G/yohannes A. Blood safety & prevalence of transfusion transmissible viral infections among donors at the Red Cross Blood Bank in Gondar University hospital. *Ethiop Med J.* 2008;46(1):7–13.

17. Matee MIN, Magesa PM, Lyamuya EF. Seroprevalence of human immunodeficiency virus, hepatitis B and C viruses and syphilis infections among blood donors at the Muhimbili National Hospital in Dar Es Salaam, Tanzania. *BMC Public Health.* 2006;6:21. doi:10.1186/1471-2458-6-21

18. Fessehaye N, Naik D, Fessehaye T. Transfusion transmitted infections – a retrospective analysis from the National Blood Transfusion Service in Eritrea. *Pan Afr Med J.* 2011;9:40. doi:10.4314/pamj.v9i1.71219

19. Nada HA, Atwa M. Seroprevalence of HBV, HCV, HIV and syphilis markers among blood donors at Suez Canal University Hospital Blood Bank. *J Blood Disord Transf.* 2013;5(1):177.

20. Yang S, Jiao D, Liu C, et al. Seroprevalence of human immunodeficiency virus, hepatitis B and C viruses, and Treponema pallidum infections among blood donors at Shiyan, Central China. *BMC Infect Dis.* 2016;16(1):531. doi:10.1186/s12879-016-1845-z

21. Jary A, Dienta S, Leducq V, et al. Seroprevalence and risk factors for HIV, HCV, HBV and syphilis among blood donors in Mali. *BMC Infect Dis.* 2019;19:1064. doi:10.1186/s12879-019-4699-3

22. Kposowa AJ. Marital status and HIV/AIDS mortality: evidence from the US National Longitudinal Mortality Study. *Int J Infect Dis.* 2013;17(10):e868–e874. doi:10.1016/j.ijid.2013.02.018

23. Yambasu EE, Reid A, Owiti P, Manzi M, Murray M, Edwin A. Hidden dangers-prevalence of blood borne pathogens, hepatitis B, C, HIV and syphilis, among blood donors in Sierra Leone in 2016: opportunities for improvement: a retrospective, cross-sectional study. *Pan Afr Med J.* 2018;30:44. doi:10.11604/pamj.2018.30.44.14663

24. Bonja F, Hussein M, Alemu J, Gemechu D, Birhaneselassie M. The prevalence of transfusion transmitted infections: a focus on hepatitis B virus among blood donors at Hawassa blood bank center, Southern Ethiopia. *Int J Blood Transfus Immunohematol.* 2017;7:7–14. doi:10.5348/ijbti-2017-29-OA-2

25. Bekele T, Adera A, Degu G, et al. Seroprevalence and diagnosis of HIV, HBV, HCV and syphilis infections among blood donors. *Hum Antibodies.* 2017;25(1):39–55.

26. Teklemariam Z, Mitiku H, Weldegebreal F. Seroprevalence of transfusion transmissible viral infections (HIV, HBV and HCV) among voluntary blood donors at University of Gondar Comprehensive Specialized Hospital, Gondar; Northwest Ethiopia. *BMC Hematol.* 2018;18:24. doi:10.1186/s12878-018-0115-2

27. Heyredin I, Mengistie B, Weldegebreal F. Sero-prevalence of transfusion-transmittable infections and associated factors among blood donors in Eastern Ethiopia: an Institutional-based cross-sectional study. *SAGE Open Med.* 2019;7:2050312119834468. doi:10.1177/2050312119834468

Antifungal Prophylaxis with Posaconazole versus Fluconazole in Children with Neutropenia Following Allogeneic Hematopoietic Stem Cell Transplantation: Single Center Experience

Chayamon Takpradit, Chonthida Wangkittikal, Supattra Rungmaitree, Jassada Buaboonnam, Nattee Narkbunnam, Kamon Phuakpet, Nassawee Vathana, Kleebsabai Sanpakit & Bunchoo Pongtanakul

ORIGINAL RESEARCH

Antifungal Prophylaxis with Posaconazole versus Fluconazole in Children with Neutropenia Following Allogeneic Hematopoietic Stem Cell Transplantation: Single Center Experience

Chayamon Takpradit [1]
Chonthida Wangkittikal [2]
Supattra Rungmaitree [3]
Jassada Buaboonnam [1]
Nattee Narkbunnam [1]
Kamon Phuakpet [1]
Nassawee Vathana [1]
Kleebsabai Sanpakit [1]
Bunchoo Pongtanakul [1]

[1]Division of Hematology and Oncology, Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; [2]Division of Hematology and Oncology, Department of Pediatrics, Chonburi Hospital, Chonburi, Thailand; [3]Division of Pediatric Infectious Diseases, Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Background: Invasive fungal diseases (IFDs) are common and contribute to mortality in patients undergoing hematopoietic stem cell transplantation (HSCT). The relative efficacies of posaconazole (POS) and fluconazole (FLU) as primary antifungal prophylaxes are uncertain.

Methods: A retrospective study was performed on children treated with allogeneic HSCT who received POS or FLU during the early neutropenic period. The efficacies, safety, and tolerabilities of the prophylaxes were compared.

Results: Data on 78 HSCT recipients were analyzed. Most had thalassemia (58%). Pre-engraftment, POS and FLU were administered to 41 and 37 cases, respectively. There were no proven cases of IFD. However, 2 POS cases and 1 FLU case had probable IFDs. The IFD incidences of the POS (5%) and FLU (3%) groups demonstrated no statistical difference ($p = 0.620$). Of the 75 surviving cases receiving FLU post-engraftment (including 39 cases previously given POS), 3 had proven IFDs whereas 3 had probable IFDs (total, 6 [8%]) within 1 year post-HSCT. No cases discontinued the prophylaxes due to drug intolerance. The common adverse events with POS and FLU were not significantly different. Only 19% of the patients achieved the therapeutic POS level, with a starting dose of 4 mg/kg thrice daily.

Conclusion: POS and FLU demonstrate comparable levels of effectiveness, safety, and tolerability as IFD prophylaxes for neutropenic children treated with allogeneic HSCT. Determination of the optimum POS dose and duration requires larger studies.

Keywords: antifungal prophylaxis, children, fluconazole, hematopoietic stem cell transplantation, posaconazole

Introduction

Invasive fungal diseases (IFDs) are leading causes of mortality in patients receiving hematopoietic stem cell transplantations (HSCTs). Several studies reported that the IFD incidence in HSCT recipients ranged from 6% to 33% and was associated with grave prognoses.[1–4] *Candida* and *Aspergillus* species, the 2 most commonly encountered fungal pathogens,[1,2] have high mortality rates (49% and 67%, respectively).[5] Our hospital has also reported the incidence of IFD among adult patients with hematological malignancies. Candidiasis was the most common pathogen (63%), followed by aspergillosis (26%).[6]

Correspondence: Bunchoo Pongtanakul
Division of Hematology and Oncology, Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkok Noi, Bangkok, 10700, Thailand
Tel +66 2-419-5971
Fax +66 2-866-3021
Email pongtanakul@yahoo.com

Since the 1990s, fluconazole (FLU) has been the drug of choice as the primary prophylaxis against invasive candidiasis for allogeneic HSCT recipients.[7] However, the major drawback of FLU is that it demonstrates no antimicrobial activity against molds. To overcome this problem, posaconazole (POS), an oral suspension of an azole mold-active prophylaxis agent, has been utilized. Several studies (mostly in adult HSCT recipients) reported that POS produced superior outcomes to FLU—including a lower mortality rate—for the prevention of IFDs.[8,9]

On the other hand, POS may exhibit erratic absorption, and it requires therapeutic drug monitoring. The pharmacokinetics of POS in the pediatric population remains limited.[10,11] Promising results were shown for immunocompromised pediatric patients, with no serious adverse events related to POS being observed.[12–14] There is a recommendation to administer a mold-active agent with an echinocandin or a mold-active azole when systemic antifungal prophylaxis is warranted. For children younger than 13 years of age, echinocandin, voriconazole, or itraconazole is suggested. POS may also be used in those aged 13 years or older.[15] The oral suspension of POS has been available at our institute since 2012. We report the incidence and outcomes of IFDs, and the safety and tolerability of using either POS or FLU, for pediatric patients (aged 0–18 years) who underwent allogeneic HSCTs at our center.

Patients and Methods

We retrospectively analyzed the medical records of 78 Siriraj Hospital patients aged 0–18 years who underwent allogeneic HSCTs, with either FLU or POS as their primary IFD prophylaxis, between 2000 and 2019. A combination of calcineurin inhibitors and short-course methotrexate was administered to the patients as a graft-versus-host disease (GvHD) prophylaxis. Active surveillance to identify the adverse events during the HSCTs was undertaken. The trough level of cyclosporine (CsA), between 200 and 400 ng/mL, was monitored after 2004, and cytomegalovirus viral-load testing was available from 2006. Filgrastim was given once daily at 10 mcg/kg IV from Day +1 until the absolute neutrophil count reached $\geq 1 \times 10^3$/uL for at least 2 consecutive days. Broad-spectrum antibiotics were administered for the treatment of febrile neutropenia. An antifungal prophylaxis—either POS or FLU—was commenced on Day +1 after the stem cell infusion and continued until engraftment. Between 2000 and 2011, FLU (10 mg/kg) was administered orally or intravenously (depending on a patient's status) once daily for all patients receiving HSCTs. After 2012, however, all HSCT patients were given POS as an oral suspension at the dose of 4 mg/kg, thrice daily with meals. The POS level was determined on Day +5 post-HSCT and adjusted dose to the target trough levels of ≥ 0.7 mg/L. Post-engraftment, all patients in the POS group were changed to FLU until cessation of immunosuppression due to financial constraints. The post-engraftment period for patients with FLU started one day after evidence of neutrophil engraftment.

The primary analysis compared the incidences of proven and probable IFDs during the pre-engraftment phase. The diagnosis of IFDs was made by a pediatric infectious-disease specialist team. The secondary analysis focused on 2 aspects. The first was identification of adverse events that were possibly related to the antifungal agents, such as nausea, diarrhea, exanthema, elevated transaminase levels, and renal insufficiency. We categorized the subgroup analysis to identify liver toxicity and CsA level, based on antifungal exposure and POS levels: (1) POS ≥ 0.7 ng/mL group; (2) POS < 0.7 ng/mL group; and (3) FLU group. The second aspect was the incidence of IFDs during the post-engraftment period among 2 sets of patients: those administered POS pre-engraftment and FLU post-engraftment, and those receiving FLU from the outset. Approval for the study was obtained from Siriraj Institutional Review Board/Privacy Board (716/2560 [EC2]). The need for consent forms was waived, given that the study was conducted retrospectively. We declared that the patient data confidentiality complied with the Declaration of Helsinki.

Definitions

Adverse events were graded according to the Common Terminology Criteria for Adverse Events (version 5.0).[16] IFDs were categorized as proven and probable, as per the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) 2019 definitions.[17] The IFD outcomes followed the criteria specified in the EORTC/MSGERC criteria.[18]

Statistical Analysis

The statistical analyses were performed using IBM SPSS Statistics for Windows (version 26.0; IBM Corp., Armonk, NY, USA). In comparisons of the 2 treatments, a chi-squared test or Fisher's exact test was used for qualitative variables, whereas a two-sample *t*-test and the Mann–Whitney *U*-test were used for quantitative variables with

and without normal distribution, respectively. Kaplan–Meier curves were used to present the cumulative incidence of time-to-event data from the dates of the HSCTs, and the curves were compared using the Log rank test. All *p* values were 2-tailed, and $p < 0.05$ was considered statistically significant. The Kruskal–Wallis test was used to compare the baseline and maximum levels of total bilirubin, direct bilirubin, alanine transaminase, and aspartate transaminase. The CsA levels during the antifungal prophylaxis were analyzed using one-way ANOVA. Graphs were created with GraphPad Prism for Mac (version 8.4; GraphPad Software, San Diego, CA, USA).

Results
Patient Characteristics

The analysis used data from 78 patients (49 males and 29 females), with a median age at HSCT of 7.5 years (range, 1.4 to 17.7). During the pre-engraftment period, 41 patients (53%) received POS (the POS group), while 37 (47%) received FLU (the FLU group). The patient characteristics are listed in Table 1. Hematological malignancy was significantly higher for the POS group ($p = 0.043$), while non-hematological malignancy was higher for the FLU group ($p = 0.043$). The stem cell source differed between the 2 groups ($p = 0.002$), as did the median

Table 1 Patient and Transplant Characteristics

Variable	POS	FLU	Total	*P* value
Number of patients; no. (%)	41 (53)	37 (47)	78	
Male sex; no. (%)	26 (63)	23 (62)	49 (63)	0.909
Age; median (range) in year	7.7 (1.4–17.7)	7.0 (1.4–14.7)	7.5 (1.4–17.7)	0.337
Primary disease; no. (%)				
Hematological malignancy	15 (37)	6 (16)	21 (27)	0.043[a]
Relapsed/refractory ALL	5 (12)	3 (8)	8 (10)	0.631
AML	4 (10)	0	4 (5)	0.280
Relapsed/refractory AML	2 (5)	1 (3)	3 (4)	0.843
Biphenotypic leukemia	1 (2)	0	1 (1)	0.517
Histiocytic sarcoma	1 (2)	0	1 (1)	0.517
CML	0	1 (3)	1 (1)	0.286
JMML	1 (2)	1 (3)	2 (3)	0.5
Lymphoma	1 (2)	0	1 (1)	0.517
Non-hematological malignancy	25 (61)	31 (84)	56 (72)	0.043
Thalassemia	20 (49)	25 (68)	45 (58)	0.952
Aplastic anemia	3 (7)	4 (11)	7 (9)	0.919
Immunodeficiency diseases	1 (2)	1 (3)	2 (3)	0.877
IBMFS	1 (2)	1 (3)	2 (3)	0.877
Solid tumors	1 (2)	0	1 (1)	0.345
Donor; no. (%)				
MRD	23 (56)	28 (75)	51 (65)	0.179
MMRD	2 (5)	0	2 (3)	
MUD	12 (29)	8 (22)	20 (26)	
MMUD	4 (10)	1 (3)	5 (6)	
CMV serostatus; no. (%)	41	29[b]	70	
D+ R-	4 (10)	2 (7)	6 (9)	0.444
D+ R+	25 (61)	13 (45)	38 (54)	
D- R+	9 (22)	11 (38)	20 (28)	
D- R-	3 (7)	3 (10)	6 (9)	

(*Continued*)

Table 1 (Continued).

Variable	POS	FLU	Total	*P* value
Stem cell source; no. (%)				
Bone marrow	19 (46)	30 (81)	49 (63)	0.002
Peripheral blood	22 (54)	7 (19)	29 (37)	
Conditioning regimen; no. (%)				
MAC	36 (88)	34 (92)	70 (90)	0.714
RIC	5 (12)	3 (8)	8 (10)	
GvHD prophylaxis; no. (%)				
MTX+CsA	37 (90)	35 (95)	72 (92)	0.677
MTX+MMF+Tacro	4 (10)	2 (5)	6 (8)	
Duration of neutropenia ANC < 500/cumm; median (range) in days	16 (6–35)	14 (3–33)	16 (3–35)	0.323
Day of engraftment; median (range)	Day +19 (11–44)	Day +24 (14–35)	Day +21 (11–44)	0.002
Hospitalization; median number of days (range)	79 (33–232)	88 (45–258)	80 (33–258)	0.126
Acute GvHD; no. (%)	11 (27)	10 (27)	21 (27)	0.984
Grades I–II	7 (64)	9 (90)	16 (76)	0.311
Grades III–IV	4 (36)	1 (10)	5 (24)	
Chronic GvHD; no. (%)[c]	10 (26)	4 (11)	14 (19)	0.142
Limited	1 (10)	2 (50)	3 (21)	0.176
Extensive	9 (90)	2 (50)	11 (79)	
Outcome; no. (%)				
Alive	31 (76)	30 (81)	61 (78)	0.594
Death	10 (24)	7 (19)	17 (22)	
Follow-up time; median (range) in years	1.9 (0.6–7.7)	9.9 (1–20)	3.3 (0.6–20)	< 0.001

Notes: [a]Two-tailed *p* value compared no. with hematological malignancy vs non-hematological malignancy, [b]CMV status in FLU group available for 29 of 37 patients, [c]Posaconazole n = 39; fluconazole = 36; total = 75.

number of days to engraftment (*p* = 0.002). The durations of neutropenia in the 2 groups were comparable, with 16 days (range, 6 to 35) for the POS group and 14 days (range, 3 to 33) for the FLU group (*p* = 0.323). Graft failure was evident for 2 severe aplastic anemia patients (one from each group).

Toxicity, Complications, and Outcomes

There were no significant differences in the acute toxicity of the POS and FLU groups except for cytomegalovirus-infection reactivation (*p* = 0.038; Table 2). The most common complications were febrile neutropenia, mucositis, and elevated liver enzymes.

The CsA trough level was available for 58 of the total of 72 patients (81%) who received methotrexate plus CsA as a GvHD prophylaxis. The median CsA level was 125.5 ug/L (range, 31 to 533) for the POS group, and 119 ug/L (range, 37 to 333) for the FLU group. Nine of the 33 POS patients (27%) and 3 of the 25 FLU patients (12%)

achieved the therapeutic CsA level; however, most patients in both groups had a CsA level of < 200 ug/L. Three patients (9%) in the POS group had a maximum CsA level exceeding 400 ug/L, but without evidence of hypertension or renal insufficiency; all 3 reached the therapeutic range after a dose reduction.

The POS trough level was checked in 37 of 41 patients (90%) in the POS group. Their median POS level was 0.5 μg/mL (range, 0.1 to 1.02). Seven of the patients (19%) reached the therapeutic level from the starting dose. After a dose adjustment, a further 7 patients (23%) reached the target level.

The 2-year IFD-free overall survival rates of the POS and FLU groups were comparable at 87.8% and 88%, respectively (*p* = 0.507; Figure 1A). However, subgroup analyses showed significant differences in the overall survival rates of 4 groups of patients: (1) POS with a hematological malignancy, 73.9%; (2) POS with a non-hematological malignancy (NHMD), 78.6%; (3) FLU with

Table 2 Patient-Reported, and Treatment-Emergent, Complications During the Pre-Engraftment Phase

Variable — no. (%)	POS (N = 41)	FLU (N = 37)	Total (N = 78)	P value
Oral mucositis	29 (71)	24 (65)	53 (68)	0.632
Nausea/vomiting	3 (7)	2 (5)	5 (6)	0.731
Non-infectious diarrhea	12 (29)	8 (22)	20 (26)	0.604
Exanthema	5 (12)	3 (8)	8 (10)	0.715
Hypertension	16 (39)	9 (24)	25 (32)	0.225
Engraftment syndrome	5 (12)	2 (5)	7 (9)	0.436
Sinusoidal obstruction syndrome	7 (17)	7 (19)	14 (18)	0.832
GI bleeding	3 (7)	2 (5)	5 (6)	0.731
AST/ALT increased	21 (51)	20 (54)	41 (53)	0.824
Hypokalemia	3 (7)	3 (8)	6 (8)	0.896
Acute kidney injury	13 (32)	8 (22)	21 (27)	0.444
Febrile neutropenia	30 (73)	22 (59)	52 (67)	0.235
Septicemia	16 (39)	13 (35)	29 (37)	0.816
Gram-negative septicemia	14 (34)	9 (24)	23 (29)	0.457
Escherichia coli	5 (36)	2 (22)	7 (31)	0.657
Escherichia coli ESBL	4 (29)	5 (56)	9 (39)	0.383
Klebsiella pneumoniae	1 (7)	1 (11)	2 (9)	0.742
Pseudomonas aeruginosa	1 (7)	0	1 (4)	0.412
Stenotrophomonas maltophilia	0	1 (11)	1 (4)	0.391
Acinetobacter baumannii	3 (21)	0	3 (13)	0.253
Gram-positive septicemia	6 (15)	7 (19)	13 (17)	0.763
MRCNS	1 (17)	2 (29)	3 (23)	0.611
MRSA	0	1 (14)	1 (8)	0.335
MSSA	1 (17)	0	1 (8)	0.462
Corynebacterium	2 (32)	0	2 (15)	0.192
Streptococcus pneumoniae	1 (17)	2 (29)	3 (23)	0.611
Streptococcus viridans	0	1 (14)	1 (8)	0.335
Bacillus cereus	1 (17)	1 (14)	2 (15)	0.906
Mucocutaneous candidiasis	5 (12)	7 (19)	12 (15)	0.534
CMV infection reactivation	21 (51)	10 (27)	31 (39)	0.038
Herpes simplex reactivation	4 (10)	4 (11)	8 (10)	0.878
Shingles	3 (7)	3 (8)	6 (8)	0.896
Pneumonia	11 (27)	10 (27)	21 (27)	0.984
Urinary tract infection	7 (17)	6 (16)	13 (17)	0.919
Infectious diarrhea	7 (17)	3 (8)	10 (13)	0.317
Clostridium difficile	1 (14)	1 (33)	2 (20)	0.490
Rotavirus	3 (43)	NA	3 (30)	NA
Salmonella	2 (29)	2 (67)	4 (40)	0.500
Aeromonas hydrophila	1 (14)	0	1 (10)	0.490

Abbreviation: NA, not available.

a hematological malignancy, 33.3%; and (4) FLU with NHMD, 96.8% ($p = 0.021$; Figure 1B).

Overall, 17 of the 78 patients (21.8%) died after their HSCTs. Relapse was the leading cause of death in patients with a malignant disease, while bacterial septicemia was the major cause of death in patients with NHMD.

IFDs in Pre-Engraftment Period

The incidences and characteristics of breakthrough IFDs during the pre-engraftment period are detailed in Tables 3 and 4 (overall incidence: 4%; 5% with POS vs 3% with FLU; $p = 0.620$). No proven IFD was detected in any patient. Two POS patients (5%) and 1 FLU patient (3%) had probable IFDs ($p = 0.620$). All 3 patients with IFDs had prolonged
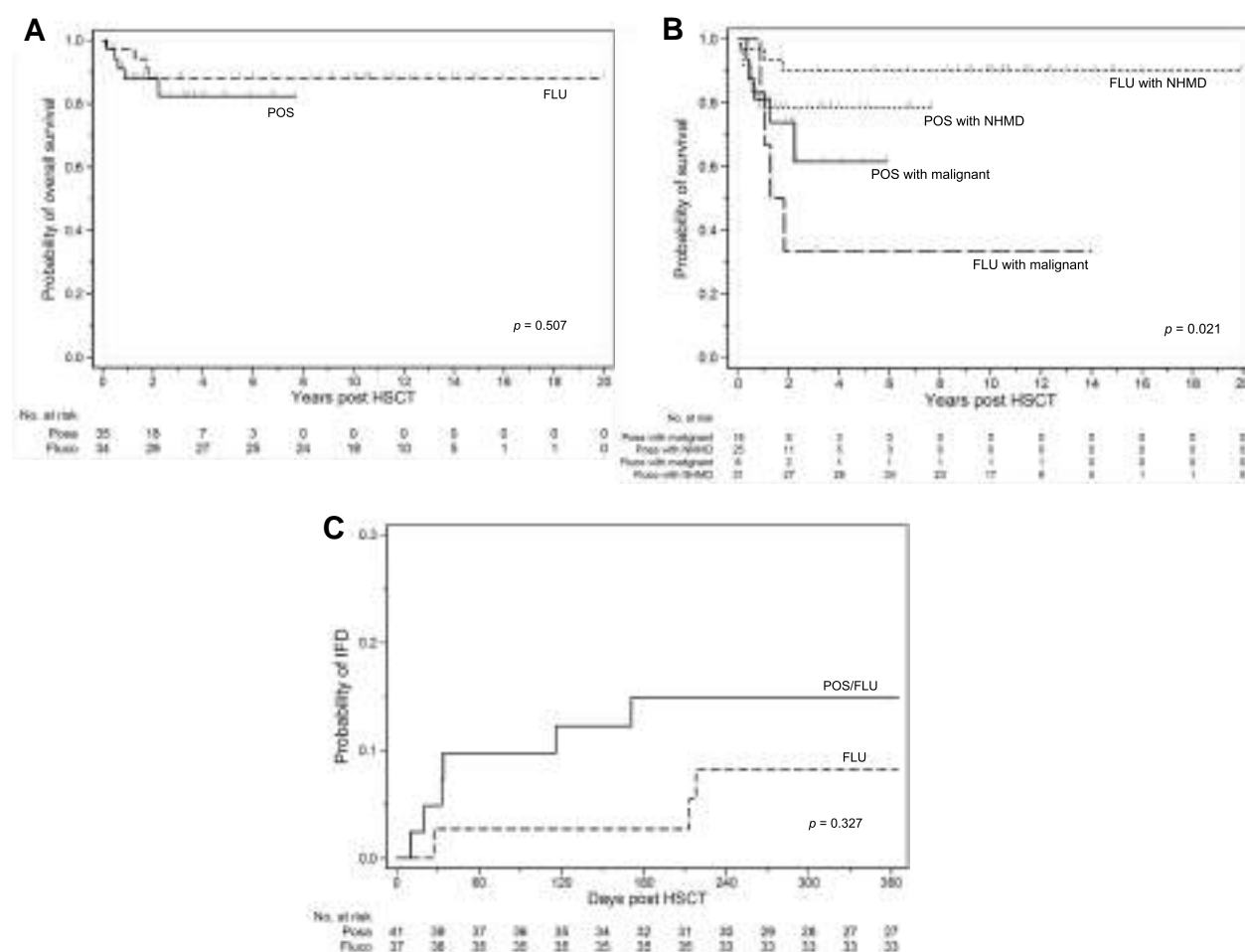
Figure 1 Outcomes of patients receiving allogeneic HSCT. (**A**) IFD-free overall survival. (**B**) Overall survival for patients with hematological malignancy versus non-hematological malignancy (NHMD). (**C**) Incidence of IFDs during the first year post-HSCT.

neutropenia. One patient from each group experienced primary graft failure, while 1 patient with POS had neutrophil engraftment on Day +14. The IFD was diagnosed on Day +10 and Day +20 for the 2 patients with POS, and on Day +27 for the 1 patient with FLU. The 2 POS patients with IFDs had POS levels of 0.6 and 0.5 µg/mL.

All 3 probable-IFD patients had pulmonary symptoms, with evidence of consolidation by CT scan plus a positive serum galactomannan assay. Patient 1 with POS had a partial response after 4 weeks of broad-spectrum antifungal therapy. However, he suffered a primary graft failure and died 2 months post-HSCT because of *Acinetobacter baumannii* septicemia. Patient 2 with POS developed grade 4 aGvHD on Day +32 post-HSCT. She required a combination of corticosteroid plus CsA to treat the aGvHD. A CT scan revealed the disappearance of lung infiltrates after 6 weeks of voriconazole administration. Unfortunately, 4 months post-HSCT, she developed

extensive cGvHD (skin, liver, eyes, and gastrointestinal tract). These symptoms were refractory to extracorporeal photopheresis, and she died 15 months post-HSCT. Patient 3 with FLU experienced a primary graft failure and had a poor response to amphotericin B, with progressive infiltration on both lungs evident in a follow-up CT scan. He died from massive hemoptysis 5 months post-HSCT. The mortality rates related to IFDs of the POS group, the FLU group, and the total were 0%, 100%, and 33%, respectively ($p = 0.333$).

Adverse Events Related to the Primary Antifungal Therapies

No patient had to discontinued POS or FLU due to drug intolerance. There were also no significant differences in the common adverse events that were related to POS and FLU—namely, nausea (7% vs 5%), diarrhea (29% vs

684

Journal of Blood Medicine 2021:12

Table 3 Comparison of Efficacies of Posaconazole and Fluconazole as Primary Antifungal Prophylaxes During Pre-Engraftment Period

	POS (N = 41)	FLU (N = 37)	Total (N = 78)	P value
Invasive fungal infection				
Total — no. (%)	2 (5)	I (3)	3 (4)	0.620
Proven	0	0	0	
Probable	2	I	3	0.620
Evidence of fungus				
Culture for fungus	0	0	0	0.359
CT/MRI	2	I	3	
Serum galactomannan	2	I	3	
Broad spectrum antifungal agent				
Amphotericin B	0	I	I	0.613
Voriconazole	I	0	I	
Micafungin	I	0	I	
Outcome				
Alive	2	0	2 (3)	0.494
Deceased	0	I	I (I)	0.474

22%), exanthema (12% vs 8%), and elevated transaminase levels (51% vs 54%), respectively (Table 2).

We categorized these 74 cases into 3 subgroups: (1) a POS ≥ 0.7 ng/mL group (n = 14); (2) a POS < 0.7 ng/mL group (n = 23); and (3) an FLU group (n = 37). All patients had significantly elevated total bilirubin, direct bilirubin, and liver enzyme levels during the 30 days post-HSCT ($p < 0.05$; Supplementary Figure 1 – 1A, 1B, 1C and 1D). However, there were no statistical differences in the rates of rise in the liver profiles of the FLU and the 2 POS groups.

Regarding the available CsA levels, the 58 patients were classified into 3 subgroups: (1) a POS ≥ 0.7 ng/mL group (n = 14); (2) a POS < 0.7 ng/mL group (n = 19); and (3) an FLU group (n = 25). The median CsA levels of the 3 groups were comparable (Figure 2; $p = 0.270$). No patients in the POS ≥ 0.7 group or the FLU group experienced a CsA overdose. On the other hand, 3 patients (16%) in the POSA < 0.7 group had CsA levels > 400 ug/L; following a 25% dose reduction, their levels fell to the therapeutic range. Renal insufficiency occurred in 4 patients (29%) from the POS ≥ 0.7 group and 2 patients (8%) from the FLU group; all 6 patients had CsA levels within the therapeutic range. They responded when the CsA was discontinued and replaced with MMF.

Post-Engraftment Period

IFDs occurred in 4 of the 39 patients who were given POS at the beginning of their HSCTs, and in 2 of the 36 patients who were administered FLU at the time of their HSCTs (overall incidence rate, 8%; Figure 1C). Three patients had proven IFDs, whereas the other 3 had probable IFDs (Table 4). Two patients developed IFDs during the early post-engraftment period, while the other 4 cases were
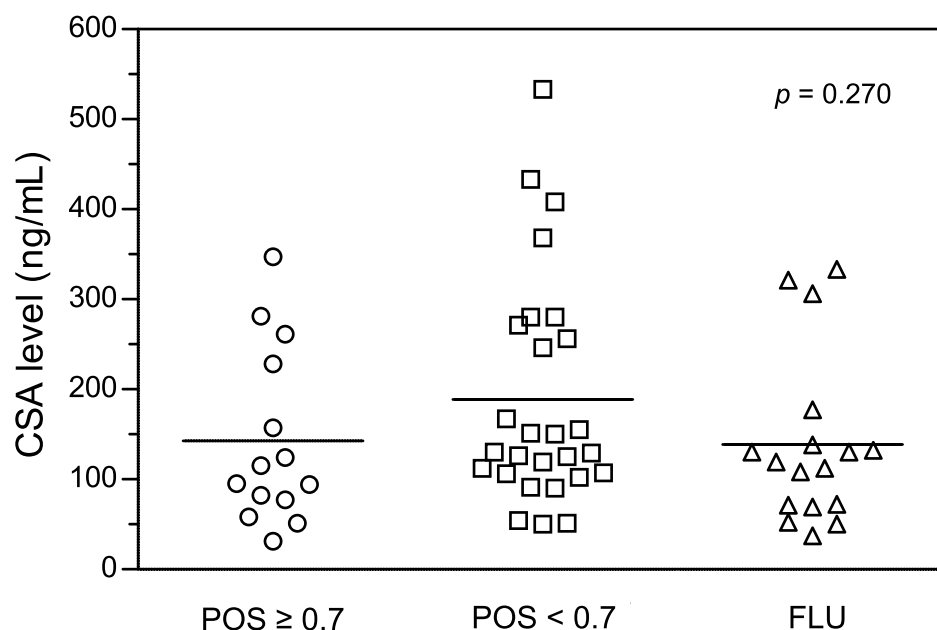
Figure 2 Cyclosporine levels of patients receiving azole antifungal drugs.

Table 4 Overall Breakthrough Invasive Fungal Disease in Patients Receiving HSCTs

Variable	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
Sex/age (yr)	M/7.7	M/5.9	M/5.8	F/7.7	M/16.6	M/14.8	F/11.7	M/5.7	M/10.8
Primary disease	SAA	Relapsed ALL	SAA	HbE/β^0	Relapsed ALL	Relapsed BAL	HbE/β^0	Relapsed AML	Relapsed ALL
Donor/grafts	MMSD/ BM	MUD/PB	MUD/PB	MSD/BM	MSD/PB	MSD/PB	MUD/PB	MSD/BM	MSD/BM
Engraftment	Graft failure	Day +14	Graft failure	Day +24	Day +14	Day +16	Day +32	Day +16	Day +33
Time-point post-HSCT	Day +10	Day +20	Day +27	Day +33	Day +34	Day +116	Day +171	Day +213	Day +218
CMV reactivation (grade)	Grade 2	Grade 3	None	None	Grade 2	Grade 3	Grade 2	None	None
Host factors									
Neutropenia > 10 days	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Hematological malignancy	No	Yes	No	No	Yes	Yes	No	Yes	Yes
Use of corticosteroids	No	Yes	No	No	No	Yes	Yes	No	Yes
Immunosuppressants	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Acute/chronic GvHD	None	Grade IV/ cGvHD	None	None	None	Grade III	Grade IV/ cGvHD	None	Grade II
Pre-engraftment prophylaxis	POS	POS	FLU	POS	POS	POS	POS	FLU	FLU
Post-engraftment prophylaxis (day of switching post-HSCT)	–	–	–	FLU (Day +26)	FLU (Day +17)	FLU (Day +22)	FLU (Day +34)	FLU (Day +17)	FLU (Day +34)
POS level (μg/mL)	0.6	0.5	–	0.52	0.58	0.35	0.5	–	–
IFD category	Probable	Probable	Probable	Probable	Proven	Probable	Proven	Probable	Proven
Pathogen	-	-	-	-	Mucor	-	*Aspergillus*	-	Candida albicans
Site	Lung (imaging, GM)	Lung (imaging, GM)	Lung (imaging, GM)	Lung (imaging, GM)	Small bowel (histology); lung (imaging)	Lung (imaging, GM)	Skin, lung (pus culture, imaging)	Lung (imaging, GM)	Blood (culture)
IFD outcome	PR	CR	PD	CR	PD	PD	PD	PD	PD
Status	Death unrelated to IFD	Death unrelated to IFD	Death related to IFD	Alive	Death related to IFD	Death related to IFD and relapsed	Death related to IFD	Death related to IFD and relapsed	Death related to IFD and relapsed

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BAL, biphenotypic acute leukemia; BM, bone marrow; CMV, cytomegalovirus; CR, complete response; FLU, fluconazole; GM, serum galactomannan; GvHD, graft-versus-host disease; HbE/β^0, hemoglobin E-beta-thalassemia disease; HSCT, hematopoietic stem cell transplant; IFD, invasive fungal disease; MMSD, mismatched sibling donor; MSD, matched sibling donor; MUD, matched unrelated donor; PB, peripheral blood; PD, progressive disease; POS, posaconazole; PR, partial response; SAA, severe aplastic anemia.

detected after Day +100 post-HSCT. The mortality rate related to IFDs was 83%.

Patient 5 experienced fever with acute respiratory distress syndrome, abdominal pain, and hematochezia 6 weeks after the HSCT. Laparotomy was performed, and a small bowel resection showed evidence of a mucormycosis infection from histology. A chest CT scan showed typical fungus lung infiltration, and a serum galactomannan assay was positive. However, there was no mycologic evidence to define the pathogen. He

demonstrated no response to a combination of liposomal amphotericin B and voriconazole, and he died 10 weeks post-HSCT. Patient 7 developed cGvHD (skin and liver) 5 months post-HSCT and was treated with CsA plus prednisolone. She was readmitted with febrile neutropenia, left-knee swelling, and multiple abscesses on both legs. A pus culture from her skin and knee-joint fluid revealed *Aspergillus* spp. Despite receiving amphotericin B and voriconazole, she developed massive hemoptysis and died 2 weeks after her re-admission. Patient 9 had a relapse of acute lymphoblastic leukemia at 8 months post-HSCT and underwent salvage therapy. He suffered from fever and was given amphotericin B. Nevertheless, his blood cultures were persistently positive for *Candida albicans*, and he died 1 month after his re-admission.

As to probable IPA, only Patient 4 demonstrated a complete response to voriconazole. However, the 2 other cases had a disease relapse at 3 and 4 months post-HSCT, and died within a month of commencing treatment with voriconazole.

Discussion

Currently, there is a clinical practice guideline for the use of echinocandin or a mold-active azole as an antifungal prophylaxis for pediatric patients receiving HSCTs.[15] Some investigators have reported that mold-active azoles such as itraconazole, voriconazole, and POS have better efficacies in the pediatric population than FLU.[19,20] From our data, the incidences of IFDs of the POS and FLU groups were comparable, at 5% and 3%, respectively ($p = 0.620$). Doring also reported that POS, FLU, and itraconazole have comparable degrees of effectiveness in preventing IFD in patients with neutropenia.[19] However, our study showed a significantly higher proportion of patients with a hematological malignancy in the POS group than the FLU group (37% vs 16%; $p = 0.043$). In addition, the rates of grades III and IV aGvHD were higher for the POS group than the FLU group (36% vs 10%; $p = 0.311$). Regarding the host factors which increased the risk for IFDs, POS seemed to be more effective than FLU as an antifungal agent for the recipients during the pre-engraftment period. Moreover, 8% of patients receiving FLU post-engraftment had IFDs, and the mortality rate related to IFDs was 83%. Whether using either POS or other mold-active agents during the post-engraftment period could further decrease the IFD incidence is an interesting research question.

The US Food and Drug Administration has approved the use of the oral suspension of POS as an antifungal agent for children older than 13 years.[21] Many studies have since reported that the off-label use of POS for children younger than 13 demonstrated promising efficacy with a safety profile comparable to that for the adult population.[13,14,22] Doring reported the adverse events associated with using POS in pediatric patients with HSCTs.[23,24] Almost 7% of cases needed to discontinue the drug due to nausea and vomiting; furthermore, most patients developed abnormal liver function and required a CsA dose reduction of 22%. In the current study, while 7% of our POS patients experienced nausea and vomiting, none had to discontinue POS. We also found that about 50% of our POS patients had increased levels of liver enzymes. Only 9% of our patients had a CsA overdose, which required a 25% dose reduction. The other 9% of our patients with CsA levels < 400 ug/L had renal insufficiency and needed to cease the use of CsA.

Although a trough level of 0.7 ng/mL is required for POS, most studies reported lower levels (0.383–0.438 ng/mL), with only 10–15% of their patients achieving the therapeutic level.[24–27] The median POS level in our study was 0.5 ng/mL; 19% of our patients reached a POS level ≥ 0.7 ng/mL. Several investigators have attempted to solve the problem of failure to achieve the therapeutic level. Boonsathorn reported that the suspension form of POS had an inferior bioavailability—as low as 30% of the target level—to the tablet form.[13] Tragiannidis also reported that 90% of patients attained the therapeutic target with the use of delayed-release POS tablets, and had no serious adverse events.[28] Although those results are impressive, a further large study in the pediatric population is needed to clarify the pharmacokinetics of delayed-release POS tablets and to determine their effectiveness as an IFD prophylaxis.

The chief limitation of our study is that it was a retrospective study. It was conducted over a 20-year period, during which different levels of supportive care were provided and the protocols for surveillance of adverse events varied. Moreover, the index of suspicion for IFD might have been lower during the first of the 2 decades, resulting in IFDs being under-detected. Furthermore, the higher percentage of hematological malignancy with POS might have increased the risk of IFDs, relative to FLU, during the pre-engraftment period. Lastly, the switching from POS to FLU after engraftment made it difficult to clarify the extent to

which each drug contributed to the efficacy of IFD prophylaxis during the interchange period.

Conclusions

In summary, our study shows that POS and FLU have comparable levels of effectiveness, safety, and tolerability as IFD prophylaxes for children with allogeneic HSCTs during the pre-engraftment phase. Establishing the optimum dose and duration of POS in this setting requires further investigation using a larger pediatric population.

Acknowledgments

The authors acknowledge the expert care provided to patients by the staff of the Department of Pediatrics at Siriraj Hospital.

Disclosure

The abstract of this paper was presented at the 45th Annual Meeting of the European Society for Blood and Marrow Transplantation. Entitled "Antifungal prophylaxis with posaconazole versus fluconazole in children with neutropenia following hematopoietic stem cell transplantation", it was given as a poster presentation with interim findings. The abstract was published in the "Physicians–Poster Session" of the 24–27 March 2019 publication of the journal, "Bone Marrow Transplantation": https://doi.org/10.1038/s41409-019-0559-4. All authors reported no conflicts of interest for this work.

References

1. Kriengkauykiat J, Ito JI, Dadwal SS. Epidemiology and treatment approaches in management of invasive fungal infections. *Clin Epidemiol*. 2011;3:175–191.
2. Camps IR. Risk factors for invasive fungal infections in haematopoietic stem cell transplantation. *Int J Antimicrob Agents*. 2008;32(Suppl 2):S119–23.
3. Safdar A, Rodriguez GH, Mihu CN, et al. Infections in non-myeloablative hematopoietic stem cell transplantation patients with lymphoid malignancies: spectrum of infections, predictors of outcome and proposed guidelines for fungal infection prevention. *Bone Marrow Transplant*. 2010;45(2):339–347.
4. Kontoyiannis DP, Marr KA, Park BJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin Infect Dis*. 2010;50(8):1091–1100.
5. Neofytos D, Horn D, Anaissie E, et al. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. *Clin Infect Dis*. 2009;48(3):265–273.
6. Phikulsod P, Suwannawiboon B, Chayakulkeeree M. Invasive fungal infection among febrile patients with chemotherapy-induced neutropenia in Thailand. *Southeast Asian J Trop Med Public Health*. 2017;48 (1):159–169.

7. Goodman JL, Winston DJ, Greenfield RA, et al. A controlled trial of fluconazole to prevent fungal infections in patients undergoing bone marrow transplantation. *N Engl J Med*. 1992;326(13):845–851.
8. Ullmann AJ, Lipton JH, Vesole DH, et al. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. *N Engl J Med*. 2007;356(4):335–347.
9. Cornely OA, Maertens J, Winston DJ, et al. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. *N Engl J Med*. 2007;356(4):348–359.
10. Dekkers BGJ, Bakker M, van der Elst KCM, et al. Therapeutic drug monitoring of posaconazole: an update. *Curr Fungal Infect Rep*. 2016;10:51–61.
11. Petitcollin A, Boglione-Kerrien C, Tron C, et al. Population pharmacokinetics of posaconazole tablets and monte carlo simulations to determine whether all patients should receive the same dose. *Antimicrob Agents Chemother*. 2017;61(11):e01166–e01217.
12. Chen L, Krekels EHJ, Verweij PE, et al. Pharmacokinetics and pharmacodynamics of posaconazole. *Drugs*. 2020;80(7):671–695.
13. Boonsathorn S, Cheng I, Kloprogge F, et al. Clinical pharmacokinetics and dose recommendations for posaconazole in infants and children. *Clin Pharmacokinet*. 2019;58(1):53–61.
14. Vicenzi EB, Calore E, Decembrino N, et al. Posaconazole oral dose and plasma levels in pediatric hematology-oncology patients. *Eur J Haematol*. 2018;100(3):315–322.
15. Lehrnbecher T, Fisher BT, Phillips B, et al. Clinical practice guideline for systemic antifungal prophylaxis in pediatric patients with cancer and hematopoietic stem-cell transplantation recipients. *J Clin Oncol*. 2020;38(27):3205–3216.
16. U.S. Deprment of Health and Human Services NioH, National Cancer Institute. Common Terminology Criteria for Adverse Events (CTCAE). Version 5.0; 2017 [cited January 4, 2021]. Available from: https://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_8.5x11.pdf. Accessed July 20, 2021.
17. Donnelly JP, Chen SC, Kauffman CA, et al. Revision and update of the consensus definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. *Clin Infect Dis*. 2020;71(6):1367–1376.
18. Segal BH, Herbrecht R, Stevens DA, et al. Defining responses to therapy and study outcomes in clinical trials of invasive fungal diseases: mycoses Study Group and European organization for research and treatment of cancer consensus criteria. *Clin Infect Dis*. 2008;47(5):674–683.
19. Döring M, Eikemeier M, Cabanillas SKM, et al. Antifungal prophylaxis with posaconazole vs. fluconazole or itraconazole in pediatric patients with neutropenia. *Eur J Clin Microbiol Infect Dis*. 2015;34 (6):1189–1200.
20. Science M, Robinson PD, MacDonald T, et al. Guideline for primary antifungal prophylaxis for pediatric patients with cancer or hematopoietic stem cell transplant recipients. *Pediatr Blood Cancer*. 2014;61 (3):393–400.
21. FDA. U.S. Noxafl instruction 2015. U.S.: FDA; [cited January 4, 2021]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/205053s1lbl.pdf. Accessed July 20, 2021.
22. Döring M, Cabanillas SKM, Klinker H, et al. Posaconazole plasma concentrations in pediatric patients receiving antifungal prophylaxis during neutropenia. *Med Mycol*. 2017;55(4):375–384.
23. Döring M, Blume O, Haufe S, et al. Comparison of itraconazole, voriconazole, and posaconazole as oral antifungal prophylaxis in pediatric patients following allogeneic hematopoietic stem cell transplantation. *Eur J Clin Microbiol Infect Dis*. 2014;33 (4):629–638.
24. Döring M, Müller C, Johann PD, et al. Analysis of posaconazole as oral antifungal prophylaxis in pediatric patients under 12 years of age following allogeneic stem cell transplantation. *BMC Infect Dis*. 2012;12:263.

25. Vanstraelen K, Colita A, Bica AM, et al. Pharmacokinetics of posa-conazole oral suspension in children dosed according to body surface area. *Pediatr Infect Dis J.* 2016;35(2):183–188.
26. McMahon J, Théorêt Y, Autmizguine J, et al. Posaconazole plasma monitoring in immunocompromised children. *J Pediatric Infect Dis Soc.* 2017;6(4):389–392.
27. Heinz WJ, Cabanillas Stanchi KM, Klinker H, et al. Posaconazole plasma concentration in pediatric patients receiving antifungal pro-phylaxis after allogeneic hematopoietic stem cell transplantation. *Med Mycol.* 2016;54(2):128–137.

28. Tragiannidis A, Herbrüggen H, Ahlmann M, et al. Plasma exposures following posaconazole delayed-release tablets in immunocompro-mised children and adolescents. *J Antimicrob Chemother.* 2019;74 (12):3573–3578.

Incidence and Risk Factors of Thrombocytopenia in Neonates Admitted with Surgical Disorders to Neonatal Intensive Care Unit of Tikur Anbessa Specialized Hospital: A One-Year Observational Prospective Cohort Study from a Low-Income Country

Hana Abebe Gebreselassie, Hanna Getachew, Amezene Tadesse, Tihitena Negussie Mammo, Woubedel Kiflu, Fisseha Temesgen & Belachew Dejene

Dovepress

ORIGINAL RESEARCH

Incidence and Risk Factors of Thrombocytopenia in Neonates Admitted with Surgical Disorders to Neonatal Intensive Care Unit of Tikur Anbessa Specialized Hospital: A One-Year Observational Prospective Cohort Study from a Low-Income Country

Hana Abebe Gebreselassie[1]
Hanna Getachew[2]
Amezene Tadesse[2]
Tihitena Negussie Mammo[2]
Woubedel Kiflu[2]
Fisseha Temesgen[2]
Belachew Dejene[2]

[1]St. Paul's Hospital Millennium Medical College, Department of Surgery, Addis Ababa, Ethiopia; [2]Addis Ababa University School of Medicine, Department of Surgery, Paediatrics Surgery Unit, Addis Ababa, Ethiopia

Background: Thrombocytopenia is one of the most common hematologic disorders affecting neonates admitted to the neonatal intensive care unit. The aim of this study was to determine the incidence and associated risk factors of neonatal thrombocytopenia in neonates admitted with surgical disorders.

Methods: An observational prospective cohort study was conducted and all neonates admitted to neonatal intensive care unit of Tikur Anbessa Specialized Hospital with surgical disorders were included. Data were collected using a checklist and analyzed by SPSS version 23. Chi square test and independent sample *t*- test were used to assess the association among different variables.

Results: A total of 210 neonates were included in the study, out of which 56.2% were males. The incidence of thrombocytopenia was 55.8%. Among neonates with thrombocytopenia, 90.9% had late onset thrombocytopenia and half were in the severe range (<50,000/μL). The presence of sepsis (P = 0.000) and atresia (P = 0.000) were found to be significantly associated with the development of thrombocytopenia. The mean non feeding hours were found to be significantly longer for patients with thrombocytopenia (t [199], 5.81, P = 0.000).

Conclusion: The incidence of thrombocytopenia is high in our institution. Prevention methods towards neonatal sepsis should be given due emphasis.

Keywords: thrombocytopenia, incidence, risk factors

Background

Platelets first appear in the human fetus at five weeks post conception and progressively increase in number attaining the normal adult range in the second trimester around 22 weeks of gestation.[1,2] Hence, in the neonate thrombocytopenia is defined as a platelet count of less than 150,000/μL similar to adults, which also corresponds to values at or below the fifth percentile.[3] The severity of thrombocytopenia can also be graded as follows: mild (a platelet count of 100,000–150,000/μL), moderate (a platelet count of 50,000–100,000 /

Correspondence: Hana Abebe Gebreselassie
St. Paul's Hospital Millennium Medical Collage, Department of Surgery, 1271 Swaziland Street, Addis Ababa, Ethiopia
Tel +251 913 766 868
Email hanaabebe23@gmail.com

µL), severe (a platelet count of 30,000–50,000/µL) and very severe (a platelet count of < 30,000/µL).[4]

Thrombocytopenia in healthy term neonates is uncommon, with a reported incidence of 1–5% and warrants evaluation only in case of a platelet count less than 50,000 per µL, bleeding diathesis, persistent thrombocytopenia, or subsequent worsening of the degree of thrombocytopenia.[5–7]

Unlike healthy neonates, thrombocytopenia in the setup of neonatal intensive care unit is quite common being one of the most common hematologic disorders with a reported incidence of 18–35%.[8–10]

Several maternal, perinatal and neonatal factors risk factors are identified for neonatal thrombocytopenia. Maternal factors include age, number of pregnancies, maternal auto immune disease, malignancies, maternal medication including nonsteroidal anti-inflammatory drugs and heparin, and pregnancy-induced hypertensive disorders. Perinatal factors include multiple birth, prematurity, being small for gestational age, and intrauterine growth restriction. Neonatal factors include gender, sepsis, necrotizing enterocolitis (NEC), toxoplasmosis, rubella, cytomegalo virus, and herpes viruses (TORCH) infections, asphyxia, neonatal immunologic disorders, chromosomal disorders and metabolic diseases.[11]

Based on the timing of development after birth, thrombocytopenia can be classified as early onset (<72hrs of life) and late onset (>72hrs) thrombocytopenia. This classification can possibly indicate the underlying cause for thrombocytopenia as early onset thrombocytopenia is usually considered to be due to antenatal and maternal factors while late onset thrombocytopenia is most likely associated with postnatally acquired infection. Moreover, this classification also helps to predict the course and the severity of thrombocytopenia as early onset thrombocytopenia tends to be mild or moderate in range and resolves spontaneously whereas late onset thrombocytopenia usually tends to be more severe and prolonged.[3,12]

The clinical consequences of thrombocytopenia can be followed both clinically and with laboratory monitoring by assessing haemostatic function. The main clinical concern for neonates with severe thrombocytopenia is the occurrence of major bleeding especially intracranial hemorrhage into the intraventricular and periventricular space (IVH-PVH). The risk of major IVH-PVH is particularly high in premature neonates with a reported incidence of more than 25%.[10,13,14]

This study is aimed to generate an objective data about the incidence of thrombocytopenia in neonates admitted to neonatal intensive care unit of Tikur Anbessa Specialized Hospital with surgical disorders. Thrombocytopenia is a common finding in these neonates and is one of the major causes delaying surgical intervention in our institution from our observation. This study has also evaluated the associated risk factors for the development of thrombocytopenia in these neonates so that some preventive and therapeutic measure can be taken before the development of complications. There has been no study done on the incidence and associated risk factors of neonatal thrombocytopenia in the Ethiopian setup so far. Thus, this study will be helpful to understand this common condition better in order to improve the outcome of neonates with this disorder.

Materials and Methods

Study area: Addis Ababa University, School of Health Sciences, Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia. This study was conducted in Tikur Anbessa Specialized Hospital which is the largest referral hospital and teaching center in Ethiopia. The paediatric Surgery Unit in this hospital is the major center in the country providing surgical care for neonates and has been the sole center for the country providing neonatal surgical care until recently.

A prospective observational cohort study was conducted to assess the incidence and risk factors associated with neonatal thrombocytopenia. All neonates with surgical disorders who were admitted to NICU of TASH for one-year period (January 1, 2019–December 31, 2019) were included in the study. All neonates were followed daily from their day of admission to discharge or death using various clinical and laboratory parameters.

Data were collected by structured interview and chart review using a checklist developed for the study by the principal investigators. Written informed consent was taken from parents of enrolled neonates and each neonate's clinical data was kept in confidential manner using a coding system. Ethical clearance for this study was obtained from institutional review board of the Department of Surgery of Addis Ababa University. This study was conducted in accordance with the Declaration of Helsinki.

Data were analyzed using SPSS 23. The association of different variables with regard to the development of thrombocytopenia was tested for significance by chi

square analysis and independent samples *t*-test. Considering a confidence interval of 95%, a P-value of <0.05 was considered significant in all statistical comparisons.

Operational Definitions

Sepsis is defined as systemic inflammatory response to infectious cause and diagnosed based on the following parameters and also a positive acute phase reactant like the c reactive protein.[15]

Two or more of the following conditions:

- Temperature instability <35°C (101.3°F) or >38.5°C (101.3°F),
- Respiratory dysfunction: Tachypnea >2 SD above the mean for age, Hypoxemia (PaO2< 2 SD above the mean for age),
- Cardiac dysfunction: Tachycardia >2 SD above the mean for age, Delayed capillary refill >3 sec Hypotension >2 SD below the mean for age,
- Perfusion abnormalities: Oliguria (urine output <0.5 mL/kg/hour), Lactic acidosis (elevated plasma lactate and/or arterial pH), altered mental status.

Necrotizing enterocolitis (NEC) is an acquired inflammatory disease that affects the gut of newborns. For this study, The Bell's criteria was used to define NEC which is graded as follows.[16]

Stage 1, suspected NEC

- Temperature instability, apnea, bradycardia, lethargy, gastric retention, abdominal distention, emesis, heme-positive or grossly bloody stool, plain abdominal X-ray normal or intestinal dilation.

Stage 2, definite NEC

- Features of stage one plus absent bowel sounds, abdominal tenderness with or without abdominal cellulitis or right lower quadrant mass, ascites, mild metabolic acidosis and thrombocytopenia, plain abdominal X-ray Intestinal dilation, pneumatosis intestinalis.

Stage 3, complicated NEC

- Features of stage 2 plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic

acidosis, DIC, and neutropenia, ascites, signs of peritonitis like marked tenderness and abdominal distention, plain abdominal X-ray as stage 2 plus pneumoperitoneum.

Result

A total of 210 neonates who were admitted to the NICU of TASH with surgical diagnosis were included in the study. The neonates were followed from their admission to their discharge day via various clinical parameters and platelet count.

Maternal Characteristics

The mean maternal age was 27.17±4.9 years with a range of 16 to 38 years. Primiparous mothers accounted for 40% of the maternal population. A great majority (96.67%) of the mothers claimed that they had antenatal follow up among whom 9 (4.4%) were diagnosed to have gestational hypertension, 2 (0.96%) type 2 diabetes mellitus, 1 (0.49%) gestational diabetes mellitus and 1 (0.49%) retroviral infection. Among the mothers who were hypertensive, three were taking methyl dopa in the third trimester and one was on magnesium sulphate. The rest of the mothers claimed that they had not taken any medication during pregnancy.

Neonatal Characteristics

Males accounted for more than half of the study population (118; 56.2%). Term neonates constituted the majority (165; 78.6%), preterm neonates accounted for 39 (18.6%). As to the birth weight, 63 (29.9%) of the neonates were underweight, of which 40 (19%) had low birth weight, 20 (9.5%) had very low birth weight and 3 (1.4%) had extremely low birth weight (Figure 1).

Among the neonates, 11 (5.2%) were the result of multiple pregnancies with the majority being twins and one triplet pregnancy. Intrauterine growth restriction was observed in 31 (14.8%) of the neonates. APGAR score was not recorded in the referral paper for 174 (82.9%) of the neonates but the mothers claimed that the neonates had cried immediately after birth.

Perinatal asphyxia was observed in 5 (2.4%) of the neonates all being stage 2. Only one neonate was screened for TORCH infection and the rest were not. A diagnosis of chromosomal abnormality merely based on gross phenotypic characteristics was made in 13 neonates: 11 Down's syndrome, 1 Patau syndrome and 1 turner/Klippel foli syndrome. NEC was diagnosed in one preterm neonate. The most

FREQUENCY
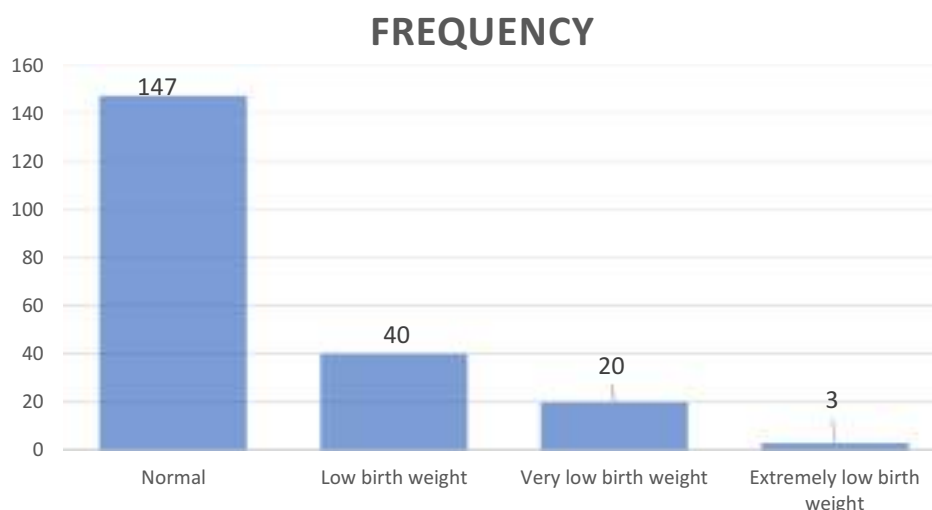
Figure 1 Birthweight of the study population.

common admission diagnosis was gastro intestinal atresia accounting for 95 (45.2%) of the cases followed by anorectal malformations 48 (22.9%) (Table 1). The diagnosis of neonatal sepsis was made in 116 (55.2%) of the study population out of which the great majority were of early onset 109 (51.9%) while late onset neonatal sepsis was found in 7 (3.3%) of them.

The mean duration of non-feeding (NPO) hours was found to be different in the group of neonates with thrombocytopenia (114.3±119.14 hours) and without

thrombocytopenia (33.88±55.07 hours). The duration of intravenous drugs excluding maintenance fluid and electrolytes were compared between neonates with and without thrombocytopenia and found to be longer for the group with thrombocytopenia. The mean duration of hospital stay was 10.2±9.4 days.

Thrombocytopenia

Based on the platelet count determined at admission, 35.2% of the neonates had thrombocytopenia and on their subsequent days of admission, 43 (20.6%) additional neonates were found to be thrombocytopenic making the overall incidence of thrombocytopenia 55.8% in the study period.

Among neonates with thrombocytopenia, a great share of them had a late onset thrombocytopenia (90.9%). As to the degree of thrombocytopenia, 60 (28.4%) had severe, 29 (13.7%) moderate, and 29 (13.7%) mild thrombocytopenia.

Associations
Risk Factors for Thrombocytopenia

A chi square analysis was made considering a confidence interval of 95%, to see the association of the various maternal, perinatal, and neonatal factors with development of thrombocytopenia and the factors which were found to have significant association with the development of thrombocytopenia were sepsis (0.000) and atresia (P = 0.000) (Table 2).

An independent samples *t*-test was done to compare the difference in the means of the non-feeding (NPO) hours

Table 1 Admission Diagnosis Versus Thrombocytopenia

Surgical Diagnosis	Thrombocytopenia		Total
	Yes	No	
Atresia (Esophageal, stomach, small bowel and colonic)	76	19	95
Anorectal malformations	17	31	48
Omphalocele	9	12	21
Hirschsprung disease	7	6	13
Bladder exstrophy	1	5	6
Gastroschisis	3	3	6
Congenital diaphragmatic hernia	2	1	3
Cloacal exstrophy	0	2	2
Sacrococcygeal teratoma	0	2	2
Others	4	11	15
Total	118	92	210

694

Journal of Blood Medicine 2021:12

Table 2 Percentage and Chi Square Test Result for Various Risk Factors of Thrombocytopenia

Variable	Thrombocytopenia			Total	Percentage (%)	p- value
		Yes	No			
Gestational age	Preterm Term Post term	20 93 5	19 72 1	39 165 6	18.6 78.6 2.8	0.473
Pregnancy induced hypertension	Yes No Unknown	5 111 2	4 84 4	9 195 6	4.3 92.8 2.9	0.450
Maternal drug	Yes No	4 114	6 86	10 200	5.2 94.8	0.859
Low birth weight	Yes No	37 81	26 66	63 147	30 70	0.627
IUGR	Yes No	18 100	13 79	31 179	14.8 85.2	0.820
Multiple pregnancy	Yes No	5 113	6 86	11 199	5.2 94.8	0.461
First minute APGAR score <5	Yes No	2 18	1 15	3 33	8.3 91.7	0.686
Fifth minute APGAR score <5	Yes No	0 20	1 15	1 33	5.56 94.4	0.257
Sex	Male Female	63 55	55 37	118 92	56.2 43.8	0.354
Perinatal asphyxia	Yes No	2 116	3 89	5 205	2.4 97.6	0.460
Chromosomal abnormalities	Yes No	8 110	5 87	13 197	6.2 93.8	0.620
Sepsis	Yes No	80 38	36 56	116 94	55.2 44.8	0.000
GI Atresia	Yes No	76 42	19 73	95 115	45.2 54.8	0.000

between the group of neonates with and without thrombocytopenia and the difference was found to be statistically significant (t [199], 5.81, P = 0.000).

Discussion

Thrombocytopenia is one of the most common hematologic findings among neonates admitted to NICU with up to 30% of them having a low platelet count detected at some point during their hospital stay. This study is aimed to determine the incidence of this disorder which is commonly observed in our institution. The neonates who

were included in this study were those admitted with surgical diagnosis. Thrombocytopenia is one of the factors commonly responsible for delaying surgery and also complicates the post-operative course of such patients. To the best of our knowledge, there is no published literature so far on neonates with surgical diagnosis as a study subject.

The mean maternal age in our study was 27.17±4.9 years, which was comparable to a study from Saudi Arabia in which it was 30±5.5 years.[17] The most common maternal comorbidity in our study was pregnancy induced hypertension which was found in 4.4% of the mothers.

This figure is significantly lower compared to other reports from India (13.5%), Iran (17.7%), Indonesia (24.3%) and Iran (57.1%).[18–21]

Male neonates constituted for more than half (56.2%) of our study population which is comparable to a study from Iran (51.3%).[19] However, this figure was found to be higher than reports from Indonesia (48.6%) and Nigeria (42.4%) but lower than a study from Saudi Arabia (70.4%).[17,20,22] A great majority of our patients were born at term (78.6%) which is comparable to a study from Turkey (73.6%) and Indonesia (85.7%) but significantly higher than that of Iran (34.1%) and Saudi Arabia (51.9).[17,20,21,23] Neonates with low birth weight constituted for one fifth of our study population which compares well to a study from Indonesia (20.7%) but higher than a report from Nigeria (13.6%) and significantly lower than India (62.5%).[18,20,22]

In our study the incidence of thrombocytopenia was found to be 55.8% which is comparable to a report from Nigeria 53% but significantly higher from studies from India (16.7%), Iran (17.9%), Indonesia (12.1%), and the Netherlands (27%).[11,19,20,24,25] This difference may be explained by the variability of the types and magnitude of the risk factors involved in the development of thrombocytopenia in each set up. In the great majority (90.9%) of the neonates with thrombocytopenia, the onset was >72 hrs from birth. This figure was significantly higher compared to other studies from Nigeria (15.8%), India (56%), Iran (24.7%), Indonesia (11.8%).[18,20–22] This difference may reflect the underlying etiology for thrombocytopenia which was sepsis in our study.

The incidence of severe thrombocytopenia (< 50,000/µL) in our study was 28.6% accounting for half of the cases of thrombocytopenia. This figure was found to be lower than studies from India (37.5%) and Iran (30.5%) but higher compared to that of Nigeria (13.6%), the UK (6%) and the US (2.4%).[18,19,22,26,27] This difference may be explained by the difference in the underlying cause of thrombocytopenia.

In our study one of the risk factors that was found to be associated with the development of thrombocytopenia among the various maternal, neonatal and perinatal factors analyzed was sepsis. The incidence of neonatal sepsis in our study was higher compared to two systematic reviews done in Ethiopia.[28,29] This association of neonatal sepsis with thrombocytopenia was also present in similar studies from Nigeria, Turkey, India, Iran, Indonesia and Austria.[18–20,22,23,30] The other factors like maternal

hypertension, prematurity and low birth weight which were found to be significant risk factors for thrombocytopenia in other studies were not significantly associated in our study.

Abbreviations

EOS, early onset sepsis; EOT, early onset thrombocytopenia; IVH, intraventricular haemorrhage; LOS, late onset sepsis; LOT, late onset thrombocytopenia; NEC, necrotizing enterocolitis; NT, neonatal thrombocytopenia; PT, platelet transfusions; NICU, neonatal intensive care unit; TORCH, toxoplasmosis, rubella, cytomegalo virus, herpes simplex virus; TASH, Tikur Anbessa Specialized Hospital.

Funding

Disclosure

All authors reported no conflicts of interest for this work.

References

1. Sola-Visner M. Platelets in the neonatal period: developmental differences in platelet production,function, and hemostasis and the potential impact of therapies; American Society of Hematology. *Hematology.* 2012;2012(1):506–511. doi:10.1182/asheducation.V2012.1.506.3798 532

2. Ferrer-Marin F, Liu Z-J, Gutti R, Sola-Visner M. Neonatal thrombocytopenia and megakaryocytopoiesis. *Semin Hematol.* 2010;47 (3):281–288. doi:10.1053/j.seminhematol.2010.04.002

3. Roberts I, Murray NA. Neonatal thrombocytopenia: causes and management. *Arch Dis Child Fetal Neonatal Ed.* 2003;88(5):F359–F364. doi:10.1136/fn.88.5.F359

4. Fernandez CJ. *Causes of Neonatal Thrombocytopenia.* UpToDate®; 2017:28.

5. Ulusoy E, Tüfekçi Ö, Duman N, Kumral A, Irken G, Ören H. Thrombocytopenia in neonates: causes and outcomes. *Ann Hematol.* 2013;92(7):961–967. doi:10.1007/s00277-013-1726-0

6. Wong W, Glader B. Approach to the newborn who has thrombocytopenia. *NeoReviews.* 2004;5(10):e444–e450. doi:10.1542/neo.5-10-e444

7. Sola-Visner M, Saxonhouse MA, Brown RE. Neonatal thrombocytopenia: what we do and don't know. *Early Hum Dev.* 2008;84 (8):499–506. doi:10.1016/j.earlhumdev.2008.06.004

8. Cremera M, Sallmon H, Kling PJ. Thrombocytopenia and platelet transfusion in the neonate. *Semin Fetal Neonatal Med.* 2015;1:10–18.

9. Roberts IAG, Murray NA. Neonatal thrombocytopenia: new insights into pathogenesis and implications for clinical management. *Curr Opin Pediatr.* 2001;13(1):16–21. doi:10.1097/00008480-200102000-00003

10. Antonio DV. Evaluation and management of thrombocytopenic neonates in the intensive care unit. *Early Hum Dev.* 2014;90(Supplement 2):S51–S55. doi:10.1016/S0378-3782(14)50014-X

11. Roberts I, Stanworth S, Murray NA. Thrombocytopenia in the Neonate. *Blood Rev.* 2008;22(4):173–186. doi:10.1016/j.blre.2008.03.004

12. Gunnink SF, Vlug R, Fijnvandraat K, van der Bom JG, Stanworth SJ, Lopriore E. Neonatal thrombocytopenia: etiology, management and outcome. *Expert Rev Hematol.* 2014;7(3):387–395. doi:10.1586/17474086.2014.902301

13. Bonifacio L, Petrova A, Nanjundaswamy S, Mehta R. Thrombocytopenia related neonatal outcome in preterms. *Indian J Pediatr.* 2007;74(3):269–274. doi:10.1007/s12098-007-0042-x

14. Josephson CD, Su LL, Christensen RD. Platelet transfusion practices among neonatologists in the United States and Canada: results of a survey. *PEDIATRICS.* 2009;123(1):278–285. doi:10.1542/peds.2007-2850

15. Adams-Chapman I, Stoll BJ. Systemic inflammatory response syndrome. *Semin Pediatr Infect Dis.* 2001;12(1):5–16. doi:10.1053/spid.2001.19230

16. Sylvester KG, Liu GY, Albanese CT. Necrotizing enterocolitis. In: *Coran pediatrics Surgery.* 7th ed. chapter 94. 2011:1187–1207.

17. Eltawel M, AlHarbi T, AlJamaan K, Alsaif S, Ali Y, Salam M. A prospective study on the incidence and outcomes of neonatal thrombocytopenia at a tertiary care facility in central Saudi Arabia. *Adv Neonatal Care.* 2018;18(5):E3–E12. doi:10.1097/ANC.0000000000000539

18. Tirupathi K, Swarnkar K, Vagha J. Study of risk factors of neonatal thrombocytopenia. *Int J Contemp Pediatr.* 2017;4(1):191–196. doi:10.18203/2349-3291.ijcp20164603

19. Khalessi N, Khosravi N, Sanii S. The prevalence and risk factors for neonatal thrombocytopenia among new-borns admitted to intensive care unit of aliasghar children's hospital. *IJBC.* 2013;2:41–45.

20. Kusumasari N, Rohsiswatmo R, Gatot D. Incidence and risk factors of neonatal thrombocytopenia: a Preliminary Study. *Paediatr Indones.* 2010;50(1):31–37.

21. Eslami Z, Lookzadeh M, Noorishadkam M, Hashemi A, Ghilian R, Pirdehghan A. Thrombocytopenia and associated factors in neonates admitted to NICU during years 2010–2011. *Iran J Pediatr Hematol Oncol.* 2013;3(1):205–215.

22. Jeremiah ZA, Oburu JE. Pattern and prevalence of neonatal thrombocytopenia in Port Harcourt, Nigeria. *Arch Pathol Lab Med.* 2010;2:27–31. doi:10.2147/PLMI.S9968

23. Oren H, Irken G, Oren B, Olgun N, Özkan H. Assessment of clinical impact and predisposing factors for neonatal thrombocytopenia. *Indian J Pediatr.* 1994;61(5):551–558. doi:10.1007/BF02751717

24. Gupta AK, Kumari S, Singhal A, Bahl A. Neonatal thrombocytopenia and platelets transfusion. *Asian J Transfus Sci.* 2012;6(2):161. doi:10.4103/0973-6247.98924

25. von Lindern JS, van den Bruele T, Lopriore E, Walther FJ, Thrombocytopenia in neonates and the risk of intraventricular haemorrhage: a Retrospective Cohort Study. *BMC Pediatr.* 2011;11(1):16. doi:10.1186/1471-2431-11-16

26. Murray NA, Howarth LJ, Mc Cloy MP, Letsky EA, Roberts IAG. Platelet transfusion in the management of severe thrombocytopenia in neonatal intensive care unit patients. *Transf Med.* 2002;12(1):35–41. doi:10.1046/j.1365-3148.2002.00343.x

27. Baer VL, Lambert DK, Henry E, Christensen RD. Severe thrombocytopenia in the NICU. *Pediatrics.* 2009;124(6):e1095–e1100. doi:10.1542/peds.2009-0582

28. Belachew A, Tewabe T. Neonatal sepsis and its association with birth weight and gestational age among admitted neonates in Ethiopia: systematic review and meta-analysis. *BMC Pediatr.* 2020;20(1):55. doi:10.1186/s12887-020-1949-x

29. Assemie MA, Alene M, Yismaw L, et al. Prevalence of neonatal sepsis in ethiopia: a systematic review and meta-analysis. *Int J Pediatr.* 2020;2020:9. Article ID 6468492. doi:10.1155/2020/6468492

30. Resch E, Hinkas O, Urlesberger B, Resch B. Neonatal thrombocytopenia—causes and outcomes following platelet transfusions. *Eur J Pediatr.* 2018;177(7):1045–1052. doi:10.1007/s00431-018-3153-7

Economic Burden Associated with Major Surgery in Patients with von Willebrand Disease: A United States Retrospective Administrative Database Analysis

Abiola Oladapo, Yanyu Wu, Mei Lu, Sepehr Farahbakhshian & Bruce Ewenstein

ORIGINAL RESEARCH

Economic Burden Associated with Major Surgery in Patients with von Willebrand Disease: A United States Retrospective Administrative Database Analysis

Abiola Oladapo[1]
Yanyu Wu[2]
Mei Lu[3]
Sepehr Farahbakhshian[3]
Bruce Ewenstein[2]

[1]Baxalta US Inc., a Takeda Company, Cambridge, MA, USA; [2]Takeda Development Center Americas, Inc., Cambridge, MA, USA; [3]Takeda Development Center Americas, Inc., Lexington, MA, USA

Purpose: To estimate the incremental economic burden of major surgeries in patients with von Willebrand disease (VWD).

Patients and Methods: This was a retrospective analysis of the IBM Health MarketScan® database (2008–2018). Patients with at least two healthcare visits for VWD in the database who had undergone at least one major surgery unrelated to VWD (identified via International Classification of Diseases, Ninth and Tenth Revisions procedure codes) were included. Patients without VWD with major surgeries were selected from a 1% random database sample. All patients had ≥12 months of continuous healthcare plan enrollment before and following their first major surgery. Patients with VWD were matched (1:1) with patients without VWD using propensity score matching. Regression models compared healthcare resource utilization and costs between the matched cohorts over a 12-month period after patients' index major surgery.

Results: After propensity score matching, 2972 pairs were selected. Musculoskeletal and digestive surgeries were the two most common major surgeries (patients with VWD, 39.6% and 25.0%; without VWD, 37.1% and 23.4%, respectively). Patients with VWD were significantly more likely (p<0.0001) to have an inpatient admission (odds ratio = 1.71; 95% confidence interval [CI] 1.52–1.92) or emergency room visit (odds ratio = 1.41; 95% CI 1.25–1.59) than patients without VWD. The numbers of inpatient admissions (incidence rate ratio [IRR] = 1.47; 95% CI 1.35–1.60), emergency room visits (IRR = 1.44; 95% CI 1.31–1.59), and outpatient visits (IRR = 1.16; 95% CI 1.11–1.21) per patient were also significantly greater for patients with VWD than for those without VWD (p<0.0001). Patients with VWD incurred significantly higher (p<0.0001) total healthcare costs (medical and pharmacy) per patient than patients without VWD ($50,733.89 versus $30,154.84, respectively).

Conclusion: Healthcare resource utilization and associated costs among patients undergoing major surgeries were significantly higher for those with VWD than for patients without VWD.

Keywords: bleeding, healthcare costs, retrospective studies, healthcare resource utilization

Correspondence: Yanyu Wu
Takeda Development Center Americas, Inc., 40 Landsdowne Street, Cambridge, MA, 02139, USA
Tel +1 617-955-6408
Email yanyu.wu@takeda.com

Introduction

von Willebrand disease (VWD) is an autosomal inherited blood clotting disorder that manifests most commonly as recurrent mild-to-moderate mucocutaneous

bleeding and excessive bleeding after surgery or trauma.[1,2] Patients with VWD have impaired hemostasis owing to a quantitative or qualitative deficit in von Willebrand factor (VWF), a plasma glycoprotein that mediates platelet adhesion and aggregation and stabilizes coagulation factor VIII (FVIII) in the circulation.[1–4] Although VWD is classified as a rare disease, it is considered the most common bleeding disorder, with an estimated prevalence of between 0.01% and 1.00% depending on the population and diagnostic approach.[5–8]

The first-line treatment of bleeding events in patients with VWD is desmopressin, especially in those with type 1 disease, the mildest form.[1–3] Desmopressin increases plasma levels of endogenous VWF and FVIII by provoking the release of stored VWF.[3] For patients with more severe disease (such as those with type 2 subtypes or type 3), severe bleeding events, or an inadequate response to desmopressin, VWF replacement therapies are the mainstay of treatment.[9–11] In patients with VWD who undergo major surgical procedures, the risk of potentially life-threatening hemorrhage means that hemostatic measures to normalize functional VWF and FVIII levels are obligatory.[11–14]

Although major surgical procedures impose a clinical and economic burden on patients in general, patients with VWD who undergo major surgery may be exposed to the additional burden of impaired hemostasis.[15] There is a paucity of published data regarding the economic burden associated with major surgical procedures in patients with VWD in the United States, including gaps in knowledge regarding the extent of all-cause healthcare resource utilization and associated healthcare costs. A retrospective analysis of a large US database (the Healthcare Cost and Utilization Project National Inpatient Sample) detected a statistically significant higher risk of post-operative hemorrhage in patients with VWD undergoing major noncardiac surgery relative to patients without VWD undergoing major surgery.[15] However, this study did not capture information on the costs and resources expended on managing these patients.

Therefore, the objective of this study was to estimate the overall economic burden associated with major surgeries in patients with VWD compared with matched patients without VWD who had major surgery in the United States.

Methods
Data Source
In this retrospective administration data analysis, we utilized data from the commercial IBM® MarketScan® Commercial Claims and Encounters (denoted MarketScan) database and the Medicare Supplemental and Coordination of Benefits database for the period of January 2008 to June 2018. The MarketScan database includes medical and procedural claims for outpatients and inpatients, and outpatient pharmaceutical claims for millions of individuals with employer-sponsored health insurance, including their spouses and dependents.[16] All VWD-related diagnoses (for patient identification) and procedures (for patient identification and economic analysis) were identified by medical claims with codes from the International Classification of Diseases, Ninth/Tenth Revision, Clinical Modification (ICD-9-CM/ICD-10-CM), Current Procedural Terminology (CPT), Healthcare Common Procedure Coding System, and National Drug Code.

As this analysis used de-identified patient data from the MarketScan® database, ethical approval was not required. The MarketScan® database is designed to meet the requirements of the Health Insurance Portability act (HIPAA) of 1996 for a limited-use dataset, and has also undergone statistical analysis by a third party to confirm that the data meet HIPAA requirements for fully de-identified datasets.

Patient Identification
Patients in the VWD cohort were identified from the MarketScan database using the following eligibility criteria: VWD diagnoses (ICD-9-CM = 286.4; ICD-10-CM = D68.0) from two separate healthcare visits (≥1 day apart and excluding laboratory and radiology orders), no diagnosis of acquired coagulation factor deficiency, including acquired VWD (ICD-9-CM = 286.7; ICD-10-CM = D68.32, D68.4) at any time, and major surgery on or after the first diagnosis of VWD (ie, after the first VWD claim and before the end of the observation period).

Patients in the non-VWD cohort with major surgeries were selected from a 1% random sample from the database and had no diagnosis of VWD or acquired coagulation factor deficiency at any time. To control for potential selection bias, patients with VWD were matched 1:1 with patients without VWD on the basis of baseline demographic and clinical characteristics (age, sex, US region, comorbidities

[anemia, anxiety, depression, fatigue, and obesity], and Charlson Comorbidity Index [CCI] scores[17]) using a propensity score matching method. Matching was performed using a preset caliper size of 0.01 to maintain the maximum sample size using the smallest caliper width.

A major surgery was defined as a medical claim associated either with an ICD-9/10 procedure coding system (PCS) code classified by the Healthcare Cost and Utilization Project as a major therapeutic operating room procedure (Procedure Class 4) or with a CPT code classified by the Centers for Medicare & Medicaid Services as a major procedure (Global Surgical Indicator = 090). Types of major surgical procedures are listed in Supplementary Table 1. In both the VWD and non-VWD cohorts, major surgeries associated with VWD treatment (ie, hysterectomy, nasal ablation, or uterine ablation; ICD-9-PCS = 21.69, 68.0, 68.23; ICD-10-PCS = 09BL*ZZ, 09TL*ZZ, 0U99**Z, 0UC9*ZZ, 0UJD*ZZ, 0U5B*ZZ, 0UDB*ZZ; CPT = 30801, 30802, 58150–58294, 58353, 58541–58554, 58563, 58570–58573) that were conducted at any time during the observation period were excluded.

The first medical claim for a major surgery during the identification period was designated the index date, defined as 1 day before the inpatient admission date (if the claim was identified in an inpatient setting) or 1 day before the procedure date (if the claim was identified in an outpatient setting). An emergency room (ER) visit also served to define the index date (ie, 1 day before ER visit) if the patient's visit was associated with a medical claim for a major surgical event. Patients in both groups were required to have had continuous healthcare plan enrollment for ≥12 months before the index date (baseline period) and after the index date (observation period following and including their first major surgery) and no capitated healthcare plan in the 12-month observation period.

Patient demographics (age, sex, and geographic region) as of the index date were extracted for the VWD cohort with major surgeries and the non-VWD cohort with major surgeries. Patient-related clinical characteristics, including CCI scores and comorbidities (anemia, anxiety, depression, fatigue, and obesity; identified using ICD-9-CM and ICD-10-CM codes; Supplementary Table 2 [18]), were extracted for the 12-month baseline period.

Outcome Measures

All types of major surgery performed during the 12-month observation period, including the index surgery, were

evaluated in both cohorts, with the three most common types of surgery reported.

The economic burden of major surgery in patients with and without VWD was evaluated by comparing healthcare resource utilization (HCRU) and costs in VWD cases with matched controls during the 12-month observation period from the index date. HCRU included the proportion of patients with inpatient admissions, ER visits, or any outpatient visits, as well as the number of visits per patient by visit type. Total healthcare costs represented the sum of pharmacy and medical costs (sum of inpatient, ER, and outpatient costs). All costs reflected reimbursed amounts from payers to healthcare providers and were adjusted to 2018 US dollars using the medical component of the Consumer Price Index.

Statistical Methods

Baseline patient demographics, clinical attributes, and outcome measures were summarized descriptively as the mean ± standard deviation (SD) and median (range) for continuous variables and frequency (percentage) for categorical variables.

After propensity score matching, comparisons between the VWD and non-VWD cohorts were conducted for each of the study endpoints (proportion and frequency of inpatient, outpatient, and ER visits and medical and pharmacy costs) using generalized linear regression models with the appropriate link function (eg, identity, log, and logit), controlling for age, sex, region, health plan, index year, CCI, comorbidities (anemia, anxiety, depression, fatigue, and obesity), and HCRU (inpatient, ER, and outpatient) during the baseline period. Comparisons were made on the basis of the type (categorical or continuous) and data distribution of the study endpoints (eg, normal, Poisson, binomial, categorical, and gamma). The Pearson scale was utilized when applying the Poisson model to account for over-distribution of the data.

All statistical analyses were performed using SAS (version 9.3; SAS Institute Inc., Cary, NC).

Results
Patient Disposition and Baseline Characteristics

Overall, 25,653 patients with VWD and 1,638,475 patients without VWD were identified from the MarketScan database (Figure 1). Of these, 2973 and 65,627 patients with and without VWD, respectively, met the inclusion criteria for this retrospective analysis.
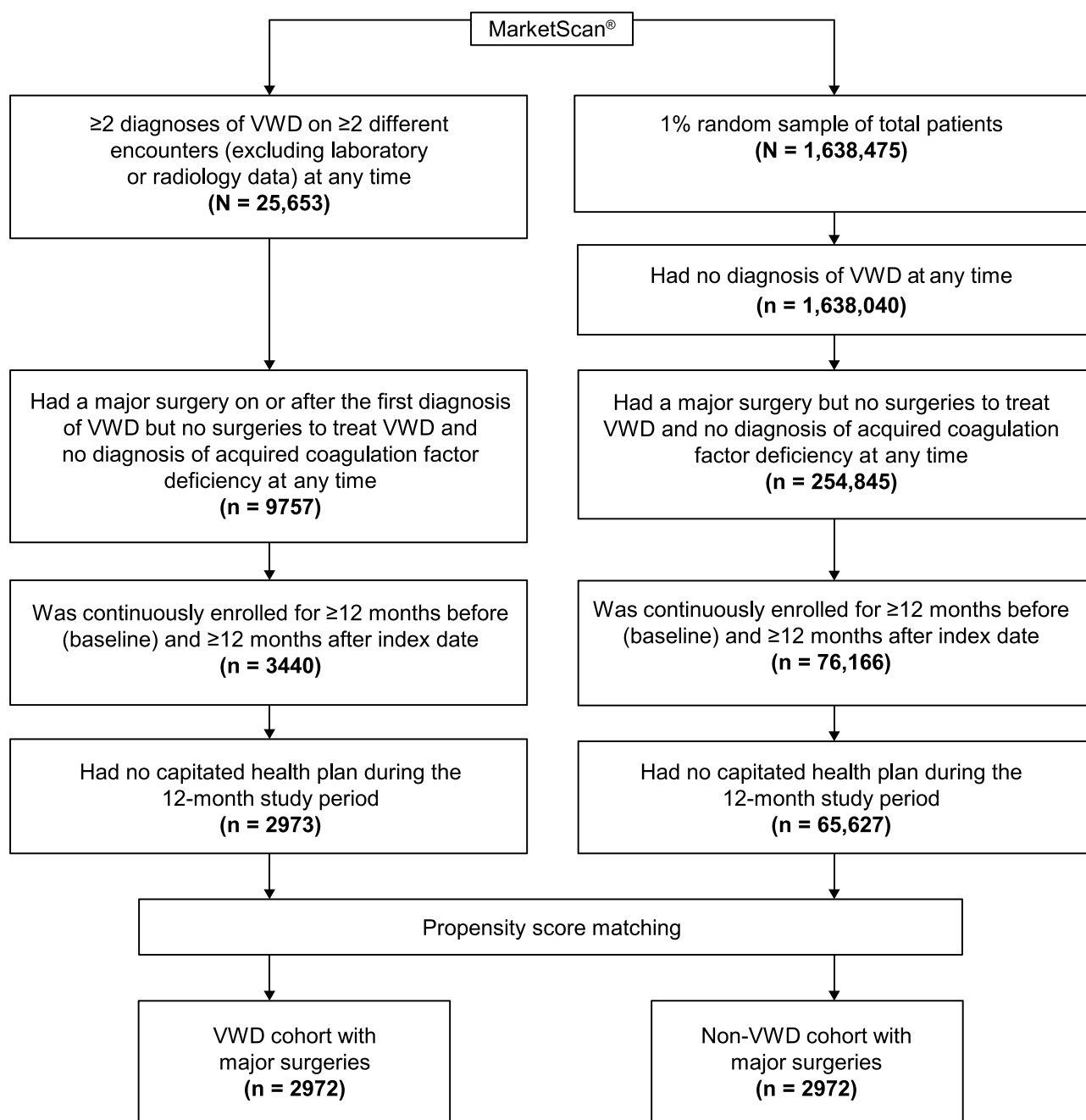
Figure 1 Patient selection for the VWD and non-VWD study cohorts with major surgeries.
Notes: Surgeries to treat VWD included uterine ablation, nasal ablation, and hysterectomy. The index date was defined as the date preceding the admission date for the first major surgery for cases identified in the hospital or as the date preceding the date of the first procedure for major surgery for cases identified in other settings.
Abbreviation: VWD, von Willebrand disease.

Baseline Characteristics

After propensity score matching, 2972 patients with VWD and 2972 patients without VWD who had undergone at least one major surgery were selected for analysis (Table 1). Mean (SD) age was 40.5 (20.6) and 40.9 (20.3) years in the VWD and non-VWD matched

cohorts, respectively. The matched study population was predominantly female, with female patients accounting for nearly three-quarters of the patients in each cohort (73.3% and 73.6% for the VWD and non-VWD cohorts, respectively). Mean (SD) CCI score was 0.7 (1.3) in the VWD cohort and 0.6 (1.3) in the non-

Table 1 Baseline Demographic and Clinical Characteristics of the VWD and Non-VWD Cohorts

Status	Before Propensity Score Matching			After Propensity Score Matching		
	VWD Cohort (n = 2973)	Non-VWD Cohort (n = 65,627)	p-value	VWD Cohort (n = 2972)	Non-VWD Cohort (n = 2972)	p-value
Age, years			<0.0001			0.4267
Mean (SD) Median (range)	40.5 (20.6) 42 (1–94)	45.8 (21.0) 49 (0–103)		40.5 (20.6) 42 (1–94)	40.9 (20.3) 42 (0–96)	
Age group, years, n (%)			<0.0001			0.6448
0–11 12–17 18–54 >55	240 (8.1) 274 (9.2) 1582 (53.2) 877 (29.5)	4656 (7.1) 3980 (6.1) 31,458 (47.9) 25,553 (38.9)		240 (8.1) 274 (9.2) 1581 (53.2) 877 (29.5)	235 (7.9) 247 (8.3) 1599 (53.8) 891 (30.0)	
Sex, n (%)			<0.0001			0.769
Female Male	2178 (73.3) 795 (26.7)	35,026 (53.4) 30,601 (46.6)		2177 (73.3) 795 (26.7)	2188 (73.6) 784 (26.4)	
US geographic region, n (%)			<0.0001			0.7946
Midwest Northeast South West Unknown	762 (25.6) 906 (30.5) 858 (28.9) 399 (13.4) 48 (1.6)	17,097 (26.1) 12,750 (19.4) 25,632 (39.1) 9260 (14.1) 888 (1.4)		761 (25.6) 906 (30.5) 858 (28.9) 399 (13.4) 48 (1.6)	761 (25.6) 946 (31.8) 842 (28.3) 378 (12.7) 45 (1.5)	
CCI			<0.0001			0.1067
Mean (SD) Median (range)	0.7 (1.3) 0 (0–12)	0.5 (1.0) 0 (0–13)		0.7 (1.3) 0 (0–12)	0.6 (1.3) 0 (0–10)	
Comorbidity, n (%)						
Anemia Anxiety Depression Fatigue Obesity	296 (10.0) 351 (11.8) 360 (12.1) 537 (18.1) 227 (7.6)	2677 (4.1) 3882 (5.9) 4414 (6.7) 6758 (10.3) 3904 (5.9)	<0.0001 <0.0001 <0.0001 <0.0001 0.0002	295 (9.9) 350 (11.8) 359 (12.1) 536 (18.0) 227 (7.6)	282 (9.5) 362 (12.2) 365 (12.3) 546 (18.4) 212 (7.1)	0.5991 0.6604 0.8428 0.7623 0.4875

Abbreviations: CCI, Charlson Comorbidity Index; SD, standard deviation; VWD, von Willebrand disease.

VWD cohort. Baseline comorbidities were comparable between the two matched cohorts.

Clinical Outcomes

The most common major surgeries over the observation period were musculoskeletal, digestive, and integumentary in both patients with VWD and without VWD (39.6%, 25.0%, and 8.6% versus 37.1%, 23.4%, and 9.0%, respectively). The percentages of major surgeries that were related to the female genital organs or were obstetric procedures (including Cesarean sections) were 7.0% and 6.1%, respectively, in the matched VWD cohort and 7.7%

and 7.6% in the non-VWD cohort. Further details on the types of major surgery undertaken in the matched cohorts are provided in Supplementary Table 3.

Economic Outcomes
HCRU

During the 12-month observation period, patients with VWD had significantly greater HCRU than those without VWD (Figure 2A and B). The proportions of patients having an inpatient admission (43.0% versus 29.4%; p<0.0001), ER visit (34.5% versus 25.7%; p<0.0001), and outpatient visit (99.5% versus 99.1%; p=0.0302)

A Proportion of patients by visit type

B Number of visits per patient by visit type
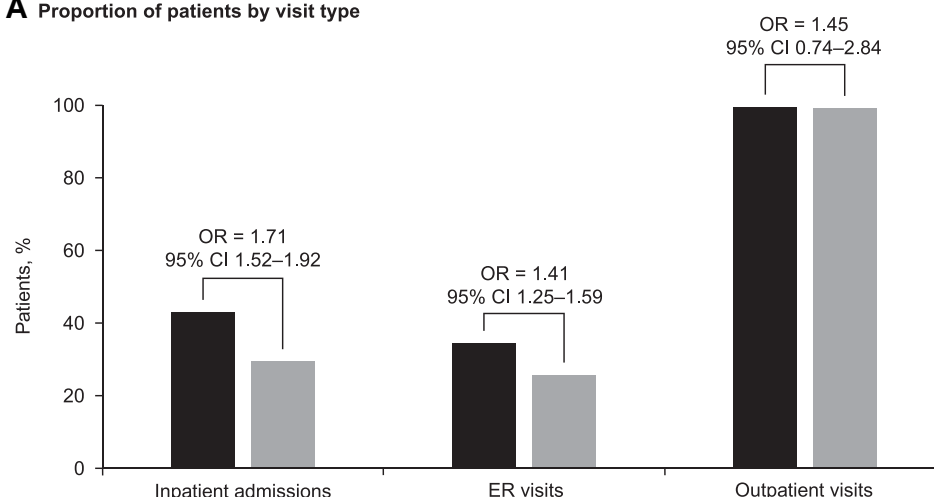
■ Patients with VWD (n = 2972) ■ Patients without VWD (n = 2972)
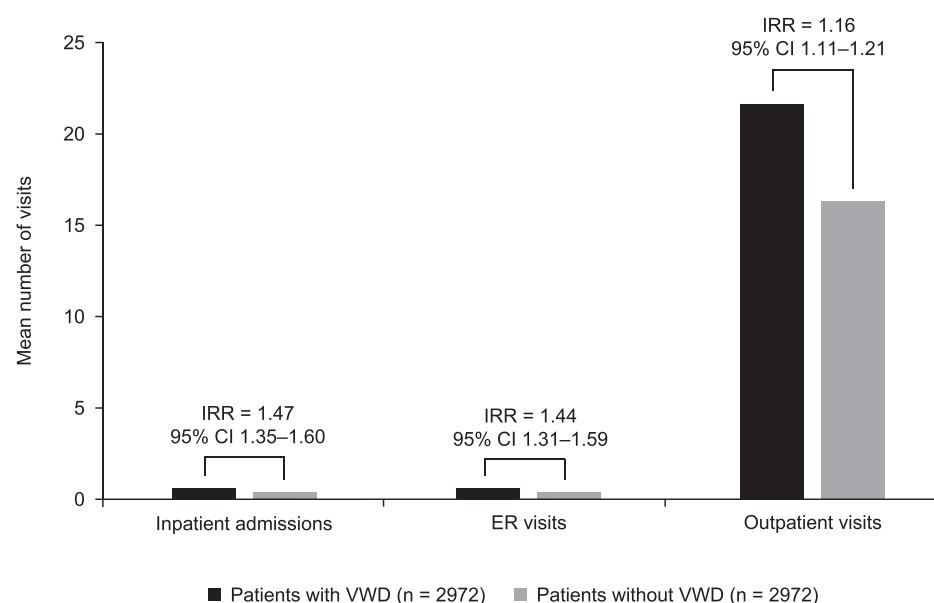
Figure 2 Comparison of all-cause HCRU in the 12-month observation period between matched cohorts of patients with and without VWD who had major surgery, showing (**A**) proportion of patients by visit type and (**B**) number of visits per patient by visit type.
Notes: HCRU was measured during the observation period, defined as the 12-month period beginning from the index date. ORs were evaluated for binary variables (ie, at least one visit) using logistic regression. IRRs were evaluated for count variables (ie, number of visits and total length of inpatient stay) using Poisson regression. Models were controlled for age, sex, region, health plan, index year, CCI, comorbidity (anemia, anxiety, depression, fatigue, and obesity), and HCRU (inpatient, ER, and outpatient) during the baseline period. ORs >1 indicate higher odds for patients with VWD and major surgeries compared with propensity score matched patients without VWD who had major surgeries. IRRs >1 indicate increased incidence rate for patients with VWD and major surgeries compared with propensity score matched patients without VWD who had major surgeries. Inpatient visits were identified with a service location of inpatient hospital; ER visits were identified with a service location of emergency department; and outpatient visits were identified with a service location of clinic, office, or outpatient hospital.
Abbreviations: CCI, Charlson Comorbidity Index; CI, confidence interval; ER, emergency room; HCRU, healthcare resource utilization; IRR, incidence rate ratio; OR, odds ratio; VWD, von Willebrand disease.

were significantly higher in the VWD cohort than in the non-VWD cohort, respectively. Patients with VWD were 71.0% and 41.0% more likely (p<0.0001) to have an inpatient admission (odds ratio [OR] = 1.71; 95%

confidence interval [CI] 1.52–1.92) and/or ER visit (OR = 1.41; 95% CI 1.25–1.59), respectively, compared with those without VWD. No statistically significant between-cohort difference was detected regarding the odds of

having an outpatient visit (OR = 1.45; 95% CI 0.74–2.84; p=0.2824) (Figure 2A).

Patients with VWD had significantly more healthcare visits than those without VWD (p<0.0001): inpatient admissions (mean [SD] = 0.59 [0.93] vs 0.38 [0.73]; incidence rate ratio [IRR] = 1.47; 95% CI 1.35–1.60), ER visits (mean [SD] = 0.58 [1.14] vs 0.40 [1.21]; IRR = 1.44; 95% CI 1.31–1.59), and outpatient visits (mean [SD], 21.6 [18.75] vs 16.3 [16.22]; IRR = 1.16; 95% CI 1.11–1.21) (Figure 2B).

Healthcare Costs

Over the 12-month observation period, patients with VWD incurred significantly higher adjusted total healthcare costs ($50,734 versus $30,155; p<0.0001), pharmacy costs ($10,581 versus $4632; p<0.0001), and medical costs ($41,943 versus $26,234; p<0.0001) than patients without VWD, respectively, after adjusting for baseline covariates: age, sex, region, health plan, index year, CCI, comorbidity profile (anemia, anxiety, depression, fatigue, and obesity), and HCRU (inpatient, ER, and outpatient; Figure 3). Medical costs accounted for the greatest proportion (83% and 87%) of overall costs in both cohorts.

Discussion

This retrospective cohort analysis of claims data assessed the economic impact of VWD among patients undergoing major surgery in a real-world US setting. Our study results suggest that HCRU and total healthcare costs in the 12-month period following and including the index major surgical procedure were significantly higher for the VWD cohort than for the non-VWD cohort. Among patients undergoing major surgery, those with VWD were significantly more likely to have an inpatient admission and ER visit and required significantly more inpatient admissions and ER visits. As would be expected in patients undergoing major surgery, most (99%) patients in both the VWD and non-VWD cohorts had at least one outpatient visit; however, the number of outpatient visits per patient was significantly higher in patients with VWD than in patients without VWD. Not surprisingly, the increased level of post-surgical healthcare engagement by patients with VWD relative to patients without VWD translated primarily into increased medical costs. Given that the mean age of patients with VWD included in this analysis was 40.5 years, it may be expected that the greater burden faced by patients with VWD will have
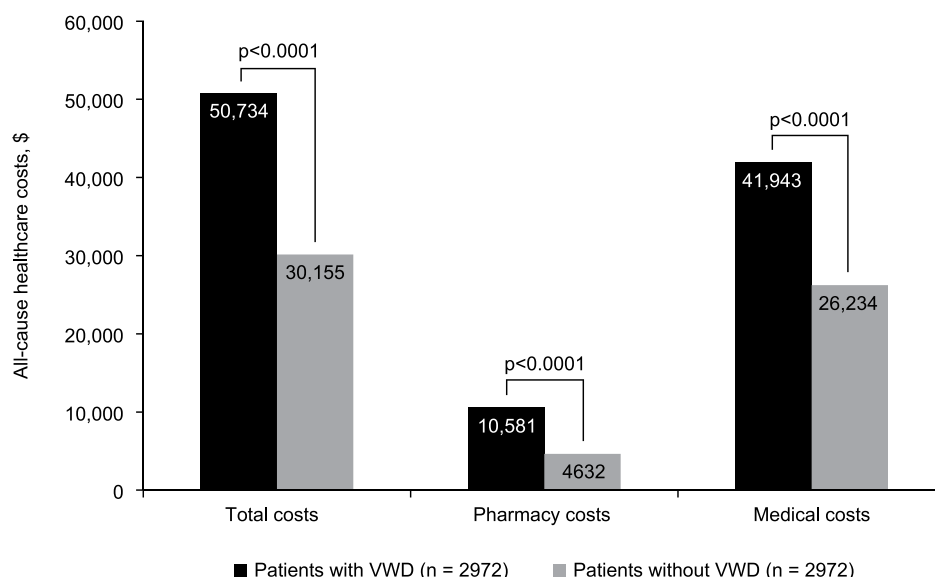
Figure 3 Comparison of adjusted healthcare costs in the 12-month observation period between matched cohorts of patients with and without VWD who had major surgery.
Notes: Healthcare costs were measured during the observation period, defined as the 12-month period beginning from the index date. All costs were measured as reimbursed amounts from payers to healthcare providers and adjusted to 2018 US dollars using the medical component of the Consumer Price Index. Predicted means for all costs were estimated using a generalized linear model with a gamma distribution and log link. All models were controlled for age, sex, region, health plan, index year, CCI, comorbidity (anemia, anxiety, depression, fatigue, and obesity), and HCRU (inpatient, ER, and outpatient) during the baseline period.
Abbreviations: CCI, Charlson Comorbidity Index; ER, emergency room; HCRU, healthcare resource utilization; VWD, von Willebrand disease.

a major social and economic impact on individuals' productivity and family life.

A probable explanation for the incremental economic burden associated with major surgery among patients with VWD is complications related to their bleeding propensity. In a separate analysis of the MarketScan database (2008–2016) that involved 19,785 patients with documented VWD, 15.1% of patients experienced at least one major bleeding event during a median 4-year observation period (mean [SD] rate = 0.11 [0.64] major bleeding events per year).[18] Furthermore, within this VWD cohort, patients with major bleeding events were significantly more likely to have an inpatient admission (OR = 4.1; 95% CI 3.4–5.0), ER visit (OR = 1.8; 95% CI 1.5–2.1), or outpatient visit (OR = 4.9; 95% CI 1.8–13.4); they also had more frequent inpatient admissions (OR = 3.2; 95% CI 2.8–3.8), ER visits (OR = 2.0; 95% CI 1.8–2.3), and outpatient visits (OR = 1.3; 95% CI 1.2–1.3) relative to patients without major bleeding events (all $p<0.01$).[18] As a result, patients with VWD and major bleeding events incurred significantly higher total healthcare costs (adjusted mean difference $20,890; 95% CI $15,524–29,254; $p<0.01$) than patients with VWD without major bleeding events.[18] These findings are also consistent with data from the Swedish VWD Prophylaxis Network, a population-based registry, which showed, between 1987 and 2009, a two-fold higher rate of inpatient hospitalizations among 2790 patients with VWD versus age- and sex-matched controls.[19]

The 2008 National Heart, Lung, and Blood Institute guidelines recommend various strategies for the treatment of VWD, with the appropriate therapy depending on the type and severity of VWD, the severity of the hemostatic challenge, and the nature of the actual or potential bleeding event.[3] The guidelines recommend evaluating the risks and benefits of prophylaxis with VWF replacement therapies when considering long-term therapy for VWD.[3] There is evidence from the observational VWD Prophylaxis Network supporting prophylaxis as a means to reduce hospitalizations in VWD patients with severe and frequent bleeds,[19–22] but data are limited regarding the use of prophylaxis among patients with VWD undergoing elective surgical procedures.[12]

Limitations

Our findings should be interpreted with due consideration to the methods used for data collection. Findings from an analysis by Sidonio et al[23] have raised questions concerning the reliability of using ICD-9 claims data alone to identify patients with VWD. Sidonio et al also utilized ICD-9 codes (at least two

claims for VWD) to identify patients with VWD, and found that less than two-thirds of patients had a diagnostic laboratory test within the 2 years before or after diagnosis. Our analysis did include criteria such as exclusion of laboratory and radiology claims to minimize false positives; however, this may not have completely eliminated this issue.

As VWD was identified via ICD code, it was not possible to identify the specific type of VWD. Comorbidities were identified using ICD-9/10 diagnosis codes, which may be underestimated or mislabeled in administrative claims databases. Owing to the observational design, the analysis may have been affected by unobserved differences between comparison cohorts. As the data used in this analysis are limited to patients in a US commercial plan, findings may not be generalizable to populations beyond those covered by commercial medical insurance plans in the United States. Future research, however, could utilize the methodology described in this publication to undertake a similar analysis in other patient cohorts, including in other countries.

Conclusion

This retrospective analysis of a large US commercial healthcare database suggests that patients with VWD who had major surgeries incurred significantly higher HCRU and associated costs (particularly medical costs) compared with patients without VWD who had major surgeries.

Previous Presentation

Poster presentation (#4602) at 61st American Society of Hematology (ASH) Annual Meeting, December 7–10, 2019, Orlando, FL, USA.

Abbreviations

CCI, Charlson Comorbidity Index; CI, confidence interval; CPT, Current Procedural Terminology; ER, emergency room; FVIII, factor VIII; HCRU, healthcare resource utilization; IRR, incidence rate ratio; OR, odds ratio; PCS, procedure coding system; SD, standard deviation; VWD, von Willebrand disease; VWF, von Willebrand factor.

Data Sharing Statement

Data are the proprietary property of IBM.

Ethics Approval and Informed Consent

Not applicable; no institutional review board approval was required for this retrospective claims database analysis

706

Journal of Blood Medicine 2021:12

because only de-identified data were used. All data analyzed in the present study complied with the requirements of the Health Insurance Portability and Accountability Act (HIPAA) of 1996 for fully de-identified datasets.

Consent for Publication

Not applicable.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas (AO, YW, ML, SF, and BE contributed to the study design, interpretation of the data, and preparation of the manuscript. YW analyzed the data); took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Disclosure

Abiola Oladapo was an employee of Baxalta US Inc., a Takeda company, at the time the analysis was completed and the manuscript developed and is an owner of Takeda stock. Yanyu Wu, Mei Lu, Sepehr Farahbakhshian, and Bruce Ewenstein are employees of Takeda Development Center Americas, Inc., and are owners of Takeda stock.

References

1. Leebeek FW, Eikenboom JC. Von Willebrand's disease. *N Engl J Med*. 2016;375(21):2067–2080. doi:10.1056/NEJMra1601561

2. Sadler JE, Budde U, Eikenboom JC, et al; Working Party on von Willebrand Disease Classification. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103–2114. doi:10.1111/j.1538-7836.2006.02146.x

3. Nichols WL, Hultin MB, James AH, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171–232. doi:10.1111/j.1365-2516.2007.01643.x

4. Stockschlaeder M, Schneppenheim R, Budde U. Update on von Willebrand factor multimers: focus on high-molecular-weight multimers and their role in hemostasis. *Blood Coagul Fibrinolysis*. 2014;25(3):206–216. doi:10.1097/MBC.0000000000000065

5. Bowman M, Hopman WM, Rapson D, Lillicrap D, James P. The prevalence of symptomatic von Willebrand disease in primary care practice. *J Thromb Haemost*. 2010;8(1):213–216. doi:10.1111/j.1538-7836.2009.03661.x

6. Flood VH, Gill JC, Friedman KD, Bellissimo DB, Haberichter SL, Montgomery RR. Von Willebrand disease in the United States: a perspective from Wisconsin. *Semin Thromb Hemost*. 2011;37(5):528–534. doi:10.1055/s-0031-1281039

7. Sadler JE, Mannucci PM, Berntorp E, et al. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost*. 2000;84(2):160–174. doi:10.1055/s-0037-1613992

8. Werner EJ, Broxson EH, Tucker EL, Giroux DS, Shults J, Abshire TC. Prevalence of von Willebrand disease in children: a multiethnic study. *J Pediatr*. 1993;123(6):893–898. doi:10.1016/S0022-3476(05)80384-1

9. Peyvandi F, Kouides P, Turecek PL, Dow E, Berntorp E. Evolution of replacement therapy for von Willebrand disease: from plasma fraction to recombinant von Willebrand factor. *Blood Rev*. 2019;38:100572. doi:10.1016/j.blre.2019.04.001

10. Franchini M, Mannucci PM. Von Willebrand factor (Vonvendi®): the first recombinant product licensed for the treatment of von Willebrand disease. *Expert Rev Hematol*. 2016;9(9):825–830. doi:10.1080/17474086.2016.1214070

11. Peyvandi F, Mamaev A, Wang J-D, et al. Phase 3 study of recombinant von Willebrand factor in patients with severe von Willebrand disease who are undergoing elective surgery. *J Thromb Haemost*. 2019;17(1):52–62. doi:10.1111/jth.14313

12. Windyga J, Dolan G, Altisent C, Katsarou O, López Fernández MF, Zülfikar B; EHTSB. Practical aspects of factor concentrate use in patients with von Willebrand disease undergoing invasive procedures: a European survey. *Haemophilia*. 2016;22(5):739–751. doi:10.1111/hae.12955

13. Berntorp E. Replacement therapy during surgery in von Willebrand disease needs personalization. *Haemophilia*. 2018;24(3):338–340. doi:10.1111/hae.13488

14. Hazendonk HCAM, Heijdra JM, de Jager NCB, et al; "OPTI-CLOT" and "WIN" study group. Analysis of current perioperative management with Haemate® P/Humate P® in von Willebrand disease: identifying the need for personalized treatment. *Haemophilia*. 2018;24(3):460–470. doi:10.1111/hae.13451

15. Smilowitz NR, Gupta N, Guo Y, Bangalore S, Berger JS. Perioperative bleeding and thrombotic risks in patients with von Willebrand disease. *J Thromb Thrombolysis*. 2017;44(1):67–70. doi:10.1007/s11239-017-1504-2

16. Kulaylat AS, Schaefer EW, Messaris E, Hollenbeak CS. Truven Health Analytics MarketScan databases for clinical research in colon and rectal surgery. *Clin Colon Rectal Surg*. 2019;32(1):54–60. doi:10.1055/s-0038-1673354

17. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis*. 1987;40(5):373–383. doi:10.1016/0021-9681(87)90171-8

18. Lu M, Oladapo A, Wu Y, Farabakhshian S, Ewenstein B. Economic burden of major bleeding events in commercially insured patients with von Willebrand disease based on claims data from the United States. *J Manag Care Spec Pharm*. 2021;27(2):175–185. doi:10.18553/jmcp.2020.20327

19. Holm E, Carlsson KS, Lövdahl S, Lail AE, Abshire TC, Berntorp E. Bleeding-related hospitalization in patients with von Willebrand disease and the impact of prophylaxis: results from national registers in Sweden compared with normal controls and participants in the von Willebrand Disease Prophylaxis Network. *Haemophilia*. 2018;24 (4):628–633. doi:10.1111/hae.13473

20. Abshire TC, Federici AB, Alvárez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). *Haemophilia*. 2013;19 (1):76–81. doi:10.1111/j.1365-2516.2012.02916.x

21. Abshire T, Cox-Gill J, Kempton CL, et al. Prophylaxis escalation in severe von Willebrand disease: a prospective study from the von Willebrand Disease Prophylaxis Network. *J Thromb Haemost*. 2015;13(9):1585–1589. doi:10.1111/jth.12995

22. Holm E, Abshire TC, Bowen J, et al. Changes in bleeding patterns in von Willebrand disease after institution of long-term replacement therapy: results from the von Willebrand Disease Prophylaxis Network. *Blood Coagul Fibrinolysis*. 2015;26(4):383–388. doi:10.1097/MBC.0000000000000257

23. Sidonio RF, Haley KM, Fallaize D. Impact of diagnosis of von Willebrand disease on patient outcomes: analysis of medical insurance claims data. *Haemophilia*. 2017;23(5):743–749. doi:10.1111/hae.13292

Knowledge and Attitudes of Young Adults Towards Donation of COVID-19 Convalescent Plasma and Its Therapeutic Properties

Lidia Perenc & Ryszard Pęczkowski

ORIGINAL RESEARCH

Knowledge and Attitudes of Young Adults Towards Donation of COVID-19 Convalescent Plasma and Its Therapeutic Properties

Lidia Perenc [ID] [1]
Ryszard Pęczkowski [2]

[1]Institute of Health Sciences, University of Rzeszow, Rzeszow, 35-310, Poland; [2]Institute of Education, University of Rzeszow, Rzeszow, 35-959, Poland

Purpose: The aim of this study is to investigate the level of knowledge of the young adults towards the possible use of the convalescent plasma (CP) in the treatment of COVID-19 infection and their attitudes towards its donation.

Methods: Cross-sectional questionnaire was administered online to 1058 university students, representing 4 different colleges. The questionnaire included demographic data and 20 questions related to the knowledge and attitudes of respondents about possible use of the convalescent plasma in the treatment of COVID-19 infection and its donation. Comparative analyses were made using the Mann–Whitney and Kruskal–Wallis tests and the Spearman correlation coefficient.

Results: Significant relationships were found between dependent variables (level of knowledge and intensity of attitude) and gender, age, and student's college affiliation. There was no statistically significant correlation between dependent variables and respondents' social background and religious commitment. Studied young adults show a satisfactory knowledge relating to the therapeutic and preventive properties of the COVID-19 convalescent plasma. They also express a sufficient intensity of positive attitude towards CP donation.

Conclusion: This study confirms the need for appropriate health promotional campaigns and educational programs aimed at popularization of CP donation in the general public, which would increase the chances of involving more patients recovered from COVID-19 disease.

Keywords: COVID-19 convalescent plasma, knowledge, attitude, donation

Introduction

Currently, the world is going through a period of very serious crisis caused by the coronavirus pandemic (SARS-CoV-2), which has induced disturbances in almost all areas of life. The disease induced by this virus, popularly called COVID-19, causes acute inflammation of the respiratory system and is characterized by a high transmissibility and infection fatality rate (IFR) estimated at 0.68%.[1]

The very first observations showed that exposure to SARS-CoV-2 does not cause infection in all people belonging to a given population, and not all infected people suffer from acute respiratory system inflammation.[2] This variation in the course of infection and disease may depend on the individual's immune response. For these reasons, clinically, SARS-CoV-2 infection has been classified into two categories: mild and severe.[2] There are many reasons to believe that the progression

Correspondence: Lidia Perenc
Email la.perenc@gmail.com

709

of the disease depends on the genetic predisposition that makes the immune systems of different people react to infection in a specific way.[3]

In the face of the existing threats, the international scientific community is working intensively to develop an effective method of treating patients with COVID-19. In this context, high hopes are attached to passive and active immunotherapy strategies. As is known, specific active immunization can take place in a natural way, consisting in acquiring immunity after a specific infectious disease. It is also possible to generate specific immunity by artificial means, by administering a specified protective vaccination, according to the route and schedule of administration established by the manufacturer. The vaccine contains an antigen, specific to a particular microorganism, capable of producing an immune response and memory.[4] In this respect, an advantage has the passive immunotherapeutic strategy of administering "ready-made" antibodies that modify the inflammatory response, thanks to the properties achieved during the previous immune reaction.[5] An example of a passive immunotherapeutic strategy is convalescent plasma therapy, widely used in the treatment of patients suffering from SARS-CoV-2 infection. The observations to date show that it is regarded as an effective form of treatment used in many countries.[6,7] However, there are some risks associated with the administration of CP that have been categorized as known and theoretical risks. The known risks include reactions against the plasma constituents and unintended infections induced by several infectious agents that might be present in the serum. The theoretical risks include phenomena like antibody-dependent enhancement of infection, in which the severity of a viral disease is enhanced in the presence of specific antibodies.[8] For example, more recent studies show that risk factors for patients with COVID-19 are concomitant hematological malignancies (HM), especially acute leukemia (AL), which are associated with the need to stay in intensive care units (ICU) and even with increased mortality in case of complications.[9–11] Therefore, this group of patients should be of special concern during the ongoing COVID-19 pandemic. Nonetheless, it can be assumed that the number of patients treated with convalescent plasma (CP) will be much higher, which will increase the demand for donors in the longer term.[12] This assumption is supported by the current trend in the development of the discussed pandemic. Recent studies on the characteristics of CP donors have shown significant differences compared to whole blood (WB) and plasma/platelet apheresis (SA)

donors. The CP donors were more likely to be first-time and female donors than WB or SA donors. Also, CP donations had a higher rate of donor adverse reactions, deferrals, and product loss than SA donations.[13] The cited authors concluded that improved knowledge of the characteristics of CP donors and donations could be used to guide donor retention strategies and in-production planning. Also, the establishment of a comprehensive database, including clinical and laboratory data from COVID-19 plasma donors, may be helpful in planning subsequent steps including convalescent plasma collection and much safer experimental and therapeutic interventions.[14]

Within the general population of Poles, young adults constitute a large group of potential blood plasma donors. In Poland, there is a lack of research assessing the knowledge, attitudes and behavior of this group of people with regard to the therapeutic role of CP and their motivation to be a donor. Recently performed research has focused on the analysis of the effectiveness of convalescent plasma for COVID-19 patients only.[15] A closer look at young people's attitudes and the cognitive and motivational mechanisms underlying donor decisions can be helpful in inspiring these persons to donate blood plasma for therapeutic purposes. It seems that this is a sufficient argument to justify the purposefulness of research undertaken by the authors of this article.

Materials and Methods
Study Design
It was a cross-sectional study conducted in four colleges belonging to the University of Rzeszow during the period of January–February 2021. The aim of the study was to investigate the knowledge, opinions and attitudes of the students towards possible use of the convalescent plasma in the treatment of COVID-19 infection and their willingness to its donation. Permission from the relevant authorities of the university was obtained before actual data collection.

Participants
The study sample consisted of 1058 students from the University of Rzeszow. For the selection of respondents, a purposive sampling technique was used to select the students from the College of Humanities, College of Medical Sciences, College of Social Sciences, and College of Natural Sciences.

Ethical Consideration

This study was conducted in accordance with the Declaration of Helsinki. Ethical approval was granted by the Bioethics Commission of the University of Rzeszow (Resolution no. 01/02/2021). Participation was voluntary and anonymous (no personal identification was recorded), and confidentiality of the participants was ensured. They were informed that the data obtained were anonymous and will be utilized only for research purposes. Details about the study objectives were provided in the questionnaire instruction, and if the respondent returned the questionnaire, it indicated informed consent.

Instrument

The questionnaire used in the study contained 3 sections that contained dependent and independent variables. The first section contained sociodemographic characteristics. The second and third sections were related, respectively, to the measurement of the knowledge and attitudes of respondents about possible use of the convalescent plasma in the treatment of COVID-19 infection and its donation. Both the second and third sections contained 10 questions each. (An example of a question related to the knowledge section: 'Do you agree that, thanks to modern laboratory methods, the risk of transmission of blood-borne pathogens and the risk of serological conflict in practice do not exist in plasma transfusions?; An example of a question related to the attitude section: "Would you voluntarily donate blood plasma after recovery from COVID-19 if asked by an unknown person?"). The authors included the knowledge questions in the questionnaire based on the results of their previous research on knowledge and attitudes of young people towards organ donation and transplantation.[16] A scoring mechanism was used in order to assess the overall knowledge level and attitude of the participant regarding CP donation. A score of 1 was given for each correct response and a score of 0 for a wrong response. Respondents with all correct responses get a maximum of 10 points, both in sections two and three. Statistical analysis of the data was based on each participant's total scores. The questionnaire was electronically administered to all participants to all participants via the university portal. This was due to the fact that during the COVID-19 pandemic, all classes at the university were conducted online. Out of 4000 persons included in the study (1000 for each college), 1058 respondents returned the completed questionnaires, which gives a response rate of 26.4%.

Statistical Analysis

The analysis of quantitative variables was performed by calculating the mean, standard deviation, median and quartiles. The analysis of qualitative variables was performed by calculating the number and percentage of occurrences of each value. The comparison of the values of quantitative variables in two groups was performed using the Mann–Whitney test, and the values of quantitative variables in three or more groups using the Kruskal–Wallis test. Correlations between quantitative variables were analyzed using the Spearman correlation coefficient. A significance level of 0.05 was adopted in the analysis. Thus, all p values below 0.05 were interpreted as showing significant relationships. The analysis was performed in the R software, version 4.0.4.

Results

Table 1 shows sociodemographic characteristics of the study participants. Among the total of 1058 respondents, the largest group are students of the College of Social Sciences (N = 391), followed by College of Medical Sciences (N = 294), College of Natural Sciences (N = 213), and College of Humanities (N = 160). The average age of the respondents ranged from 21.76 (College of Natural Sciences) to 23.04 years (College of Humanities). In this respect, the difference between the compared groups of respondents was not statistically significant (p = 0.272). Larger differences between them occurred in terms of the gender of the respondents. The percentage of women in particular groups ranged from 61.50% (College of Natural Sciences) to 80.31% (College of Social Sciences), and men from 19.69% (College of Social Sciences) to 38.50% (College of Natural Sciences). In this case, the difference between the compared groups was statistically significant (p < 0.001). In the entire surveyed population, women accounted for 73.82%, while men 26.18% only. Among all respondents, 54.16% (N = 573) come from the rural social environment, and 45.84% (N = 485) from the urban. The rural social environment was most often represented in the group of students of College of Natural Sciences (65.73%), while it was the least frequent among students of College of Medical Sciences (45.58%). The largest number of students from the urban environment was in the College of Medical Sciences (54.42%), and the smallest number in the College of Natural Sciences (34.27%). In this respect, the difference between the compared groups turned out to be statistically significant (p <

Table 1 Sociodemographic Characteristics of the Studied Group

Parameter		College of Humanities (N = 160)	College of Medical Sciences (N = 294)	College of Natural Sciences (N = 213)	College of Social Sciences (N = 391)	p
Age [years]	x ± SD	23.04 ± 4.98	22.62 ± 4.72	21.76 ± 2.6	22.6 ± 5.17	p = 0.272
	Median	22	22	21	22	
	Quartiles	20–23	20–23	20–23	20–23	
Gender	Women	123 (76.88%)	213 (72.45%)	131 (61.50%)	314 (80.31%)	p < 0.001*
	Men	37 (23.12%)	81 (27.55%)	82 (38.50%)	77 (19.69%)	
Social background	Urban	74 (46.25%)	160 (54.42%)	73 (34.27%)	178 (45.52%)	p < 0.001*
	Rural	86 (53.75%)	134 (45.58%)	140 (65.73%)	213 (54.48%)	
Religious commitment	Strong	38 (23.75%)	51 (17.35%)	37 (17.37%)	83 (21.23%)	p = 0.083
	Moderate	67 (41.88%)	122 (41.50%)	106 (49.77%)	189 (48.34%)	
	Weak	31 (19.38%)	75 (25.51%)	35 (16.43%)	64 (16.37%)	
	Indifferent	24 (15.00%)	46 (15.65%)	35 (16.43%)	55 (14.07%)	

Notes: p – for quantitative variables Kruskal–Wallis test, for qualitative variables chi-square test or exact Fisher test; *Statistically significant difference (p <0.05).

0.001). However, in terms of religious commitment, there are no statistically significant differences between these groups (p = 0.083).

Table 2 shows the levels of knowledge and the intensity of attitude of all surveyed respondents toward donation of CP and its implementation in treating COVID-19 patients. The responses were summarized as a knowledge score or attitude score separately. A statistical analysis was done for gender, age, social background and religious commitment. Generally, the surveyed students showed an above-average level of knowledge on the various aspects of the use of CP in treating patients infected with the SARS-CoV-2 virus (x = 6.43). In this respect, women achieved significantly higher results compared to men (6.57 ± 1.76 vs 6.29 ± 1.84; p = 0.034). Women also presented more favorable attitudes towards CP donation for COVID-19 patients (7.34 ± 2.02 vs 6.70 ± 2.35; p < 0.001). The results show that the gender of the respondents is a significant factor influencing their knowledge and attitudes towards this important issue.

Also, the age of the respondents turned out to be a factor that statistically significantly differentiates their

knowledge and attitudes towards implementation of CP in treatment of COVID-19 patients and its donation. The age correlates significantly (p<0.05) and positively (r>0) both with the knowledge and attitude scores, so the older the age, the higher the knowledge and the more favorable the attitude of the respondents. This relationship turned out to be somewhat unexpected considering the fact that the age differences between the respondents are relatively small. There were no statistically significant differences between the respondents in terms of their social background and religious commitment. Although participants from the urban social environment achieved higher results compared to those from the rural environment, in terms of the level of knowledge (6.59 v. 6.41), the difference between them did not reach the required level of statistical significance (p = 0.280). Even smaller differences between the respondents occurred when comparing the results depending on their religious commitment (Table 2).

Table 3 presents the results obtained by students depending on their affiliation to a particular college. The comparison shows that the highest average results regarding knowledge about CP were achieved by students from the College of

Table 2 Association Between Sociodemographic Characteristics and an Overall Knowledge and Attitudes Results

Scores		Gender		p
		Women (N = 781)	Men (N = 277)	
Knowledge	x ± SD	6.57 ± 1.76	6.29 ± 1.84	p = 0.034*
	Median	7	7	
	Quartiles	6–8	5–8	Mann–Whitney test
Attitude	x ± SD	7.34 ± 2.02	6.70 ± 2.35	p < 0.001*
	Median	8	7	
	Quartiles	6–9	5–9	

Scores	Age
	Spearman Correlation Coefficient
Knowledge	r = 0.123, p < 0.001*
Attitude	r = 0.095, p = 0.002*

Scores		Social Background		p
		Urban (N = 485)	Rural (N = 573)	
Knowledge	x ± SD	6.59 ± 1.67	6.41 ± 1.88	p = 0.280
	Median	7	7	
	Quartiles	6–8	5–8	
Attitude	x ± SD	7.15 ± 2.13	7.19 ± 2.12	p = 0.779
	Median	8	8	Mann–Whitney test
	Quartiles	6–9	6–9	

Scores		Religious Commitment				p
		Strong (N = 209)	Moderate (N = 484)	Weak (N = 205)	Indifferent (N = 160)	
Knowledge	x ± SD	6.33 ± 1.84	6.46 ± 1.82	6.59 ± 1.64	6.68 ± 1.78	p = 0.349
	Median	7	7	7	7	
	Quartiles	6–8	5–8	6–8	6–8	
Attitude	x ± SD	7.17 ± 2.18	7.26 ± 1.96	7.19 ± 2.15	6.89 ± 2.49	p = 0.893
	Median	8	8	8	8	
	Quartiles	6–9	6–9	6–9	6–9	Kruskal–Wallis test

Note: *Statistically significant relationship.

Medical Sciences (x = 7.04). The difference turned out to be statistically significant at a high level (p < 0.001). Similar differences between medical students and others occurred in relation to the intensity of attitudes towards donation and the use of CP in the treatment of patients due to COVID-19. Medical students presented more favorable attitudes of this type than representatives of other colleges (x = 7.77). Also in this case, the difference turned out to be statistically significant (p < 0.001). The lowest level of the average results related to the knowledge was achieved by students of the College of Social Sciences (x = 6.20). Students representing the College of Natural Sciences and the College of Humanities obtained results at an average level, in both the knowledge and attitudes domains.

Table 3 Comparison of the Results Obtained by Students of Individual Colleges

Scores		College of Humanities – A (N = 160)	College of Medical Sciences – B (N = 294)	College of Natural Sciences – C (N = 213)	College of Social Sciences – D (N = 391)	p
Knowledge	x ± SD	6.32 ± 1.84	7.04 ± 1.47	6.41 ± 1.94	6.20 ± 1.81	p < 0.001 *
	Median	7	7	7	6	
	Quartile	5 –7	6–8	5–8	5–8	B > C, A, D
Attitude	x ± SD	6.72 ± 2.26	7.77 ± 1.9	7.05 ± 2.07	6.97 ± 2.18	p < 0.001 *
	Median	7	8	8	8	
	Quartile	5–9	7–9	6–9	6–9	B > C, D, A

Notes: p - Kruskal–Wallis test + post-hoc analysis (test Dunn test); *Statistically significant relationship.

Discussion

Since the outbreak of the COVID-19 pandemic, significant efforts have been made around the world to find an effective therapeutic and preventive measures that would stop the development of the infection and the resulting disease. One of them is the use of convalescent plasma collected from fully recovered patients with COVID-19 disease, which is a source of antibodies. Transfusion of plasma or its derived products containing immunoglobulin from patients who have fully recovered from COVID-19 will be a crucial intervention to be used for those who are not able to defend themselves against this pandemic virus, especially in the absence of the relevant vaccine faced in many countries. A systematic review of case reports, case series, observational studies and randomized control trials conducted by Bakhtawar et al between December 2019 and June 2020 showed that plasma therapy produces notable improvements in patients' clinical symptoms and radiological and biochemical parameters associated with COVID-19 infection. The authors emphasize that until we have concrete evidence to prove; otherwise, convalescent plasma therapy may be used as an adjuvant therapy for treating COVID-19 infection in critically ill patients.[17] Following the initial positive clinical experiences of the use of CP in COVID-19 patients, many European countries and the USA started to organize the collection of hyperimmune plasma to meet current needs and/or to store sufficient numbers of plasma units for future epidemic peaks.[18] Under these circumstances, much depends on the knowledge and attitudes of potential CP donors. In some countries, such as Italy, there have been dramatic situations due to the lack of CP donors, especially when national law does not provide any compensation for these services.[19] Based on this example, we understand why currently the collection process and transfusion of plasma derived from patients who have fully recovered from COVID-19 is of widespread interest, both nationally and internationally. Closer understanding of psychological factors, such as the motivation, knowledge and attitudes of potential CP donors, is critical to the success of their recruitment. This is confirmed by preliminary research on targeting and motivating the fully recovered COVID-19 patients to be donors.[20] The studies conducted so far indicate the existence of a limited number of potential CP donors in the general population, who differ in many respects from typical blood donors.[21] However, due to the lack of adequate research on CP donors, the results obtained in our study must be compared with the results of studies carried out on regular blood donors.[22]

This study was conducted in order to obtain information and inputs from young adults, students of various colleges, which will be useful in implementing relevant donor recruitment strategies because this population can contribute to health-promoting activities in the society. In practice, the hospitals and blood banks have two ways to achieve this goal: 1. to implement traditional policy for effective use of blood, 2. to increase blood donor recruitment by raising awareness of potential donors.[22] The latter task is related to raising their knowledge to a higher level and shaping attitudes favorable to blood donation.

The results of our study showed that the studied group of young adults has a relatively satisfactory level of knowledge about the treatment of COVID-19 disease with convalescent plasma. Significant differences in the

results occurred depending on the sex of the respondents. It turned out that women showed a higher level of knowledge about convalescent plasma and its therapeutic and preventive properties compared to men. Also, the age of the respondents turned out to be a factor that significantly determined the level of their knowledge in the discussed area. Older respondents generally presented a wider range of knowledge about convalescent plasma and its possible application in the treatment of patients suffering from COVID-19. This result seems somewhat unexpected, as the respondents only slightly differ in terms of their age of life. This can be explained by the fact that older students had more opportunities to learn about the therapeutic properties of blood plasma, including the COVID-19 convalescent plasma. However, it also turned out that factors, such as the social background of the respondents and their religiosity, did not differentiate between the level of their knowledge about the therapeutic properties of the COVID-19 convalescent plasma and the possibility of its donation. Another important factor differentiating the level of knowledge of the respondents turned out to be their affiliation to specific university colleges. As expected, the highest results were achieved by students of the College of Medical Sciences, with women in this group showing a higher level of knowledge than men. The lowest level of knowledge about convalescent plasma was found in a group of students from the College of Social Sciences. These findings are supported by the results of past studies suggesting that there is sufficient basic knowledge regarding the importance of blood donation among medical students.[23,24] Also, the results of recent studies among Polish medical students showed that they demonstrated a purposeful desire to get vaccinated against SARS-CoV-2, and COVID-19 vaccine conspiracy theories are less popular among them than in group of non-medical students.[25] For comparison, in another study on attitudes and behaviors towards SARS-CV-2 vaccination among Polish physicians and administrative healthcare assistants it turned out that the latter, in contrast to physicians, expressed their hesitancy to vaccinate. The authors rightly conclude that the fear of COVID-19 vaccine side effects and belief in conspiracy theories are a real threat to the public safety in achieving herd immunity.[26]

The analysis of the results showed a statistically significant relationship between the attitudes towards donation of the COVID-19 convalescent plasma and the gender of the respondents. It turned out that women exhibit more favorable attitudes of this type than men. This result stands somewhat in contrast to that of another study that revealed that the unwillingness to donate blood was more common among the female students and the major reasons were fear and perceived inconvenience, which were associated with blood donation.[27] Perhaps this difference is due to the fact that the cited studies were conducted in different socio-cultural conditions. In our study, the age of the respondents also modifies their attitude towards the donation of convalescent plasma, while the older respondents show greater motivation in this regard. On the other hand, there was no significant correlation between social background and religious commitment and the intensity of attitudes towards convalescent plasma donation. Nevertheless, there is reason to say that most of our students were willing to donate convalescent plasma, but they had not donated it because of the lack of an opportunity to do so. As compared to a previous study among college students, where there were a high number of respondents with a negative attitude towards blood donation,[28] in our study, we found an advantage of positive attitudes over negative ones. Our results are in line with many other studies showing that, compared to the general population, university students have a higher level of knowledge and a more positive attitude towards blood donation considering them as an important form of prevention and treatment of many diseases.[29–31] The course of the COVID-19 pandemic so far shows that it is expected to last a long time. Also so far, there has been a very low rate of donation from people who have been cured of COVID-19. In a study by Wang et al in a group of 533 convalescents, it turned out that only one person was willing to donate CP. This happened against the preliminary assumptions of the authors of the research, who expected convalescents to be more motivated to be a donor. However, the practice turned out to be different, as 1/3 of them were not interested in giving CP, many complained of post-comorbid symptoms, and the rest were not enthusiastic about it.[21] Such studies are very important as the resulting observations could help improve CP donation in current and future pandemics. In the future, efforts to find donors should be based both on providing them with appropriate knowledge and on shaping favorable attitudes. Other factors should also be taken into account because, as is known, blood donation rates are lower for some minority groups due to factors, such as fear, cultural, ethnic and religious beliefs and distrust of medical institutions.[32,33] It may be suspected that similar factors may also affect potential CP donors. In order to explain their role, the

research on the topic we have undertaken should be continued.

Like many others, this study also has some limitations. Firstly, because the study was cross-sectional, a temporal relationship between dependent and independent variables cannot be established. As is known, the true causal relationships among all the identified variables are complex and often reciprocal. Secondly, the surveyed respondents were only university students who may differ from the general population of young adults in Poland, and this would affect the generalizability of the results. Thirdly, the analyzed material does not contain data from students who did not agree to participate in the research, which may induce the possibility of a sampling bias.

Conclusions

Polish young adults, represented by university students, show a satisfactory level of knowledge relating to the therapeutic and preventive properties of the COVID-19 convalescent plasma. Factors such as gender and age of the respondents are significantly correlated with the level of their knowledge, while their social background and religious commitment do not differentiate them in this respect.

The surveyed university students generally show a sufficient intensity of positive attitude towards CP donation. Attitude scores positively correlated with such socio-demographic characteristics as gender and age of the respondents, with greater intensity of attitudes among women. On the other hand, there was no significant correlation between social background and religious commitment of the respondents and the intensity of attitudes towards CP donation.

The results achieved in this study confirm the need for appropriate health promotional campaigns and educational programs aimed at popularization of CP donation in the general public, which would increase the chances of involving more patients recovered from COVID-19 disease.

Future research should be more focused on understanding the psychological barriers to CP donation, especially in the group of young adults who have recovered from COVID-19 disease.

Acknowledgments

Author Contributions

Both authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval for the version to be published; and agreed to be accountable for all aspects of the work.

Funding

Disclosure

The authors declared no conflicts of interest for this work.

References

1. Meyerowitz-Katz G, Merone L. A systematic review and meta-analysis of published research data on COVID-19 infection fatality rates. *Int J Infect Dis*. 2020;101:138–148. doi:10.1016/j.ijid.2020.09.1464
2. Shi Y, Wang Y, Shao C, et al. COVID-19 infection: the perspectives on immune responses. *Cell Death Differ*. 2020;27(5):1451–1454. doi:10.1038/s41418-020-0530-3
3. ASM Communications. Individual genetic variation in immune system may affect severity of covid-19; 2020. Available from: https://asm.org/Press-Releases/2020/Individual-Genetic-Variation-in-Immune-System-May. Accessed July 28, 2021.
4. Clem AS. Fundamentals of vaccine immunology. *J Glob Infect Dis*. 2011;3(1):73–78. doi:10.4103/0974-777X.77299
5. Casadevall A, Pirofski L-A. Antibody-mediated regulation of cellular immunity and the inflammatory response. *Trends Immunol*. 2003;24(9):474–478. doi:10.1016/S1471-4906(03)00228-X
6. Cunningham AC, Goh HP, Koh D. Treatment of COVID-19: old tricks for new challenges. *Crit Care*. 2020;24(1):91. doi:10.1186/s13054-020-2818-6
7. Tortorici MA, Veesler D. Structural insights into coronavirus entry. *Adv Virus Res*. 2019;105:93–116.
8. Piyush R, Rajarshi K, Khan R, Ray S. Convalescent plasma therapy: a promising coronavirus disease 2019 treatment strategy. *Open Biol*. 2020;10(9):200174. doi:10.1098/rsob.200174
9. Lanza F, Agostini V, Monaco F, Passamonti F, Seghatchan J. Therapeutic use of convalescent plasma in COVID-19 infected patients with concomitant hematological disorders. *Clin Hematol Int*. 2021. doi:10.2991/chi.k.210403.001
10. Passamonti F, Cattaneo C, Arcaini L, et al. Clinical characteristics and risk factors associated with COVID-19 disease severity in patients with hematological malignancies in Italy: a multicentre, cohort study. *Lancet Haematol*. 2020;7:e737–e745. doi:10.1016/S2352-3026(20)30251-9
11. Brissot E, Labopin M, Baron F, et al. Management of patients with acute leukemia during the COVID-19 outbreak: practical guidelines from the acute leukemia working party of the European society for blood and marrow transplantation. *Bone Marrow Transplant*. 2021;56:532–535. doi:10.1038/s41409-020-0970-x
12. Wooding DJ, Bach H. Treatment of COVID-19 with convalescent plasma: lessons from past coronavirus outbreaks. *Clin Microbiol Infect*. 2020;26(10):1436–1446. doi:10.1016/j.cmi.2020.08.005

13. Lasky B, Goodhue Meyer E, Steele WR, Crowder LA, Young PP. COVID-19 convalescent plasma donor characteristics, product disposition, and comparison with standard apheresis donors. *Transfusion*. 2021;61(5):1471–1478. doi:10.1111/trf.16286

14. Lanza F, Seghatchain J. Reflection on passive immunotherapy in those who need most: some novel strategic arguments for obtaining safer therapeutic plasma or autologous antibodies from recovered COVID −19 infected patients. *Br J Haematol*. 2020;190:e1–e38. doi:10.1111/bjh.16814

15. Moniuszko-Malinowska A, Czupryna P, Zarębska-Michaluk D, et al. Convalescent plasma transfusion for the treatment of COVID-19—experience from Poland: a Multicenter Study. *J Clin Med*. 2021;10:28. doi:10.3390/jcm10010028

16. Perenc L, Radochonski M, Radochonska A. Knowledge and attitudes of Polish university students toward organ donation and transplantation. *Psychol Health Med*. 2012;17(6):667–673. doi:10.1080/13548506.2012.658818

17. Bakhtawar N, Usman M, Khan M. Convalescent plasma therapy and its effects on COVID-19 patient outcomes: a systematic review of current literature. *Cureus*. 2020;12(8):e9535.

18. Budhai A, Wu AA, Hall L, et al. How did we rapidly implement a convalescent plasma program? *Transfusion*. 2020;60(7):1348–1355. doi:10.1111/trf.15910

19. Del Fante C, Franchini M, Baldanti F, et al. A retrospective study assessing the characteristics of COVID-19 convalescent plasma donors and donations. *Transfusion*. 2020;61(3):830–838. doi:10.1111/trf.16208

20. Seghatchian J, Lanza F. Convalescent plasma, an apheresis research project targeting and motivating the fully recovered COVID 19 patients: a rousing message of clinical benefit to both donors and recipients Alice. *Transfus Apher Sci*. 2020;22:102792. doi:10.1016/j.transci.2020.102792

21. Wang HE, Ostrosky-Zeichner L, Katz J, et al. Screening donors for COVID-19 convalescent plasma. *Transfusion*. 2021;61(4):1047–1052. doi:10.1111/trf.16253

22. Allen J, Butler DD. Assessing the effects of donor knowledge and perceived risk on intentions to donate blood. *J Health Care Mark*. 1993;13:26–33. PMID: 10129813.

23. Juárez-Ocaña S, Pizaña-Venegas JL, Farfán-Canto JM, Espinosa-Acevedo FJ, Fajardo-Gutiérrez A. Factors that influence non-donation of blood in relatives of patients at a pediatric hospital. *Gac Med Mex*. 2001;137:315–322.

24. Sabu KM, Remya A, Binu VS, Vivek R. Knowledge, attitude and practice on blood donation among health science students in a University campus, South India. *Online J Health Allied Sci*. 2011;10:6.

25. Szmyd B, Bartoszek A, Karuga FF, Staniecka K, Błaszczyk M, Radek M. Medical students and SARS-CoV-2 vaccination: attitude and behaviors. *Vaccines*. 2021;9(2):128. doi:10.3390/vaccines9020128

26. Szmyd B, Karuga FF, Bartoszek A, et al. Attitude and behaviors towards SARS-CoV-2 vaccination among healthcare workers: a Cross-Sectional Study from Poland. *Vaccines*. 2021;9(3):218. doi:10.3390/vaccines9030218

27. Ahmed Z, Zafar M, Khan AA, Anjum MU, Siddique MA. Knowledge, attitude and practices about blood donation among undergraduate medical students in Karachi. *J Infect Dis Ther*. 2014;2:134. doi:10.4172/2332-0877.1000134

28. Wiwanitkit V. A study on attitude towards blood donation among people in a rural district, Thailand. *Southeast Asian J Trop Med Public Health*. 2000;31:609–611.

29. Hosain GM, Anisuzzaman M, Begum A. Knowledge and attitude towards voluntary blood donation among Dhaka University students in Bangladesh. *East Afr Med J*. 1999;74:549–553.

30. Mwaba K, Keikelame MJ. Blood donation behaviour and beliefs among a sample of high school students in Mmabatho. *Curationis*. 1995;18:2–3. doi:10.4102/curationis.v18i3.1356

31. Wiwanitkit V. Knowledge about blood donation among a sample of Thai university students. *Vox Sang*. 2002;83(2):97–99. doi:10.1046/j.1423-0410.2002.00209.x

32. Patel EU, Bloch EM, Grabowski MK, et al. Sociodemographic and behavioral characteristics associated with blood donation in the United States: a population-based study. *Transfusion*. 2019;59(9):2899–2907. doi:10.1111/trf.15415

33. Spratling R, Lawrence RH. Facilitators and barriers to minority blood donations: a systematic review. *Nurs Res*. 2019;68(3):218–226. doi:10.1097/NNR.0000000000000355

A Deep Dive into the Pathology of Gray Platelet Syndrome: New Insights on Immune Dysregulation

Ana C Glembotsky, Geraldine De Luca & Paula G Heller

Published online: 11 Aug 2021.

Submit your article to this journal

View related articles

View Crossmark data

REVIEW

A Deep Dive into the Pathology of Gray Platelet Syndrome: New Insights on Immune Dysregulation

Ana C Glembotsky[1,2]
Geraldine De Luca[1,2]
Paula G Heller [ID][1,2]

[1]Departamento Hematología Investigación, Instituto de Investigaciones Médicas "Dr. A. Lanari", Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina; [2]Departamento Hematología Investigación, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires, Instituto de Investigaciones Médicas (IDIM), Buenos Aires, Argentina

Abstract: The gray platelet syndrome (GPS) is a rare platelet disorder, characterized by impaired alpha-granule biogenesis in megakaryocytes and platelets due to NBEAL2 mutations. Typical clinical features include macrothrombocytopenia, bleeding and elevated vitamin B12 levels, while bone marrow fibrosis and splenomegaly may develop during disease progression. Recently, the involvement of other blood lineages has been highlighted, revealing the role of NBEAL2 outside the megakaryocyte-platelet axis. Low leukocyte counts, decreased neutrophil granulation and impaired neutrophil extracellular trap formation represent prominent findings in GPS patients, reflecting deranged innate immunity and associated with an increased susceptibility to infection. In addition, low numbers and impaired degranulation of NK cells have been demonstrated in animal models. Autoimmune diseases involving different organs and a spectrum of autoantibodies are present in a substantial proportion of GPS patients, expanding the syndromic spectrum of this disorder and pointing to dysregulation of the adaptive immune response. Low-grade inflammation, as evidenced by elevation of liver-derived acute-phase reactants, is another previously unrecognized feature of GPS which may contribute to disease manifestations. This review will focus on the mechanisms underlying the pathogenesis of blood cell abnormalities in human GPS patients and NBEAL2-null animal models, providing insight into the effects of NBEAL2 in hemostasis, inflammation and immunity.

Keywords: NBEAL2, gray platelet syndrome, α-granules, immune dysregulation, neutrophils

Correspondence: Paula G Heller
Departamento Hematología Investigación, Instituto de Investigaciones Médicas "Dr. A. Lanari", Facultad de Medicina, Universidad de Buenos Aires, Combatientes de Malvinas 3150, Buenos Aires, 1427, Argentina
Tel +54 11 5287 3872
Email paulaheller@hotmail.com

The gray platelet syndrome (GPS) (OMIM #139090) is a rare autosomal recessive disorder characterized by moderate macrothrombocytopenia, marked decrease or absence of platelet alpha (α)-granules, which gives platelets a grayish appearance on the blood smear, and mild to moderate bleeding.[1] Some patients have splenomegaly and develop bone marrow fibrosis during disease progression. First described in 1979,[2] the genetic abnormality leading to GPS remained unknown for a long time. Thirty years later, using linkage analysis, the underlying molecular defect was mapped to chromosome 3p21,[3,4] and shortly thereafter, mutations in *NBEAL2* were recognized as the cause of GPS by three independent groups by using different approaches.[5–7] NBEAL2 is involved in the biogenesis of megakaryocyte and platelet α-granules, although its role beyond hemostasis, particularly in immunity and inflammation, has been recently highlighted by Sims et al in a landmark international study.[8] This review will focus on the mechanisms underlying abnormalities in the megakaryocyte-platelet axis and the immune system in GPS patients and *Nbeal2*-null animal models.

Spectrum of *NBEAL2* Mutations

NBEAL2 is a large gene with 54 exons located on chromosome 3p21 encoding Neurobeachin-like 2, a scaffolding protein composed of 2754 amino acids.[9] NBEAL2 belongs to a family of proteins known as BEACH (beige and Chediak-Higashi syndrome)–domain-containing proteins (BDCP), containing armadillo (ARM)-like, Concanavalin A-like lectin, pleckstrin homology (PH), BEACH and WD40 domains. These domains represent highly conserved regions that are crucial for protein–protein interactions, membrane dynamics, vesicle trafficking, lysosome size regulation and synaptosome formation.[9] It is expressed in hematopoietic cells, including megakaryocytes, platelets, monocytes, neutrophils and NK cells.

Eighty-six different *NBEAL2* variants have been detected in 69 GPS pedigrees described to date, harbouring homozygous (65%) or compound heterozygous (35%) mutations.[5–8,10–19] Genetic defects include frameshift, nonsense, missense, splicing and small indel variants,[16] which are scattered along the gene. The spectrum of *NBEAL2* variants is depicted in Figure 1 and listed in Table 1. Most of them are private mutations, while some are found in more than one pedigree. Although no mutational hotspots have been recognized, missense variants are enriched within the BEACH domain, consistent with the essential role of this region in NBEAL2 function.[8] No genotype-phenotype correlation has been demonstrated, either regarding the type of mutation or its location.[8] In some pedigrees, three different variants have been identified and the pathogenic role of each one of them is not clear.[6,8,10,18] Current gene curation efforts for platelet disorders, such as those carried out recently for Glanzmann thrombasthenia,[20] will contribute to accurate interpretation of variants. Heterozygous *NBEAL2* variants may be found in unaffected carriers, who may display a mild decrease in α-granule content, although they are not thrombocytopenic.[10] Genetic diagnosis may be approached by Sanger sequencing of the *NBEAL2* gene, when the diagnosis of GPS is suspected based on the phenotypic features, or by high-throughput sequencing, including gene panels for inherited platelet disorders[19] or whole-exome sequencing.[17]

Alpha-Granule Deficiency in GPS Platelets and Megakaryocytes

Alpha-granule deficiency is a constant feature of GPS. Electron microscopy remains the gold standard for diagnosis. Platelets characteristically show absent or markedly reduced α-granules and frequently display prominent vacuolization of the cytoplasm, whereas the content of dense granules, lysosomes, mitochondria and peroxisomes is preserved.[21] The content of α-granules may also be quantified by immunofluorescence staining of blood smears for α-granule proteins, such as thrombospondin-1 (TSP1) and platelet factor 4 (PF4), as described.[10,22,23] The finding of a severe reduction in α-granules by this method correlates with the results of ultrastructural analysis.

Although GPS platelets lack typical α-granules, two types of abnormal α-granules may be recognized by immune electron microscopy, including normal-sized granules almost devoid of content with scant von Willebrand (vWF)-positive material and very small vestigious α-granule-like vesicular structures with intense vWF staining.[24] In contrast to the ARC (arthrogryposis, renal dysfunction, and cholestasis) syndrome, due to mutations in *VPS33B* or *VP16B*, in which platelets lack α-granules and their membranes, the granule-limiting membranes are present in GPS platelets, as well as membrane proteins, such as P-selectin and GMP-140.[25–28] These findings indicate that, whereas defects in VPS33B and VPS16B completely abrogate α-granule
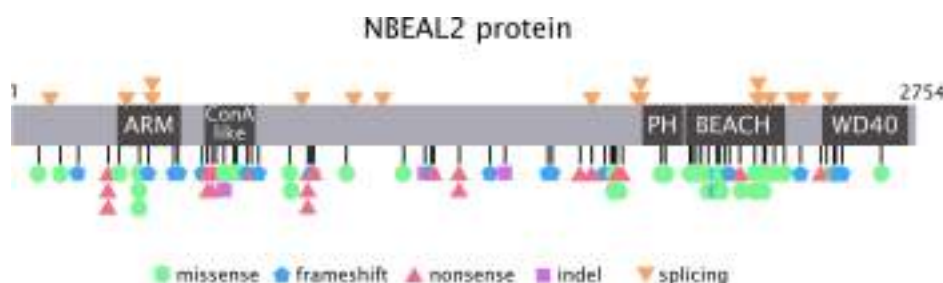
Figure 1 *NBEAL2* variants in GPS patients. Schema of the NBEAL2 protein comprising the ARM (armadillo)-like domain, spanning amino acids (aa) 377–504, the Con (concanavalin) A-like lectin domain (aa 580–730), the PH (pleckstrin homology) domain (aa 1915–2040), the BEACH (beige and Chediak-Higashi syndrome domain (aa 2053–2345) and WD40 repeat domains (2463–2722), according to Sims et al.[8] Published missense, nonsense, frameshift, indel and splicing germline *NBEAL2* variants described in GPS pedigrees (Table 1) are indicated by the symbols. Missense variants are enriched within the BEACH domain. The position of splicing variants is given according to the predicted effect at the protein level.

Table 1 Published *NBEAL2* Variants in GPS Patients

Pedigrees	Mutation state	Genotype	Predicted protein effect	Type of mutation	Ref.
1	homozygous	**c.2701C>T**	**p.R901***	nonsense	7,16
2	homozygous	**c.881C>G**	**p.S294***	nonsense	7,16
3	homozygous	**c.1163T>C**	**p.L388P**	missense	7,16
4	homozygous	c.5720+5G>A	NA	splicing	7,16
5	homozygous	c.5515C>T	p.R1839C	missense	7,16
6	homozygous	**c.1296+5G>C**	**NA**	splicing	7,16
7	homozygous	c.2257_2260delGCCC	p.A753Sfs*65	frameshift	7,16
8	homozygous	**c.1296+5G>C**	**NA**	splicing	7,16
9	homozygous	c.3819_4174del356	p.V1274Gfs*32	frameshift	7,16
10	homozygous	c.2029T>A	W677R	missense	7,16
11	homozygous	c.7604delG	p.G2535Vfs*5	frameshift	7,16
12	homozygous	c.5505T>G	p.Y1835*	nonsense	7,16
13	compund heterozygous	**c.2701C>T**	**p.R901***	nonsense	7,16
		c.6787C>T	p.H2263Y	missense	
14	compund heterozygous	c.2156delT	p.F719Sfs*100	frameshift	7,16
		c.5497G>A	**p.E1833K**	missense	
15	homozygous	c.5301+1G>A	NA	splicing	7,16
16	compund heterozygous	c.1029+1G>A	NA	splicing	5,16
		c.4371_4375dupCGTGG	p.E1459Afs*43	frameshift	
17	homozygous	**c.1820G>A**	**p.W607***	nonsense	5,16
18	compund heterozygous	c.5413dupG	p.A1805Gfs*59	frameshift	5,16
		c.1820G>A	**p.W607***	nonsense	
19	homozygous	**c.1163T>C**	**L388P**	missense	6,8,16
20	homozygous	c.2044A>T	I682F	missense	6,8,16
21	compund heterozygous	c.1928A>T	E643V	missense	6,8,16,18
		c.6299C>T	P2100L	missense	
22	compund heterozygous	c.1504_1507del	p.L502Afs*4	frameshift	10,16
		c.6801+7A>T	p.E2268Vfs*44	splicing	
23	compund heterozygous	c.5572C>T	p.R1858*	nonsense	8,10,16
		c.6652G>T	p.E2218*	nonsense	
		c.7033C>T	p.R2345W	missense	
24	homozygous	c.2187C>A	p.Y729*	nonsense	10,16
25	compund heterozygous	c.1253del	p.H418Lfs*54	frameshift	8,10,16
		c.3584G>A	p.R1195Q	missense	

(*Continued*)

Table 1 (Continued).

		c.5720+1G>A	p.M1908*	splicing	
26	homozygous	c.5299C>T	p.Q1767*	nonsense	11,16,18
27	homozygous	c.del4501-4503	p.L1501del	deletion	11,16
28	homozygous#	**c.881C>G**	**p.S294***	nonsense	11,16
		c.958C>T	p.R320W	missense	
29	homozygous	c.7440G>A	p.W2480*	nonsense	8,12,16
30	homozygous	**c.5721-1G>C**	**p.M1908***	splicing	8,12,16
31	homozygous	c.6212G>C	p.R2071P	missense	13,16
32	compound heterozygous	c.3839C>T	p.R1280*	nonsense	13,16
		c.6477C>G	p.H2159Q	missense	
33	homozygous	c.5176G>T	p.E1726*	nonsense	14,16
34	homozygous	c.7225-1G>C	NA	splicing	8,15
35	compund heterozygous	c.1870C>T	p.R624*	nonsense	16
		c.2735dupG	p.P913Sfs*3	frameshift	
36	homozygous	c.352-1_352delinsTA	NA	splicing	16
37	compound heterozygous	c.3384 + 5G>A	NA	splicing	17
		c.5965G>A	p.E1989K	missense	
38	homozygous	c.7387C>T	p.Q2463*	nonsense	8
	compound heterozygous$^\phi$	c.6657C>A	p.F2219L	missense	
39	compound heterozygous	**c.4081G>T**	**p.E1361***	nonsense	8
		c.1793G>A	p.W598*	nonsense	
40	compound heterozygous	c.3118+2T>G	NA	splicing	8
		c.6959G>C	p.R2320P	missense	
41	homozygous	**c.6432delT**	**p.F2144Lfs*23**	frameshift	8
42	homozygous	c.1725_1728dupACGT	p.A577Tfs*7	frameshift	8
43	homozygous	c.3773_3780delinsTCAGCGTTCGCCTCAGA	p.N1259_I1260delinsSVRLR	indel	8
44	homozygous	c.6460T>C	p.F2154L	missense	8
45	homozygous	c.4928_4929delAT	p.D1643Gfs*34	frameshift	8
46	homozygous	**c.4081G>T**	**p.E1361***	nonsense	8
47	homozygous	**c.881C>G**	**p.S294***	nonsense	8
48	homozygous	c.607dupA	p.I203Nfs*21	frameshift	8
49	compound heterozygous	**c.1163T>C**	**p.L388P**	missense	8
		c.6202T>C	p.W2068R	missense	
50	homozygous	c.7192_7202dupTCCTTCATCAC	p.Q2402Pfs*12	frameshift	8
51	homozygous	**c.6359G>A**	**p.R2120Q**	missense	8

(*Continued*)

722

Journal of Blood Medicine 2021:12

Table 1 (Continued).

52	homozygous	c.4890delG	p.R1631Gfs*3	frameshift	8,18
53	homozygous	c.7506delT	p.D2503Mfs*2	frameshift	8
54	compund heterozygous	c.6568delT	p.C2190Afs*23	frameshift	8,18
		c.7937T>C	p.L2646P	missense	
55	compound heterozygous	**c.2701C>T**	**p.R901***	nonsense	8
		c.2552C>T	p.P851L	missense	
56	homozygous	c.6515G>A	p.R2172H	missense	8
57	compund heterozygous	c.5935C>T	p.R1979W	missense	8
		c.6868G>T	p.G2290W	missense	
		c.6881A>G	p.E2294G	missense	
58	compound heterozygous	c.2650-1G>A	NA	splicing	8,18
		c.3058T>C	p.Y1020H	missense	
		c.7134G>A	NA	splicing	
59	compound heterozygous	c.6787C>T	p.H2263Y	missense	8
		c.6894T>A	p.N2298K	missense	
60	compound heterozygous	c.1789C>T	p.R597*	nonsense	8
		c.6359G>A	**p.R2120Q**	missense	
61	homozygous	c.6239T>A	p.M2080K	missense	8
62	homozygous	c.1476_1479dupAGGC	p.L494Rfs*59	frameshift	8
63	homozygous	c.2537T>C	p.L846P	missense	8
64	homozygous	c.7501C>T	p.H2501Y	missense	8
65	compound heterozygous	c.427G>A	p.E143K	missense	8
		c.2751dupT	p.D918*	nonsense	
66	compound heterozygous	c.256A>G	p.I86V	missense	6,8,18
		c.6805_6806insAGGGAGT	p.S2269*	splicing	
		c.6920-1G>C	NA	splicing	
67	compound heterozygous	c.4485-1G>T	NA	splicing	8,18
		c.1936_1938delTTC	p.F646del	deletion	
68	compound heterozygous	**c.6432delT**	**p.F2144Lfs*23**	frameshift	18
		c.5497G>A	**p.E1833K**	missense	
69	homozygous	c.5674C>T	p.Q1829*	nonsense	19

Notes: *NBEAL2* variants are named according to reference sequence NM_015175. Intronic variants located at splice junctions, predicted to affect splicing, are listed as splicing variants. Mutations detected in more than one pedigree are depicted in bold. All references (Ref.) where the variants are described are included. [#] both variants (p. S294* and p.R320W) were found in homozygosis in pedigree 28. [φ] in addition to the homozygous variant (p.Q2463*) found in the two affected individuals from pedigree 38, one of them harboured an additional heterozygous variant (p.F2219L).

formation, lack of NBEAL2 seems to interfere with α-granule maturation and cargo loading and retention, reflecting that NBEAL2 acts at a later stage of α-granule development.

As recently reviewed,[29] α-granules are formed by fusion of vesicles budding from the trans-Golgi network (loaded with endogenous cargo) or the cell membrane (early endosomes carrying exogenous cargo), which are directed to multivesicular bodies that represent α-granule precursors. Whereas α-granule proteins synthesized in the megakaryocyte, such as platelet factor 4 (PF4), vWF, β-thromboglobulin, thrombospondin, platelet-derived growth factor (PDGF) and fibronectin, are markedly reduced in GPS platelets, those routed to α-granules following endocytosis of plasma proteins, such as albumin, IgG and fibrinogen, are less affected,[26] indicating that NBEAL2 loss of function preferentially affects the delivery of endogenously synthesized cargo to α-granules. Interestingly, analysis of the platelet proteome revealed that, in addition to downregulation of α-granule content, unexpectedly, proteins normally resident in neutrophil granules, including myeloperoxidase and elastase, were overrepresented.[8] Although there is no clear explanation for this finding, the transfer of neutrophil proteins during emperipolesis, which occurs in a high proportion of GPS megakaryocytes, may represent a plausible mechanism.[8]

Similar to platelet findings, ultrastructural analysis of bone marrow or cultured GPS megakaryocytes disclosed the absence of typical α-granules, although very small granules, consistent with immature α-granule precursors, were evident.[24,28] In vitro differentiation of GPS primary megakaryocytes further revealed that vWF is abundantly synthesized but fails to be correctly packaged into granules and is misdirected or discharged into the lumen of the demarcation membrane system. On the contrary, P-selectin was immunodetected in multivesicular bodies in the maturing megakaryocyte and in the membrane of vacuolar structures deprived of soluble content resembling empty α-granules.[25] Altogether, these findings support the notion that NBEAL2 deficiency leads to a defect in the transfer of protein cargo into the lumen of developing α-granules or in the retention of granule content, rather than a primary impairment in α-granule biosynthesis.

Nbeal2 knockout mouse models have been generated by three different groups and all reveal a platelet phenotype which resembles human GPS.[30–32] Ultrastructural analysis of megakaryocytes disclosed greatly reduced numbers of α-granules in two of these studies.[30,32] Intracellular tracking via fluorescence microscopy showed that initial trafficking of endocytosed proteins was preserved, but they were subsequently delivered to recycling endosomes and released, and the same fate was demonstrated for endogenous proteins, reinforcing that, as shown in humans, the main function NBEAL2 in murine cells relies on the retention of molecular cargo, possibly through its interaction with P-selectin.[33] In contrast to the absence of megakaryocyte α-granules found in the two former mouse strains, Guerrero et al showed that mature α-granules were present in megakaryocytes in their *Nbeal2*-/- murine model, as shown by electron microscopy, immunostaining for vWF and Western blot analysis of α-granule proteins.[31] Furthermore, normal numbers of granules were also evident in proplatelet buds, indicating preserved granule transport along the proplatelet shaft, further suggesting that α-granules are generated, but not retained within megakaryocytes.[31]

Role of NBEAL2 in α-Granule Biogenesis

The intrinsic mechanisms and interacting proteins underlying the role of NBEAL2 in α-granule biogenesis are beginning to be revealed.[29] NBEAL2 interactome of human embryonic kidney cells, engineered to express a subdomain of NBEAL2 containing the BEACH domain and its flanking PH and WH40 regions, disclosed several interactors, including the endoplasmic reticulum-associated protein SEC16a, the phosphatidyl inositol 3,5-biphosphate regulator VAC14, and the guanidine exchange factor DOCK7.[34] Considering that SEC16a participates in the transport from the endoplasmic reticulum to the Golgi and VAC14 activates the PIKfyve complex involved in the intracellular vesicle transport, the defect in both molecules could plausibly alter endosomal trafficking and cargo sorting during α-granule formation. DOCK7 is a guanine nucleotide exchange factor for small GTPases, Rac1 and Cdc42, which are major regulators of the actin cytoskeleton. Interestingly, DOCK7-deficient mice present with a bleeding phenotype.[27,35] DOCK7 expression is decreased in GPS patients and mouse models, and *Nbeal2*-null mouse platelets show decreased actin polymerization and spreading on fibrinogen, indicating that NBEAL2 is important for cytoskeleton reorganization in platelets, most probably by modulating DOCK7.[34] In addition, dysregulation of the DOCK7 signaling pathway in megakaryocytes could be a possible cause of
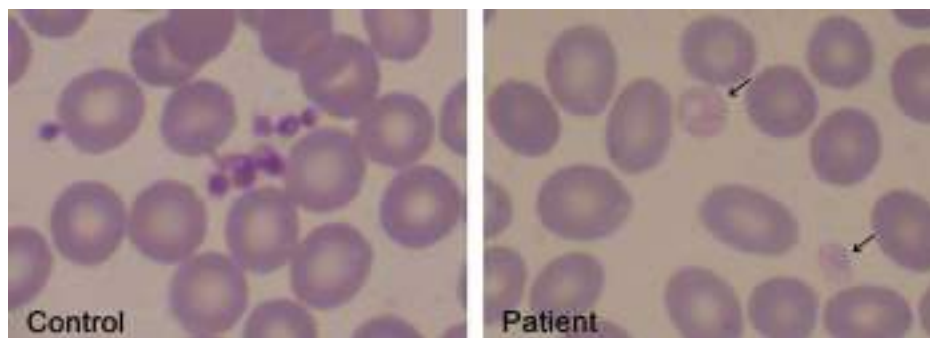
Figure 2 Pale platelets on the blood smear from a GPS patient. Large pale platelets due to absence or marked decrease in α-granules is a typical finding in GPS patients. A May-Grünwald Giemsa-stained blood smear from a GPS patient is shown on the right panel. Gray platelets are indicated by the arrows. A blood smear from a healthy subject stained simultaneously is shown on the left panel. Images were obtained at 1000x magnification.

aberrant platelet formation in GPS.[34] These findings point out that NBEAL2 function may not be entirely dependent on its ability to regulate granule biogenesis, but that other cellular processes may be affected by NBEAL2 deficiency. Another NBEAL2 interacting protein was revealed in a recent study performed on human megakaryocytes and immortalized megakaryocyte progenitors, which demonstrated that NBEAL2 binds SEC22B, an endoplasmic reticulum-resident trafficking protein.[36] Knockout of SEC22B in these cells resulted in the failure of α-granule synthesis, suggesting that interaction with SEC22B may facilitate the role of NBEAL2 in α-granule generation.[36] SEC22B binding was mapped to a region located in an N-terminal position relative to the PH-BEACH domains and was abrogated by two GPS missense variants located within this region.[36] Future research may provide further insight into the process of α-granule biogenesis and the exact place of NBEAL2 in this complex network.

Platelet Features

Thrombocytopenia is a universal feature of GPS, being of moderate degree in most patients. In a recent study performed by Sims et al, which included the largest GPS cohort gathered to date, comprising 47 patients belonging to 38 pedigrees recruited worldwide, median platelet count was 57 (28–105) x 10^9/L.[8] Notably, hemoglobin, total leukocyte counts, as well as neutrophils, eosinophils, basophils, monocytes and lymphocyte counts, were all shown to be lower in this GPS cohort compared to more than 45,000 healthy controls recruited to the INTERVAL trial,[8] indicating that abnormal blood parameters are not limited to platelets, as previously acknowledged, but involve multiple hematopoietic lineages. Thrombocytopenia may worsen with increasing age,[3] reflecting the progressive nature of this disorder. The sequential

drop in platelet counts may be due to the development of bone marrow fibrosis or, alternatively to splenomegaly and splenic sequestration. The finding of an inverse relationship between platelet counts and bone marrow fibrosis but not with splenomegaly supports the former possibility.[8] Consistent with this notion, splenectomy may lead to mild increase but not complete restoration of platelet counts.

The finding of pale large and giant platelets in the peripheral blood smear is a hallmark feature of GPS, as shown in Figure 2, and often the clue to diagnosis, although other platelet disorders associated with α-granule deficiency, such as those associated with mutations in transcription factors GATA-1 or GFI1B or the ARC syndrome, which is caused by *VPS33B* or *VPS16B* mutations, must also be considered.[37] Mean platelet volume is usually increased, although this parameter may be underestimated or may not be informed by electronic cell counters, as described for other large platelet disorders. In such cases, measurement of the platelet diameter by image analysis of blood smears may provide useful information, showing increased mean values and/or increased proportion of large platelets in GPS patients.[23,38] The bleeding diathesis is usually mild to moderate but may range from absent to severe intracranial hemorrhage.[1,16]

Significantly impaired response to different agonists such as thrombin and collagen, variable response to ADP and ristocetin and normal response to arachidonic acid were demonstrated in platelet aggregation studies,[1,14,39] although aggregation responses may be variable among different patients.[1] The expression of platelet glycoproteins was normal in most of the patients described,[39] although higher fluorescence intensity for GPIb and GPIIbIIIa due to the large size of the platelets may also be found,[14] whereas loss of GPVI was described in one patient.[40] Interestingly, impaired platelet response to

thrombin, protease-activated receptor 1 (PAR1)- and PAR4-AP (activating peptide) was a consistent finding in several studies[1,22,39] and seems to be a common defect in GPS patients, which may be paralleled by a reduction in PAR1 and PAR4 expression in megakaryocytes and platelets.[22,39] Although the total P-selectin pool may be normal or mildly reduced in GPS platelets,[1] P-selectin was found to be constitutively expressed on the platelet surface in one study,[18] whereas upregulation of P-selectin exposure in response to agonists, was markedly impaired, both in this work[18] and in a separate study.[39]

Mice lacking NBEAL2 display platelet functional abnormalities,[30–32] delayed arterial thrombus formation[30] and protection from thrombo-inflammatory brain injury following focal cerebral ischemia.[32] In addition, impaired dermal healing due to reduced transforming growth factor (TGF)-β release from mutant platelets[32] and protection against cancer metastasis[31] were other remarkable findings, pointing to the relevance of platelet α-granule content beyond hemostasis, specifically in wound healing, tumorigenesis and metastatic spread.

Megakaryocyte Abnormalities

The pathogenesis of low platelet counts in GPS has been attributed to impaired platelet production, as platelet survival is largely normal or only mildly decreased. To address the underlying mechanisms, megakaryocytes were cultured from peripheral blood and/or bone marrow hematopoietic progenitors from four GPS patients.[41] Both megakaryocyte output and maturation were normal but proplatelet formation was markedly impaired and proplatelets showed aberrant architecture with decreased branching leading to lower number of tips, indicating that thrombocytopenia is due to defective thrombopoiesis and platelet release. Interestingly, proplatelet tips were larger than control, which is consistent with the finding of large platelets in circulation. In addition, abnormal megakaryocyte interaction with extracellular matrix also shown in this work may also lead to reduced platelet production.[41] As mentioned before, bone marrow fibrosis, and, to a lesser extent, splenic sequestration may also contribute to low platelet counts. The study of megakaryocyte biology in *Nbeal2* knockout mouse models has revealed discrepant findings. Kahr et al showed altered development, polyploidization and proplatelet formation,[30] whereas Depperman et al and Guerrero et al showed normal megakaryocyte development and proplatelet formation.[31,32] Different megakaryocyte source (bone marrow vs fetal

liver) or culture techniques might account for the differences found between these animal models.

Extensive emperipolesis is a striking feature of GPS megakaryocytes and represents a prominent finding both in GPS patients and animal models.[8,30–32,39] Engulfed neutrophils are frequently found inside megakaryocytes in bone marrow biopsies. Strikingly, in vitro-differentiated megakaryocyte also showed signs of emperipolesis, indicating that this is an intrinsic cellular defect and not a consequence of altered bone marrow milieu.[41] It has been proposed that mislocalization of P-selectin at the megakaryocyte surface in GPS patients fosters interaction with neutrophils, which express the PSGL counterligand, promoting emperipolesis.[39] In addition, *Nbeal2*-/- mouse megakaryocytes show a proinflammatory gene expression profile, reflected by upregulation of several chemokines with a role in leukocyte chemotaxis. Paracrine secretion of these mediators by inflammatory megakaryocytes may attract neighbouring leukocytes favouring emperipolesis.[31]

GPS patients may develop bone marrow fibrosis during follow-up. In a recent study, myelofibrosis was found in 58% of patients at a median age of 28.5 years (range, 10–52 years).[8] Although it may remain stable for several years, in some patients increased fiber deposition may occur during follow-up, but it seldom requires treatment. Leakage of α-granule-derived fibroblast growth factors, such as PDGF and TGFβ, to the bone marrow milieu represents a likely explanation. In addition, proinflammatory megakaryocytes may promote bone marrow fibrosis by secreting cytokines and chemokines to the surrounding environment,[31] highlighting the importance of inflammation in the pathogenesis of myelofibrosis, as shown for myeloproliferative neoplasms.[42] Splenomegaly may occur in patients with bone marrow fibrosis, although it may also be found in patients without fibrosis.[8]

Innate Immune Cell Dysfunction

Although GPS has long been considered to involve exclusively the megakaryocyte-platelet lineage, more recent evidence has revealed that the innate and adaptive immune system may also be affected by NBEAL2 loss of function, as detailed in Table 2 and depicted in Figure 3, thus broadening the phenotypic spectrum of GPS abnormalities.[8,25]

The presence of granule defects in neutrophils, in addition to platelets, has been suggested several years ago by the group of Elisabeth Cramer,[25] who described four patients form two different pedigrees displaying

Table 2 Abnormalities in Innate and Acquired Immunity in GPS Patients and Animal Models

	Human GPS	*Nbeal2*-null mice
Neutrophil granule content		
All granules	Normal[3,44] (EM)	Reduced[47] (EM)
Azurophilic granules	Normal[25,43] (IEM)	Reduced[48] (EM)
Specific granules	Reduced[8,25,43] (IEM)	Reduced[48] (EM)
Gelatinase granules	Reduced[8] (MS)	Reduced[48] (EM)
Secretory vesicles	Reduced[25] (Cyto)	Reduced[48] (EM)
Surface expression of granule proteins		
CD11b (SG and SV)	Increased[25,43]	Increased[48]
CD66b (SG)	Increased[18]	NE
CD35 (SV)	Increased[25,43]	NE
Release of granule content		
Elastase (AG)	Normal[18]	Decreased[47]
Lactoferrin (SG)	Decreased[18]	NE
Proteomic analysis		
SG and GG content in neutrophils	Downregulated[8,18]	Downregulated*[47]
Neutrophil proteins in plasma	Upregulated[8]	Upregulated**[48]
Neutrophil proteins in platelets	Upregulated[18]	NE
Neutrophil functions		
Reactive oxygen species production	Normal[18,25]	Enhanced[47], Normal[48]
Bacterial killing or phagocytosis	Normal[18]	Normal[48]
Migration	Normal[25]	Enhanced[48]
NETosis	Impaired[18]	Normal[48]
Suceptibility to infection	Increased[8]	Increased[47,48]
NK cells		
NK numbers	NE	Reduced[47]
NK degranulation	NE	Impaired[47]
Autoimmunity		
Autoantibodies	Increased[8]	NE
Autoimmune disease	Increased[8]	NE
Immune response markers in CD4+ cells	Elevated[8]	NE
Inflammation		
Acute-phase reactants	Elevated[8]	NE

Notes: Techniques used to assess neutrophil granule content are detailed in parenthesis. *AG content was also shown to be downregulated. **as determined by ELISA.
Abbreviations: EM, electron microscopy; IEM, immune electron microscopy; MS, mass spectrometry; Cyto, cytochemistry; SG, specific granules; SV, secretory vesicles; AG, azurophilic granules; NE, not evaluated.

degranulated neutrophils with a grayish appearance on the blood smear. Reduced amounts of specific granules and secretory vesicles were shown by immune electron microscopy, whereas azurophilic granules, which contain neutrophil proteases such as elastase and myeloperoxidase, were preserved. Increased expression of granule membrane proteins, such as CD11b (specific granules) and CD35 (secretory vesicles) was shown at the cell surface of resting neutrophils, suggesting degranulation. However, despite these structural defects, no functional abnormalities were evident, as demonstrated by normal production of reactive oxygen species and neutrophil migration in response to stimuli. The same group then confirmed the presence of the same neutrophil abnormalities in a patient
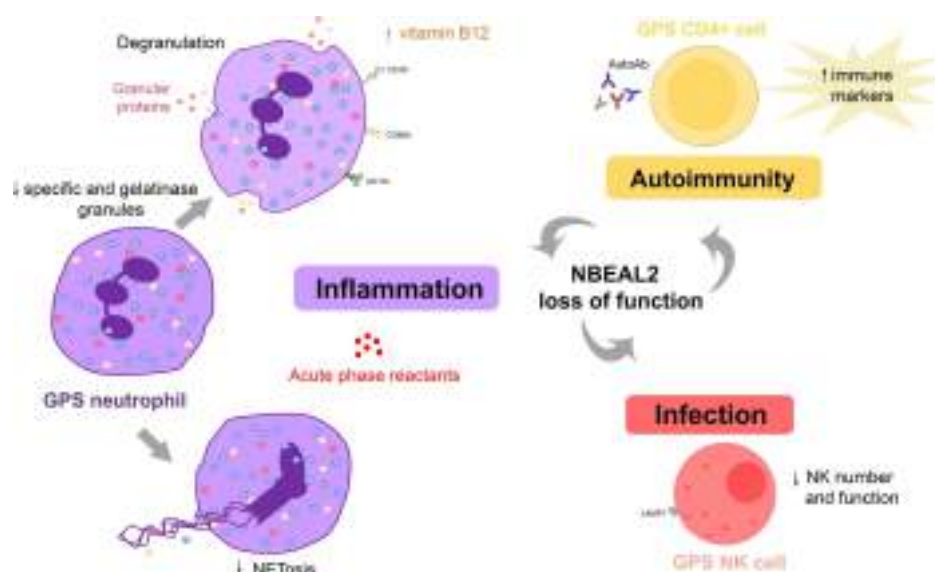
Figure 3 Abnormalities in the innate and adaptive immune response in GPS. GPS neutrophils display reduced numbers of specific (red circles) and gelatinase (yellow circles) granules. Whereas azurophilic (blue circles) granules are preserved in GPS patients, they are decreased in GPS animal models. Proteins resident in specific and gelatinase granules are expressed at the cell membrane, such as CD11b, CD66b and CD35, and elevated in plasma, indicating degranulation. Neutrophil extracellular trap formation (NETosis) is impaired. NK cell number and function are decreased in GPS animal models, although it is unknown whether it is also altered in patients. Altogether, these abnormalities may contribute to higher susceptibility to infections. Autoimmune manifestations and/or autoantibodies (AutoAb) are present in around half of GPS patients, coupled to upregulation of immune response markers in CD4+ cells. Liver-derived acute phase reactants are elevated in patient circulation, reflecting ongoing inflammation.

from an unrelated pedigree.[43] In contrast to these findings, no defect in the overall number of neutrophil granules was shown by electron microscopy in GPS patients in two other studies.[3,44]

In the study by Sims et al, neutrophil granularity, as assessed by side scatter properties, was reduced and proteomic analysis revealed selective downregulation of proteins contained in specific granules,[8] reinforcing previous findings from the French group,[25] and disclosed a concomitant decrease in the content of gelatinase granule constituents.[8] Reduced protein abundance was not due to decreased gene expression, as molecules underrepresented in the GPS proteome were expressed normally at the transcript level. The reduction in neutrophil content of specific granules was paralleled by increased levels of specific granule cargo, such as cathelicidin antimicrobial peptide, cysteine-rich secretory protein 3, and gelatinase-associated lipocalin, in circulation, as disclosed by mass spectrometry of patient plasma.[8] Interestingly, inflammatory proteins synthesized in the liver, such as C reactive protein and lipopolysaccharide-binding protein, were also found to be upregulated in the plasma proteome, reflecting a systemic proinflammatory state. Raised serum vitamin B12 levels are an almost universal finding in GPS patients,[3,8] although the mechanisms leading to B12

elevation remain undefined. Transcobalamin I, a B12 binding protein, is a major constituent of neutrophil specific granules. It might be hypothesized that its leakage to circulation could contribute to total B12 elevation, as reported for myeloproliferative neoplasms.[45] However, transcobalamins were not listed among the proteins upregulated in the GPS plasma proteome[8] and the mechanism underlying raised B12 levels remains to be determined. In addition to neutrophil abnormalities, this study also revealed decreased side scatter properties in eosinophils and reduced content of proteins which localize to granule structures in the proteome of GPS monocytes,[8] hinting to a more widespread role of NBEAL2 in granule biology.

A comprehensive study of neutrophil structure and function recently performed in 13 GPS patients further confirmed the reduction in specific and gelatinase granular compartments, as shown by immune electron microscopy and proteomic analysis, while azurophilic granules were preserved.[18] As previously shown for CD11b,[25] surface expression of CD66b, an integral membrane component of specific granules, was elevated at baseline, further pointing to degranulation and did not increase significantly upon activation. Accordingly, the release of the content of specific granules, such as lactoferrin, triggered by specific stimuli, was significantly impaired, while that

of azurophilic granules was unaffected. Intriguingly, in contrast to GPS circulating neutrophils, neutrophils derived from in vitro differentiation of patient hematopoietic progenitors displayed normal abundance of specific granules, suggesting intact granule biosynthesis during granulopoiesis but lack of retention or premature exocytosis of specific granules during egress from the bone marrow or in the bloodstream. Functional studies of blood neutrophils showed preserved production of reactive oxygen species, extracellular release of neutrophil proteases, such as elastase, and killing of gram-positive and -negative bacteria and fungi. Interestingly, however, neutrophil extracellular trap (NET) formation was blunted or substantially reduced in response to classic NET inducers, such as PMA, urate crystals and Candida albicans, which engage different NET-triggering pathways.[18] Considering that upstream steps of NET formation, such as ROS production and myeloperoxidase and elastase reactivity were not altered in GPS neutrophils, this finding suggests that, in addition to azurophilic granules, specific granules may play a previously unrecognized role in NET formation. This granule subset may be particularly important in the late phases of NETosis, as reflected by defective membrane rupture and chromatin web release, whereas initial steps such as chromatin decondensation and cell rounding proceeded normally.[18] It remains to be explored whether a particular specific granule component is responsible for this function. Increased susceptibility to infections, particularly mild upper respiratory infections and otitis media, has been previously pointed out in isolated case reports,[3,11,25,46] and was recently reported to affect 17% of GPS patients in the large international cohort mentioned above.[8] However, severe or life-threatening infections do not appear to occur frequently in GPS patients.

Immune function and pathogen defense were also assessed in *Nbeal2* knockout mice, by two different groups. By analyzing public repositories of gene expression data, Sowerby et al disclosed strong NBEAL2 expression in cells of the immune system, reaching the highest levels in neutrophils and NK cells, both of which rely heavily on their granule content to fulfill their role in innate immunity.[47] Neutrophil content of primary, specific and gelatinase granules was found to be markedly reduced in *Nbeal2* deficient mice. Proteomic analysis of *Nbeal2*-/- neutrophils revealed significant enrichment of granule- and vesicle-associated components among downregulated proteins

and, accordingly, release of granular proteins, such as elastase, was severely dampened. Although *Nbeal2*-/- neutrophils showed preserved phagocytosis and enhanced respiratory burst when challenged with gram-positive bacteria (*Staphylococcus aureus*), mice developed uncontrolled infection and had a worse outcome. In addition to neutrophil phenotypic defects, low numbers and functional abnormalities were shown for NK cells in this animal model, mainly consisting in altered degranulation, as shown by attenuated upregulation of lysosomal protein LAMP-1 triggered by specific stimuli. Considering that perforin and granzyme released from NK lytic granules are essential for NK cell-mediated cytotoxicity, the defect in NK cell degranulation could contribute to defective immunity found in these mice.[47] As shown for bacterial disease, increased susceptibility to viral (CMV) infection was demonstrated for *Nbeal2*-deficient mice, consistent with the important role of NK cells in antiviral immunity. In line with this study, Claushuis et al corroborated the decrease in all neutrophil granule subtypes in *Nbeal2* knockout mice and extended these findings by demonstrating increased levels of neutrophil granular proteins, such as MPO and elastase, in circulation.[48] Reactive oxygen species production and phagocytic capacity by *Nbeal2*−/− neutrophils were normal in this study, as well as bacterial (*K. pneumoniae*) growth, although organ damage during infection and endotoxemia was enhanced. Another study showed that the content of storage vesicles of mast cells was reduced in *Nbeal2*-/- mice,[49] suggesting the involvement of yet another cell type in the repertoire of GPS abnormalities.

Altogether, these findings clearly demonstrate that NBEAL2 deficiency leads to neutrophil abnormalities in human GPS and animal models. However, certain differences are evident between both models, highlighting that granular biosynthesis or trafficking may differ between human and mice neutrophils. The intrinsic mechanisms underlying the effect of NBEAL2 in granule formation in leukocytes and whether this process shares the same pathways and interacting proteins operating in platelets are currently unexplored issues. Increased susceptibility to infection and worse outcome has been described in *Nbeal2*-/- mice, whereas, although GPS patients do not usually suffer severe infections, an increase in mild, particularly, respiratory infections, has been reported. Defective NK cell function in *Nbeal2* knockout mice adds further complexity to the defect in innate immunity.

It would be interesting to study whether the NK compartment is also altered in human GPS.

Autoimmune Manifestations

In addition to deranged innate immunity, there is evidence of a deregulated adaptive immune response in GPS patients. The association between GPS and autoimmune disease was first suggested by the finding of *NBEAL2* variants in patients from two different pedigrees presenting with an autoimmune lymphoproliferative (ALPS)-like syndrome who concomitantly showed GPS typical platelet abnormalities.[11] Recently, autoimmune features were frequently found in GPS patients recruited by Sims et al, including autoimmune disease (26%) and the presence of at least one autoantibody (59%).[8] Autoimmune manifestations involved several systems, including Hashimoto's thyroiditis, rheumatoid arthritis, skin diseases, such as alopecia, discoid lupus erythematosus and vitiligo, and atypical autoimmune lymphoproliferative syndrome. Autoantibodies were directed against different target proteins, with rheumatoid factor, perinuclear anti-neutrophil cytoplasmic, anti-thyroperoxidase and antinuclear antibodies among the most frequently detected. Markers of immune response were upregulated in GPS CD4+ T helper cells at the protein and transcript level, as shown by the analysis of the proteomic and RNA-seq profile.[8] Overrepresented molecules were enriched in proteins with immunomodulatory function, such as Bruton tyrosine kinase, which is crucial for B cell development and has been suggested to be involved in autoimmunity.[50] On the basis of these novel findings, this study highlights the presence of immune dysregulation in GPS patients. A more detailed phenotypic and functional characterization of B and T cells and their subsets in GPS patients would be useful to gain further insight into the mechanisms underlying autoimmunity and help explain the aberrant immune pattern.

As mentioned before, a proinflammatory signature in GPS plasma proteome, as shown by upregulation of liver-derived inflammatory markers,[8] was another prominent feature of this work. This finding might reflect chronic activation of liver resident cells by myeloid-derived products leaking to the bloodstream or, alternatively, low-grade chronic infection. The potential relationship between chronic immune stimulation and the development of autoimmunity may deserve consideration. Conversely, sustained autoimmunity may, in turn, trigger further

inflammation, contributing to the ongoing proinflammatory state (Figure 3).

Treatment

Most GPS patients are treated symptomatically, according to the severity of bleeding manifestations. Patients may bleed spontaneously or secondary to surgical intervention, childbirth or trauma. Non-specific hemostatic measures, such as anti-fibrinolytics, local hemostatic measures, avoidance of non-steroidal anti-inflammatory drugs and/or platelet transfusions are usually adopted to prevent or reduce excessive bleeding. Eltrombopag has been shown to increase platelet counts in certain inherited platelet conditions, such as *MYH9*-related disorder, *ANKRD26*-related thrombocytopenia, X-linked thrombocytopenia/ Wiskott-Aldrich syndrome, monoallelic Bernard-Soulier syndrome or *ITGB3*-related thrombocytopenia.[51] The efficacy and safety of thrombopoietin receptor agonists in GPS remains unknown. Considering that these agents may promote bone marrow fibrosis, their use in GPS should be viewed with caution, especially if long-term treatment is considered. Successful hematopoietic stem cell transplantation has been achieved in a GPS patient with severe myelofibrosis and pancytopenia, with full hematopoietic recovery and resolution of bone marrow fibrosis.[52] On this basis, stem cell transplantation may represent a curative treatment option for selected patients who experience a severe clinical course.

In conclusion, recent evidence has expanded the phenotypic spectrum of GPS, providing new information which is relevant to patient care. The combined study of GPS patient samples and animal models has provided novel insight into the pathogenesis of this rare disorder. Future research will be required to further elucidate the effects of NBEAL2 in granule biogenesis and trafficking and to define its precise function in the biology of blood cells, including platelets and leukocytes, thus contributing to dissect its role in hemostasis and immunity. Increasing knowledge of the mechanisms underlying GPS will hopefully provide tools for developing appropriate therapies.

Disclosure

The authors reported no conflicts of interest for this work.

References

1. Nurden AT, Nurden P. The gray platelet syndrome: clinical spectrum of the disease. *Blood Rev.* 2007;21(1):21–36. doi:10.1016/j.blre.2005.12.003

2. Raccuglia G. Gray platelet syndrome. A variety of qualitative platelet disorder. *Am J Med.* 1971;51(6):818–828.

3. Gunay-Aygun M, Zivony-Elboum Y, Gumruk F, et al. Gray platelet syndrome: natural history of a large patient cohort and locus assignment to chromosome 3p. *Blood.* 2010;116(23):4990–5001.

4. Fabbro S, Kahr WH, Hinckley J, et al. Homozygosity mapping with SNP arrays confirms 3p21 as a recessive locus for gray platelet syndrome and narrows the interval significantly. *Blood.* 2011;117(12):3430–3434.

5. Kahr WH, Hinckley J, Li L, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. *Nat Genet.* 2011;43(8):738–740.

6. Albers CA, Cvejic A, Favier R, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. *Nat Genet.* 2011;43(8):735–737.

7. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules. *Nat Genet.* 2011;43(8):732–734.

8. Sims MC, Mayer L, Collins JH, et al. Novel manifestations of immune dysregulation and granule defects in gray platelet syndrome. *Blood.* 2020;136(17):1956–1967.

9. Cullinane AR, Schäffer AA, Huizing M. The BEACH is hot: a LYST of emerging roles for BEACH-domain containing proteins in human disease. *Traffic.* 2013;14(7):749–766.

10. Bottega R, Pecci A, De Candia E, et al. Correlation between platelet phenotype and NBEAL2 genotype in patients with congenital thrombocytopenia and -granule deficiency. *Haematologica.* 2013;98(6):868–874. doi:10.3324/haematol.2012.075861

11. Rensing-Ehl A, Pannicke U, Zimmermann SY, et al. Gray platelet syndrome can mimic autoimmune lymphoproliferative syndrome. *Blood.* 2015;126(16):1967–1969.

12. Wijgaerts A, Wittevrongel C, Thys C, et al. The transcription factor GATA1 regulates NBEAL2 expression through a long-distance enhancer. *Haematologica.* 2017;102(4):695–706.

13. Bottega R, Nicchia E, Alfano C, et al. Gray platelet syndrome: novel mutations of the NBEAL2 gene. *Am J Hematol.* 2017;92(2):E20–E22.

14. Cao L, Su J, Jiaming J, et al. A novel nonsense NBEAL2 gene mutation causing severe bleeding in a patient with gray platelet syndrome. *Platelets.* 2018;29(3):288–291.

15. Steinberg-Shemer O, Tamary H. Gray platelet syndrome mimicking atypical autoimmune lymphoproliferative syndrome: the key is in the blood smear. *Blood.* 2018;131(24):2737.

16. Pluthero FG, Di Paola J, Carcao MD, Kahr WHA. NBEAL2 mutations and bleeding in patients with gray platelet syndrome. *Platelets.* 2018;29(6):632–635.

17. Saliba AN, Ferrer A, Gangat N, et al. Aetiology and outcomes of secondary myelofibrosis occurring in the context of inherited platelet disorders: a single institutional study of four patients. *Br J Haematol.* 2020;190(5):e316–e320.

18. Aarts CEM, Downes K, Hoogendijk AJ, et al. Neutrophil specific granule and NETosis defects in gray platelet syndrome. *Blood Adv.* 2021;5(2):549–564.

19. Tariq H, Perez Botero J, Higgins RA, Medina EA. Gray Platelet Syndrome presenting with pancytopenia, splenomegaly, and bone marrow fibrosis. *Am J Clin Pathol.* 2021;aqaa229. doi:10.1093/ajcp/aqaa229

20. Ross JE, Zhang BM, Lee K, et al. Specifications of the variant curation guidelines for ITGA2B/ITGB3: clinGen Platelet Disorder Variant Curation Panel. *Blood Adv.* 2021;5(2):414–431.

21. White JG. Ultrastructural studies of the gray platelet syndrome. *Am J Pathol.* 1979;95(2):445–462.

22. De Candia E, Pecci A, Ciabattoni G, et al. Defective platelet responsiveness to thrombin and protease-activated receptors agonists in a novel case of gray platelet syndrome: correlation between the platelet defect and the alpha-granule content in the patient and four relatives. *J Thromb Haemost.* 2007;5(3):551–559.

23. Glembotsky AC, Marta RF, Pecci A, et al. International collaboration as a tool for diagnosis of patients with inherited thrombocytopenia in the setting of a developing country. *J Thromb Haemost.* 2012;10(8):1653–1661.

24. Cramer EM, Vainchenker W, Vinci G, Guichard J, Breton-Gorius J. Gray platelet syndrome: immunoelectron microscopic localization of fibrinogen and von Willebrand factor in platelets and megakaryocytes. *Blood.* 1985;66(6):1309–1316.

25. Drouin A, Favier R, Massé JM, et al. Newly recognized cellular abnormalities in the gray platelet syndrome. *Blood.* 2001;98(5):1382–1391.

26. Rosa JP, George JN, Bainton DF, Nurden AT, Caen JP, McEver RP. Gray platelet syndrome: demonstration of a-granule membranes that can fuse with the cell surface. *J Clin Invest.* 1987;80:1138–1146.

27. Pluthero FG, Kahr WHA. Gray platelet syndrome: NBEAL2 mutations are associated with pathology beyond megakaryocyte and platelet function defects. *J Thromb Haemost.* 2021;19(2):318–322.

28. Breton-Gorius J, Vainchenker W, Nurden A, Levy-Toledano S, Caen J. Defective alpha-granule production in megakaryocytes from gray platelet syndrome: ultrastructural studies of bone marrow cells and megakaryocytes growing in culture from blood precursors. *Am J Pathol.* 1981;102(1):10–19.

29. Chen CH, Lo RW, Urban D, Pluthero FG, Kahr WH. α-granule biogenesis: from disease to discovery. *Platelets.* 2017;28(2):147–154.

30. Kahr WH, Lo RW, Li L, et al. Abnormal megakaryocyte development and platelet function in Nbeal2(-/-) mice. *Blood.* 2013;122(19):3349–3358.

31. Guerrero JA, Bennett C. van der Weyden L et al. Gray platelet syndrome: proinflammatory megakaryocytes and α-granule loss cause myelofibrosis and confer metastasis resistance in mice. *Blood.* 2014;124(24):3624–3635.

32. Deppermann C, Cherpokova D, Nurden P, et al. Gray platelet syndrome and defective thrombo-inflammation in Nbeal2-deficient mice. *J Clin Invest.* 2013;123(8):3331–3342.

33. Lo RW, Li L, Leung R, Pluthero FG, Kahr W. NBEAL2 (Neurobeachin-Like 2) is required for retention of cargo proteins by α-granules during their production by megakaryocytes. *Arterioscler Thromb Vasc Biol.* 2018;38(10):2435–2447.

34. Mayer L, Jasztal M, Pardo M, et al. The BEACH-domain containing protein, Nbeal2, interacts with Dock7, Sec16a and Vac14. *Blood.* 2018;131:1000–1011.

35. Sviderskaya EV, Novak EK, Swank RT, Bennett DC. The murine misty mutation: phenotypic effects on melanocytes, platelets and brown fat. *Genetics.* 1998;148(1):381–390.

36. Lo RW, Li L, Pluthero FG, Leung R, Eto K, Kahr WHA. The endoplasmic reticulum protein SEC22B interacts with NBEAL2 and is required for megakaryocyte α-granule biogenesis. *Blood.* 2020;136(6):715–725.

37. Nurden AT, Nurden P. Should any genetic defect affecting α-granules in platelets be classified as gray platelet syndrome? *Am J Hematol.* 2016;91(7):714–718.

38. Noris P, Biino G, Pecci A, et al. Platelet diameters in inherited thrombocytopenias: analysis of 376 patients with all known disorders. *Blood.* 2014;124(6):e4–e10.

39. Larocca LM, Heller PG, Podda G, et al. Megakaryocytic emperipolesis and platelet function abnormalities in five patients with gray platelet syndrome. *Platelets.* 2015;26(8):751–757.

40. Nurden P, Jandrot-Perrus M, Combrié R, et al. Severe deficiency of glycoprotein VI in a patient with gray platelet syndrome. *Blood*. 2004;104(1):107–114.

41. Di Buduo CA, Alberelli MA, Glembotsky AC, et al. Abnormal proplatelet formation and emperipolesis in cultured human megakaryocytes from gray platelet syndrome patients. *Sci Rep*. 2016;6:25027.

42. Tefferi A, Vaidya R, Caramazza D, Finke C, Lasho T, Pardanani A. Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. *J Clin Oncol*. 2011;29 (10):1356–1363.

43. Chedani H, Dupuy E, Massé JM, Cramer EM. Neutrophil secretory defect in the gray platelet syndrome: a new case. *Platelets*. 2006;17 (1):14–19.

44. White JG, Brunning RD. Neutrophils in the gray platelet syndrome. *Platelets*. 2004;15(5):333–340.

45. Zittoun J, Zittoun R, Marquet J, Sultan C. The three transcobalamins in myeloproliferative disorders and acute leukemia. *Br J Haematol*. 1975;31(3):287–298.

46. Kahr WH, Dror Y. Gray platelet syndrome: macrothrombocytopenia with deficient α-granules. *Blood*. 2012;120(13):2543.

47. Sowerby JM, Thomas DC, Clare S, et al. NBEAL2 is required for neutrophil and NK cell function and pathogen defense. *J Clin Invest*. 2017;127(9):3521–3526.

48. Claushuis TAM, de Stoppelaar SF, de Vos AF, et al. Nbeal2 Deficiency Increases Organ Damage but Does Not Affect Host Defense During Gram-Negative Pneumonia-Derived Sepsis. *Arterioscler Thromb Vasc Biol*. 2018;38(8):1772–1784.

49. Drube S, Grimlowski R, Deppermann C, et al. The Neurobeachin-like 2 Protein Regulates Mast Cell Homeostasis. *J Immunol*. 2017;199(8):2948–2957.

50. Rip J, Van Der Ploeg EK, Hendriks RW, Corneth OBJ. The role of Bruton's Tyrosine Kinase in immune cell signaling and systemic autoimmunity. *Crit Rev Immunol*. 2018;38(1):17–62.

51. Zaninetti C, Gresele P, Bertomoro A, et al. Eltrombopag for the treatment of inherited thrombocytopenias: a Phase II clinical trial. *Haematologica*. 2020;105(3):820–828.

52. Favier R, Roussel X, Audia S, et al. Correction of severe myelofibrosis, impaired platelet functions and abnormalities in a patient with Gray Platelet Syndrome successfully treated by stem cell transplantation. *Platelets*. 2020;31(4):536–540.

Analysis of Causes of Hospitalization Among Children with Sickle Cell Disease in a Group of Private Hospitals in Jeddah, Saudi Arabia

Shereen M Abd El-Ghany, Aisha T Tabbakh, Khulud I Nur, Rayan Y Abdelrahman, Sara M Etarji & Bayan Y Almuzaini

ORIGINAL RESEARCH

Analysis of Causes of Hospitalization Among Children with Sickle Cell Disease in a Group of Private Hospitals in Jeddah, Saudi Arabia

Shereen M Abd El-Ghany [1,2]
Aisha T Tabbakh [3]
Khulud I Nur [3]
Rayan Y Abdelrahman [3]
Sara M Etarji [3]
Bayan Y Almuzaini [3]

[1]Department of Pediatrics, Ibn Sina National College for Medical Studies, Jeddah, Kingdom of Saudi Arabia; [2]Department of Pediatrics, Hematology and Oncology Unit, Ain Shams University, Cairo, Egypt; [3]College of Medicine, Ibn Sina National College for Medical Studies, Jeddah, Kingdom of Saudi Arabia

Purpose: Sickle cell anemia (SCA) is a chronic hematologic condition that requires frequent hospitalization representing a significant economic burden on the health services. The aim of this study was to explore the causes and underlying factors of hospitalization among children with SCA, as well as the factors affecting their length of hospital stay.

Patients and Methods: This retrospective study included children and adolescents less than 16 years old who were admitted in a group of private hospitals in Jeddah, Saudi Arabia, during the period from January 2018 to December 2019.

Results: A total of 94 patients were included in this study, 59.6% were males, with a mean age 7.29 ± 3.82 years. The majority of the patients (91.5%) had sickle cell disease. The most common cause of hospital admission was vaso-occlusive crises (VOC) (64.9%) followed by infection (24.5%), acute chest syndrome (ACS) (18.1%), and acute hemolytic crisis (12.8%). We found no significant difference between gender and different causes of admissions ($p > 0.05$). While in relation to age group, limb pain and back pain were found to be significantly more frequent among children ≥ 7 years old ($p = 0.03, 0.04$), while infections were significantly more frequent among children < 7 years old ($p = 0.003$). We analyzed the length of hospital stay and different factors, and we found that the mean length of hospital stay was significantly higher among children who were admitted with infections ($p = 0.01$) and ACS ($p < 0.001$) and among children who are non-compliant on hydroxyurea ($p = 0.04$).

Conclusion: The most common cause of hospitalization among children with SCD in Jeddah, Saudi Arabia, was VOC followed by infection, ACS and acute anemia. The length of hospital stay was more prolonged among children with infection and ACS, as well as children who were non-compliant to hydroxyurea.

Keywords: vaso-occlusive crisis, acute chest syndrome, infection, hydroxyurea

Introduction

Sickle cell anemia (SCA) is an autosomal recessive worldwide genetic disorder. That is caused by single point mutation in the gene encoding the β-globin chain of hemoglobin. This disorder affects many organs in the human body with high morbidity and mortality rates. Those patients also suffer a poorer quality of life than others.[1] In this disease, red blood cells become sickle shaped due to a hemoglobin gene mutation forming what is called sickle hemoglobin (Hb S). These irregularly shaped blood cells are very viscous causing them to get stuck in blood vessels at different sites and block or sluggish the circulation to the various organs. As a result of this sluggish circulation, pain and damage to the organs can occur.[2]

Correspondence: Shereen M Abd El-Ghany
Department of Pediatrics, Ibn Sina National College for Medical Studies, Ghulail District, Al Mahjar St., Jeddah, 22421, Kingdom of Saudi Arabia
Tel +966 540437238
Fax +966 126368194
Email shereen22eg@yahoo.com

733

SCA is found in almost all the world especially the Mediterranean countries, African, South and Central America in addition to Middle Eastern countries.[1] Many Arab countries reported the presence of SCA in different countries with different prevalence. In Qatar and Oman, the prevalence was (3.9% and 3.8%) respectively. In the United Arab Emirates, it reaches about 1.9%.[3] The situation in Saudi-Arabia is different. SCA is reported as one of the significant health issues among children there. The prevalence of SCA differs according to the region with the highest prevalence was reported in the Eastern regions (0.17%) while in the Southern areas, and in Al Madinah, it was only (0.01%).[4,5] Although the mutation in patients with SCD is the same. However, clinically it is very diverse, ranging from a severe, life threatening state to a benign, almost asymptomatic form. In Saudi Arabia, the clinical phenotype of SCD has two major forms. In the Eastern province, where the Saudi-Indian haplotype is prevalent, the disease has mild features in which splenic complications and bone pathology are more common. In addition, painful crisis and vasculopathy occur at a later age. However, the disease in the Western province is more severe, consistent with the Benin haplotype.[6]

Patients with SCA suffer from chronic hemolytic anemia which can lead to sudden life-threatening events. This is usually triggered by acute sickling of red blood cells and microvascular occlusion resulting in pain or damage to organs especially when there are repeated attacks.[7] Severe complications of SCA in most of cases require hospitalization which represents a burden on both the health care system and the family. Also, sickle cell patients report poorer quality of life (QoL) in comparison with the general population and other chronic non-communicable diseases.[8–10]

The hospital admission pattern of children with SCA varies in different parts of the world. The most common causes of hospitalization in these cases include painful crisis and infection. Also acute sequestration crisis and acute chest syndrome were reported in many cases. In addition, the hospitalization may be for receiving blood transfusions for anemia.[11]

One of the most useful preventive measures from SCA complications is the neonatal screening programs. These programs allow early initiation of prophylactic vaccinations. Also screening with transcranial Doppler ultrasonography annually is recommended in addition to the use hydroxyurea.[12] So, the present study was conducted to explore the causes of hospitalization among children with

SCA in a group of private hospitals in Jeddah, Saudi Arabia, as well as, the factors underlying their length of hospital stay.

Patients and Methods

This was a retrospective study which was conducted at Ibn Sina, Al Jedaani Al Safa and New Al Jedaani Hospitals in Jeddah. The present study included all children and adolescents below the age of 16 years with Sickle cell disease who were admitted to Ibn Sina Hospital and Al Jedaani Hospitals in the period from January 2018 to December 2019. We excluded patients with incomplete data and patients with sickle cell trait. Data were held confidentially. This study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. This study was approved by the Ethical committee of Ibn Sina National College for medical Sciences. Informed consent was not needed because the study was conducted retrospectively. Patients' data obtained from the medical records were kept confidential. The enrolled patients were divided into two groups: group 1: included children <7 years old and group 2: included children ≥7 years old.

Data Collection

Clinical data was obtained by reviewing patients' Hospital electronic files. Data collection sheets included: sociodemographic data (age at presentation, gender, nationality and provenance area), diagnosis, medications (Oral penicillin V [ospen], folic acid and hydroxyurea), data related to the current admission including: cause of admission (vaso-occlusive crisis, acute chest syndrome, infection, hemolytic crisis and blood transfusion), clinical examination (pallor, jaundice and hepatomegaly ± splenomegaly), complete blood count, outcome (discharge with complete recovery, discharge with disability or death), number and causes of previous hospitalizations and history of previous operations. Recurrent admissions was defined as ≥ 3 admissions /year. Vaso-occlusive crises are episodes usually present with sudden onset of severe pain, often localized to the extremities, chest, back or abdomen. VOC is the result of obstruction and reduction of the blood flow to the vital organs by the sickled red blood cells leading to ischemia, necrosis and pain.[13]

Data Management

The collected data were coded, entered, presented, and analyzed by computer using Statistical Package for Social Science (version 22, SPSS Inc., Chicago, IL) software program. Quantitative variables were expressed as the mean ± standard deviation (SD) while the qualitative variables were expressed as a number and percentage. Independent *t*-test was used to compare the difference between two means. Chi-square test was used to detect the relation between different qualitative variables. The results were considered statistically significant when the significant probability (P value) of < 0.05.

Results

This study included 94 children and adolescents with sickle cell disease. We excluded three patients with sickle cell trait and one patient who was admitted for tonsillectomy. Of these patients, 56 (59.6%) were males and 38 (40.4%) were females with a median age of 7.65 (ranged from seven months to 14 years). Patients were divided into two age groups: 40 (42.6%) patients were < 7 years old and 54 (57.4%) patients were from ≥ 7 to 14 years old. Most of the admitted patients had sickle cell anemia (91.5%) and only 8.5% of them had sickle/β thalassemia. Regarding the long-term treatment modalities; 61 (64.9%) children were on regular folic acid tablets, 37 (39.4%) children were taking oral penicillin V (ospen) daily and 30 (31.9%) children were taking hydroxyurea (HU) (Table 1). Regarding treatment given during the admission, all patients received intravenous fluids, 61 patients (64.9%) received analgesics, 23 patients (24.5%) received antibiotics and 29 patients (30.9%) needed blood transfusion. All patients were discharged safely from the hospital with complete recovery.

The causes of hospital admissions of the studied children are demonstrated in Figure 1. In this study, the most common cause of hospitalizations among our children was vaso-occlusive crisis (VOC) (64.9%), 45.7% of them presented with pain in the limbs (Figure 2). Infections represent the second cause of hospitalizations among our patients (24.5%) in the form of herpetic stomatitis, viral upper respiratory tract infections, severe gastroenteritis, encephalitis and osteomyelitis. The third cause of hospitalization was acute chest syndrome (18.1%). Acute hemolytic crisis represented 12.8% of the causes of hospitalization.

Table 1 Basic Characteristics of the Studied Group

Basic Characteristics	Study Group (n=94)	
	Number	**%**
Age		
< 7 years	40	42.6
≥ 7–14 years	54	57.4
Sex		
Male	56	59.6
Female	38	40.4
Nationality		
Saudi	51	54.3
Non-Saudi	43	45.7
Diagnosis		
SCD	84	89.4
Sickle thalassemia	10	10.6
Treatment Modalities		
Oral penicillin V (ospen)	37	39.4
Folic acid tablets	61	64.9
Hydroxyurea	30	31.9
Blood transfusion		
Regular	26	27.7
On demand	68	72.3
Length of hospitalization (days) (mean ± SD)	4.1 ± 1.94	
Minimum	1 day	
Maximum	10 days	
Hematologic parameters (mean ± SD)		
White blood cells (10^9/l)	17.1 ± 7	
Hemoglobin (g/dl)	8.2 ± 1.5	
Platelets (10^9/l)	380.7 ± 301.4	
Reticulocyte count (%)	5.5 ± 2.2	

Regarding the relation between the causes of admissions and gender, we found no significant difference between males and females regarding the different causes of hospital admissions (p >0.05). Also, we did not find any significant difference between Saudi and non-Saudi children regarding the causes of admissions (p >0.05). Analyzing the causes of admissions in relation to age groups, we found a significant higher percent of painful crises (VOC) (p = 0.02), limb pain (p = 0.027) and back pain (p = 0.042) among children > 7 to 14 years old. However, infection was significantly higher among children less than 7 years old (p=0.003) (Table 2).

The median (IQR) length hospital stay among children with SCA was 4 (2) days. We statistically analyzed the length of hospital stay in relation to age groups, gender,
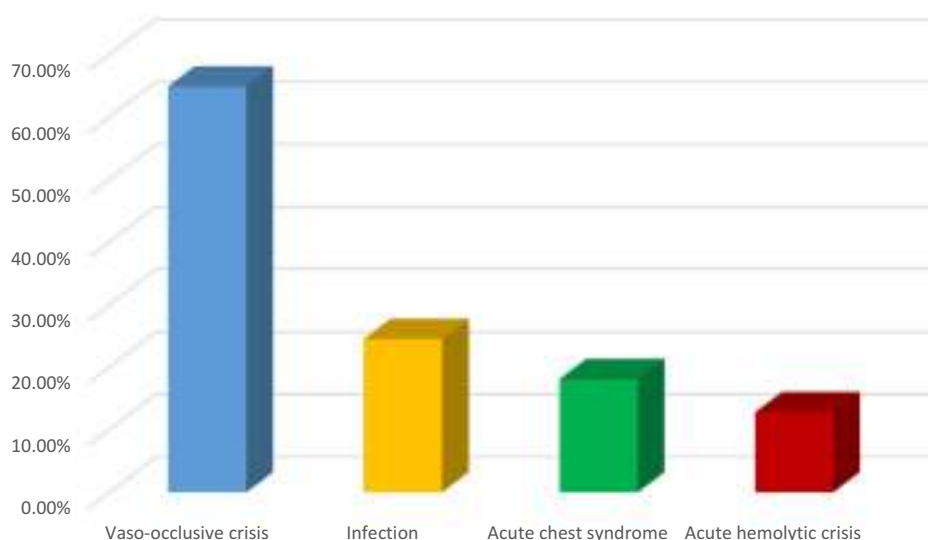
Figure 1 Bar-chart showed the different causes of admissions among children with SCA.
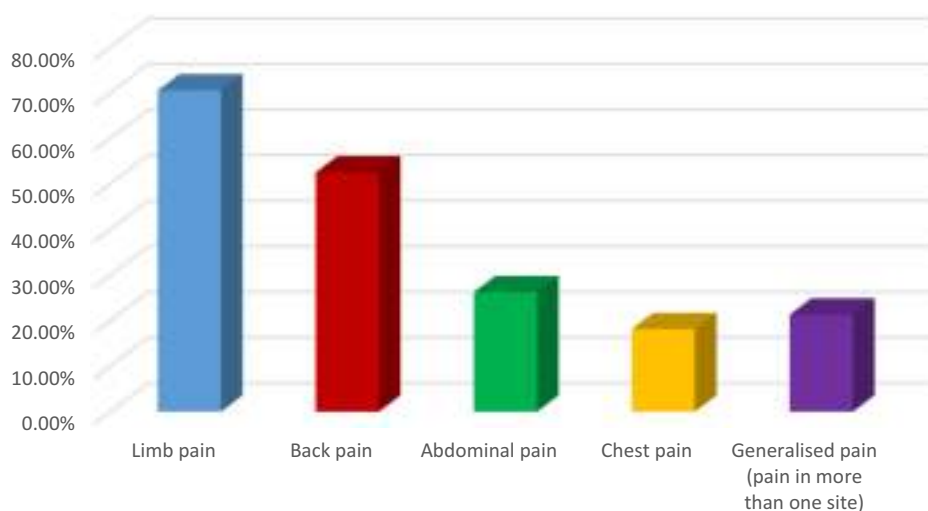
Figure 2 Bar-chart showed the frequency of vaso-occlusive crisis by body region.

causes of admission and medications. We observed a significantly higher percent of children who stayed in the hospital for more than 4 days among children admitted due to infection (p=0.01) and ACS (p < 0.001). Also, a significantly higher percent of children who were non-compliant to HU stayed for more than 4 days in the hospital (p = 0.04) (Table 3). Forty (42.6%) children had history of recurrent hospital admissions (≥3 admissions / year). We found a statistically significantly relation between recurrent hospital admissions and non-compliance to HU intake (p = 0.002). However, there was no statistical relation between regular blood transfusions and frequency of hospital admissions (p =0.98).

Discussion

Chronic conditions such as SCA require frequent utilization of the health services at different levels. These services include screening, vaccinations, drugs, blood transfusion and hospitalizations for different reasons. All these measures are provided to reduce patients' morbidity and mortality but cause significant economic burden on the health system.[14]

Vaso-occlusive crisis is due to obstruction of the microcirculation by sickled red blood cells, leading to ischemic injury of the affected organ and pain. Painful crises represent the most distinguishing clinical feature of SCD and are the leading cause of emergency department

Table 2 Relation Between Causes of Hospital Admissions and Age Group Among the Studied Group

Clinical Presentation	Age Group (n= 94)		P-value
	< 7 Years (n= 40)	≥ 7 Years (n= 54)	
Painful crisis (n=61)	21 (34.4%)	40 (65.6%)	0.02*
Limb pain (n=43)	13 (30.2%)	30 (69.8%)	0.03*
Back pain (n=32)	9 (28.1%)	23 (71.9%)	0.04*
Abdominal pain (n=16)	7 (43.8%)	9 (56.2%)	0.92
Acute chest syndrome (n=17)	9 (52.9%)	8 (47.1%)	0.34
Acute hemolytic crisis (n=12)	5 (41.7%)	7 (58.3%)	0.95
Infections (n=23)	16 (69.6%)	7 (30.4%)	0.003*
Recurrent crisis (n=40)	15 (37.5%)	25 (62.5%)	0.39

Notes: n = number, chi-square test; *Significant value.

visits and hospital admissions for affected patients.[15] The present study showed that VOC was the most common cause (64.9%) of hospitalizations among children with SCD. These results are consistent with the studies done in Nigeria, Kuwait, Iraq, and Oman who reported VOC among 61.5%, 63.2%, 73.8% and 83% of their admitted patients respectively.[7,16–18] Previous Saudi studies done in Al-Madinah Al-Munawarah and Makkah Al-Mukarramah reported similar results in which VOC was the main cause of hospitalizations among their studied group of patients with a percent of 49.7% and 55.9% respectively.[11,19] VOC is considered one of the most common causes for hospitalizations in most of studies and this may be attributed to that those children are usually physically active without taking care of proper hydration which predisposes them to dehydration and thrombosis.[20]

The majority of children included in our study were presented with limb and back pains with a significantly higher frequency among children between 7 and 14 years old. Most children with SCA experience painful crisis, of variable severity and frequency, by the age of 6 years. Pain can affect any part of the body but frequently affects the extremities, back and chest areas. Bone pains in VOC result from infarction of the bone marrow, which usually involve bones where the bone marrow is active which varies according to the age of the patient. Acute dactylitis is the most common presentation in the first 18 months of life due to affection of the metatarsals and metacarpals. As the child grows older, pain often involves the long bones, sites that retain marrow activity during childhood.

Proximity to the joints and occasional sympathetic effusions lead to the belief that the pain involves the joints.[19]

In the present study, infection was the second cause of hospitalization among our patients. This was consistent with a study done in Basra, Iraq[7] who found that infections were the second common cause of hospitalizations with a percentage of 9.3%. Regarding previous studies done in Saudi Arabia there were difference in the percentage of infection rates among patients with SCA. An earlier study done in Al-Madinah Al-Munawarah in 1998, infection was the second cause of hospital admissions with a percent of 67.9%.[5] However, in 2019 another study which was conducted at the Maternity and Children Hospital in Al-Madinah Al-Munawarah reported a regression of infection to be the third cause of admissions with a percentage of 17.5%.[11] Patients with SCA have several factors that make them more liable to severe bacterial infections, however, the most important of which is poor splenic function.[21]

ACS is the most common cause of morbidity and mortality in children and adults with SCD. It accounts for about 25% of deaths and can follow VOC. It can be triggered by hypoxia due to hypoventilation of the chest caused by VOC. Also, it can also occur as a result of fat embolism originating from the distal bone in VOC.[22] ACS was the third cause of admissions among our patients (18.1%). This is consistent with previous studies done by Hawasawi et al in Saudi Arabia,[5] and by Salman and Hassan in Iraq, who found ACS to be the third cause of admissions among their patients.[7] In another study conducted in Kuwait, acute chest syndrome was the fourth reported cause of admissions among children with SCA (6.6%).[16]

In the present study, we analyzed the relation between different causes of admissions and patient's age and sex. We did not find a significant relation between gender and different causes of hospital admissions among children with SCA. Similarly, Abd Elmoneim et al[11] reported no significant difference between males and females regarding causes of hospitalization. When considering the causes of admissions in relation to age group, we found that painful crises, limb and back pains were significantly more common among children between 7 and 14 years old. However, infections were found to be significantly more in children < 7 years old. Abd Elmoneim et al[11] reported that younger children aged < 12 years showed significantly high frequency of acute chest syndrome (26.5%) but acute painful crisis (66.4%) was significantly reported in older patients (age ≥12 years). This may be

Table 3 The Length of Hospital Stay in Relation to Different Factors

Variables		Length of Hospital Stay				p-value
		≤ 4 Days		> 4 Days		
		Number	%	Number	%	
Gender	Females	23	39.7%	15	41.7%	0.847
	Males	35	60.3%	21	58.3%	
Age groups	< 7 years	22	37.9%	18	50.0%	0.25
	≥ 7 years	36	62.1%	18	50.0%	
Nationality	Non-Saudi	26	44.8%	17	47.2%	0.821
	Saudi	32	55.2%	19	52.8%	
VOC	No	17	29.3%	16	44.4%	0.135
	Yes	41	70.7%	20	55.6%	
Limb pain	No	32	55.2%	19	52.8%	0.821
	Yes	26	44.8%	17	47.2%	
Back pain	No	34	58.6%	28	77.8%	0.057
	Yes	24	41.4%	8	22.2%	
Abdominal pain	No	49	84.5%	29	80.6%	0.622
	Yes	9	15.5%	7	19.4%	
Generalized pain	No	52	89.7%	29	80.6%	0.214
	Yes	6	10.3%	7	19.4%	
Infections	No	49	84.5%	22	61.1%	0.01*
	Yes	9	15.5%	14	38.9%	
ACS	No	55	94.8%	22	61.1%	<0.001*
	Yes	3	5.2%	14	38.9%	
Hemolytic crisis	No	48	82.8%	34	94.4%	0.099
	Yes	10	17.2%	2	5.6%	
Recurrent crisis	No	35	60.3%	19	52.8%	0.471
	Yes	23	39.7%	17	47.2%	
Ospen	No	39	67.2%	18	50.0%	0.096
	Yes	19	32.8%	18	50.0%	
Folic acid	No	19	32.8%	14	38.9%	0.55
	Yes	39	67.2%	22	61.1%	
HU	No	35	60.3%	29	80.6%	0.04*
	Yes	23	39.7%	7	19.4%	

Notes: chi-square test, *Significant value.
Abbreviations: VOC, vaso-occlusive crisis; ACS, acute chest syndrome; HU, hydroxyurea.

explained by that acute chest syndrome is probably triggered by chest infection and younger children are more susceptible to both chest infection in addition to gastroenteritis.[22]

Regarding the length of hospital stay we found a higher rate of prolonged hospitalization (>4 days) among children who were admitted with infection and ACS, as well as, among children who are non-compliant to HU. Similarly, Salman and Hassan reported that the use of HU was associated with shorter length of hospital stay and fewer hospital readmissions.[7] Also, we found that non-compliance to HU was significantly related to recurrent hospital admissions. Previous studies showed that HU improves hematological parameters and has a role in the

reduction of the complications related to SCD (mainly acute painful crises), the required hospitalizations, and the length of hospital stay.[23,24]

Conclusion

Forty (42.6%) children had history of recurrent hospital admissions (≥3 admissions /year). The most common cause of hospitalisation among children with SCD in a group of private hospitals in Jeddah, Saudi Arabia was VOC which reached up to 70%. Other causes included in order; infection, ACS and acute anemia. The length of hospital stay was more prolonged among patients with infection and ACS. Non-compliance to medications was the main underlying factor for admission and prolonged stay at hospital. Physicians should pay more attention to patient education concerning the importance of good hydration especially in countries with warm weather, and the compliance to medications like oral penicillin V and hydroxyurea.

Acknowledgments

Disclosure

The authors declare no conflicts of interest for this work.

References

1. Wali Y, Kini V, Yassin MA. Distribution of sickle cell disease and assessment of risk factors based on transcranial Doppler values in the Gulf region. *Hematology*. 2020;25(1):55–62. doi:10.1080/16078454.2020.1714113

2. Alhumaid AM, Aleidi AS, Alfakhri AS, et al. Clinical features and outcome of sickle cell anemia in a tertiary center: a retrospective cohort study. *J Appl Hematol*. 2018;9:22–28. doi:10.4103/joah.joah_48_17

3. El-Hazmi MA, Al-Hazmi AM, Warsy AS. Sickle cell disease in Middle East Arab countries. *Indian J Med Res*. 2011;134(5):597–610. doi:10.4103/0971-5916.90984

4. Memish ZA, Owaidah TM, Saeedi MY. Marked regional variations in the prevalence of sickle cell disease and β-thalassemia in Saudi Arabia: findings from the premarital screening and genetic counseling program. *J Epid and Global Health*. 2011;1:61–68. doi:10.1016/j.jegh.2011.06.002

5. Hawasawi ZM, Nabi G, Al Magamci MS, et al. Sickle cell anemia in childhood in Madina. *Ann Saudi Med*. 1998;18:293–295. doi:10.5144/0256-4947.1998.293

6. Maf EH, Warsy AS. Hemoglobinopathies in Arab countries. In: Teebi AS, Farag TL, editors. *Genetic Disorders Among Arab Populations*. New York: Oxford University Press; 1997: 83–110.

7. Salman ZA, Hassan MK. Hospitalization events among children and adolescents with sickle cell disease in Basra, Iraq. *Anemia*. 2015;25 (5):55–62.

8. McClish DK, Penberthy LT, Bovbjerg VE, et al. Health related quality of life in sickle cell patients: the PiSCES project. *Health Qual Life Outcomes*. 2005;3:50. doi:10.1186/1477-7525-3-50

9. Anie KA. Psychological complications in sickle cell disease. *Br J Haematol*. 2005;129:723–729. doi:10.1111/j.1365-2141.2005.05500.x

10. Bhagat VM, Baviskar SR, Mudey AB, et al. Poor health related quality of life among patients of sickle cell disease. *Indian J Palliat Care*. 2014;20(2):107–111. doi:10.4103/0973-1075.132622

11. Abd Elmoneim AA, Al Hawsawi ZM, Mahmoud BZ, et al. Causes of hospitalization in sickle cell diseased children in western region of Saudi Arabia. A single center study. *Saudi Med J*. 2019;40 (4):401–404. doi:10.15537/smj.2019.4.24049

12. Alsulami SH, Alotaibi MN, Alfaraj EA, et al. Evaluation of the most common complications of sickle cell anemia and management in children: simple literature review. *EJHM*. 2018;72(2):3951–3960. doi:10.21608/ejhm.2018.9076

13. Platt OS, Thorington BD, Brambilla DJ, et al. Pain in sickle cell disease. Rates and risk factors. *N Engl J Med*. 1991;325(1):11–16. doi:10.1056/NEJM199107043250103

14. Kauf TL, Coates TD, Huazhi L, et al. The cost of health care for children and adults with sickle cell disease. *AJH*. 2018;84 (6):323–327.

15. Madigan C, Malik P. Pathophysiology and therapy for haemoglobinopathies. Part I: sickle cell disease. *Expert Rev Mol Med*. 2006;8 (9):1–23. doi:10.1017/S1462399406010659

16. Akar NA, Adekile A. Ten-year review of hospital admissions among children with sickle cell disease in Kuwait. *Med Princ Pract*. 2008;17 (5):404–408. doi:10.1159/000141506

17. Jaiyesimi F, Pandey R, Bux D, et al. Sickle cell morbidity profile in Omani children. *Ann Trop Paediatr*. 2002;22(1):45–52. doi:10.1179/027249302125000148

18. Brown BJ, Jacob NE, Lagunju IA, et al. Morbidity and mortality pattern in hospitalized children with sickle cell disorders at the University College Hospital, Ibadan, Nigeria. *Niger J Pediatr*. 2013;40(1):34–39.

19. Alkot M, Almaghrabi WA, Al-Najdi N, et al. Prevalence of complications of sickle cell disease at Makkah Al-Mukaramah, Saudi Arabia, 2017. *Ann Clin Lab Res*. 2018;6(1):226. doi:10.21767/2386-5180.1000226

20. Yale SH, Nagib N, Guthrie T. Approach to the vasoocclusive crisis in adults with sickle cell disease. *Am Fam Physician*. 2000;61: 1349–1356.

21. Booth C, Inusa B, Obaro SK. Infection in sickle cell disease: a review. *Int J Infect Dis*. 2010;14:e2–e12. doi:10.1016/j.ijid.2009.03.010

22. Howard J, Hart N, Roberts-Harewood M, et al. Guideline on the management of acute chest syndrome in sickle cell disease. *Br J Haematol*. 2015;169:492–505. doi:10.1111/bjh.13348

23. Ballas SK, Bauserman RL, McCarthy WF, et al. Hydroxyurea and acute painful crises in sickle cell anemia: effects on hospital length of stay and opioid utilization during hospitalization, outpatient acute care contacts, and at home. *J Pain Symptom Manage*. 2010;40 (6):870–882. doi:10.1016/j.jpainsymman.2010.03.020

24. Mulaku M, Opiyo N, Karumbi J, et al. Evidence review of hydroxyurea for the prevention of sickle cell complications in low-income countries. *Arch Dis Child*. 2013;98(11):908–914. doi:10.1136/archdischild-2012-302387

Thrombocytopenia and Associated Factors Among HIV Infected Patients in Pre- and Post-Anti-Retroviral Therapy, North East Ethiopia

Feredegn Talargia & Lemma Getacher

ORIGINAL RESEARCH

Thrombocytopenia and Associated Factors Among HIV Infected Patients in Pre- and Post-Anti-Retroviral Therapy, North East Ethiopia

Feredegn Talargia [ID][1]
Lemma Getacher [ID][2]

[1]Department of Biomedical Science, College of Medicine, Debre Berhan University, Debre Berhan, Ethiopia;
[2]Department of Public Health, College of Health Sciences, Debre Berhan University, Debre Berhan, Ethiopia

Background: Thrombocytopenia is a common disorder of HIV (human immunodeficiency virus) infection. The magnitude of thrombocytopenia and associated factors among HIV-infected patients receiving ART (anti-retroviral treatment) are not studied well in this study area. The aim of this study was to determine the prevalence of thrombocytopenia and associated factors in pre- and post-ART patients who attended Debre Berhan Referral Hospital (DBRH) in North-East Ethiopia.

Methods: A hospital-based cross-sectional study was conducted from October to December 2020 in DBRH, North-East Ethiopia. From the total ART patients, 272 study participants were selected randomly. Socio-demographic variables and clinical characteristics of the patients were collected by standard questionnaires. Measurement of platelet count and CD4 count were made by Sysmex XT2000i hematology machine and BD FACS count analyzer, respectively. Data were analyzed with SPSS software version 23 and multivariate logistic regression was done. P-value less than 0.05 was taken as statistically significant.

Results: The prevalence of thrombocytopenia was 22.7% with 95% CI: 17.8–27.5 in pre-ART and 14.7% with 95% CI: 11.0–19.9 in post-ART HIV-infected patients with a significant difference at $P < 0.0001$. HIV patients with CD4 counts <200 cells/μL were more likely to have thrombocytopenia (35.0%) than patients with CD4 counts ≥200 with a P <0.04 in pre-ART patients. Patients on zidovudine (AZT)-based therapy were more likely to have thrombocytopenia (16.3%) than patients on tenofovir (TDF)-based therapy (14.8%) with P<0.79; however, this did not show any significant association.

Conclusion: The prevalence of thrombocytopenia decreased significantly after the beginning of ART. HIV patients with low CD4 count and on AZT-based treatment showed high risk of thrombocytopenia. According to this study, thrombocytopenic patients were observed even after the initiation of ART. As a result, to decrease thrombocytopenic associated mortality and morbidity, there should be continuous screening for HIV-infected patients.

Keywords: ART, HIV, thrombocytopenia, Ethiopia

Introduction

HIV infection is characterized by progressive damage to the body's immune system which results in a number of opportunistic infections, and immunological and hematological complications.[1] Hematological abnormalities, which can involve all lineages of blood cells and include anemia, thrombocytopenia as well as leucopenia, are the most common complications of HIV infection.[2] Among these, thrombocytopenia is a frequent complication and it can occur at any stage of HIV

Correspondence: Feredegn Talargia
P.O. Box: 445
Tel +251921792491
Email ftalarge@gmail.com

infection.[3] Thrombocytopenia is the second most common complication next to anemia and is found in 3–40% of individuals with HIV infection.[4] The cause of thrombocytopenia has not yet been established.[5] The possible mechanism may include immune-mediated platelet destruction, impaired megakaryocytes or a direct attack of megakaryocytes by HIV virus, hypersplenism, opportunistic infections, malignancy, and myelosuppression effect of medication.[5–7]

Thrombocytopenia has been linked to adverse sequelae and is regarded as an independent predictor of morbidity and mortality among HIV-infected group, owing to increased risk of bleeding, which may occur in mucus membranes, skin, soft tissue and intracranial sites.[8] It is associated with increased morbidity and mortality, accelerated deterioration in CD4 counts and accelerated progression to full-blown acquired immunodeficiency syndrome (AIDS).[9]

Immune thrombocytopenic purpura (ITP) is the most common cause of thrombocytopenia in HIV-infected individuals, and often occurs at the initial stages of infection.[10] The pathogenesis of ITP is still not clear, but both antibody mediated and T-cell mediated processes seem to be involved in ITP-associated platelet destruction.[10] HIV-associated ITP remains an important clinical problem in the era of the widespread use of ART. Although most patients respond to primary ITP treatment and there are few treatment-related complications, nearly all patients require retreatment for recurrent ITP.[11]

Several studies reported that the prevalence of thrombocytopenia was higher in ART naive patients compared with patients who were on ART.[4,8,12] For instance, a study conducted in Uganda showed that the prevalence of thrombocytopenia was 17.8% in ART naive patients and 13.0% in patients on ART.[8] Another study conducted in Addis Ababa, Ethiopia reported that the prevalence of thrombocytopenia was 25% in ART naïve patients and 5.7% in patients who took ART at least for six months.[12]

The highest prevalence of thrombocytopenia was associated with low CD4 counts,[2,8] advanced stages of HIV/AIDS, and patents on zidovudine (AZT) based therapy,[8,12] but the prevalence of thrombocytopenia did not differ by sex, ethnicity or age.[2,13] Many commonly used antiviral drugs affect platelet count and activation. Combined ART (cART) alone is often sufficient to correct thrombocytopenia, and the development of recurring thrombocytopenia upon cART discontinuation in some patients further strengthens the correlation.[14] The ability of the nucleoside

analog reverse transcriptase inhibitor (NRTI) azidothymidine (AZT, zidovudine) to affect platelet number has been extensively studied alone and in cART and AZT is reported to produce myelodysplastic syndrome with thrombocytopenia in mice.[15] Although hematologic abnormalities have been widely reported in HIV-related infection, there are few data on the prevalence of thrombocytopenia and associated factors among HIV-infected patients in pre- and post-antiretroviral treatment in Ethiopia. This study provides further information on HIV-associated thrombocytopenia, and it can serve as a baseline for future studies. The aim of this study is to determine the prevalence of thrombocytopenia and associated factors in pre- and post-ART among HIV patients who attended the ART clinic at DBRH, North-East Ethiopia.

Methods and Materials
Study Area
This study was conducted at the ART clinic of DBRH, which is located in Debre Berhan town, North Shewa zone, Amhara region, North East Ethiopia. This institution was selected based on the availability of patients from all parts of the zone and this hospital is the only hospital serving as both a teaching and referral hospital in this zone. Beginning from September to December 2020, about 2015 patients were on ART at DBRH.

Study Period
The study was conducted from September to December 2020.

Study Design
A hospital-based cross-sectional study was conducted.

Source Population
All adult HIV-infected people who were enrolled at the ART clinic of DBRH.

Study Population
All adult HIV-infected people who fulfilled the inclusion criteria were included in this study.

Inclusion and Exclusion Criteria
Age above or equal to 18 years old, HIV-infected individuals who have been taking ART for at least six months, in DBRH were included in this study. Pregnant women,

referred patients, patients with hematologic disorders, or severely sick patients during the data collection period were excluded from this study.

Sample Size Determination

The sample size was calculated by a single population statistical formula as follows:

N = z^2 (p (1-p)/d^2, we consider the prevalence of thrombocytopenia = 19% from a study done previously.[2] 5% level of precision(d), with 95% confidence interval and 15% non-response rate were added. Substituting the values on the above-mentioned formula, n = 1.96^2 $(0.19*0.81)/0.05^2$ 236.48 after the non-response rate was added, gave the final sample size of approximately 272.

Sampling Technique and Procedures

The samples in this study were collected randomly by a lottery method until the desired sample was reached from the study population who can fulfill the inclusion criteria at the time of study period.

Study Variables

Dependent Variable

Prevalence of thrombocytopenia.

Independent Variables

Age, sex, clinical stages of HIV infection, CD4 count, and types of ART drugs.

Data Collection and Procedures

The standard questionnaire was prepared after observing of several literatures, and data were collected by trained ART clinic staff nurses. Socio-demographic information and clinical characteristics of the patients were collected in face to face interviews and a review of medical registration books. Blood samples were then collected by laboratory technicians and sent for hematology analysis. According to standard procedures, platelet count and CD4 cell counts were done by using Sysmex XT2000i hematology analyzer and BD FACS count system, respectively. To enhance the quality of data, the standard procedures were followed in every aspect of the procedures and quality of CD4 and hematology analyzer was checked by running the quality control along with each patient's sample. Beside that we trained the data collectors and a pre-test of the questionnaire was made plus the collected data was checked each day by the principal investigators.

Thrombocytopenia is defined as platelet counts less than 150,000 cells/µL. It was further classified as mild (100,000–150,000 cells/µL), moderate (50,000–100,000 cells/µL) and severe thrombocytopenia (platelet counts <50,000 cells/µL).[12]

Data Processing and Analysis

Data were coded and entered by EPI data software version 3.1 and exported to SPSS software version 23 for analysis. The results of descriptive data were presented as frequency and percentages, whereas the continuous variables were expressed as mean ± standard deviation. Univariate analysis was performed to determine the associations between dependent and independent variables using crude odds ratio (COR) with a 95% confidence interval (CI). Those independent variables with a P <0.2 in univariate analysis were included in multivariate logistic regression models. P <0.05 in multivariate logistic regression was considered as a statistically significant association.

Result

General Characteristics of the Study Participants

Among the 272 study participants, 110 (40.4%) were males and 162 (59.6%) were females. The mean age of the study participants was 40.494±10.88 years. Most of the study participants were WHO stage I category in pre-ART patients.

Platelet Counts and CD4 Counts of Study Participants

The mean platelet count was 184.94±58.02X10^3/µL in pre-ART patients and 294.32±83.7/µL in patients on ART (P<0.0001). The mean CD4 count showed an increment from 264.75±184.5 cells/µL in pre-ART patients to 544.0261.3cells/µL in post-ART patients (P<0.0001). The most widely used ART regimen in this study was 1j (TDF-3TC-DTG) (Table 1).

Thrombocytopenia and Associated Factors in Pre-ART Patients

The overall prevalence of thrombocytopenia in pre-ART patients was 62 (22.7% with 95% CI: 17.8–27.5), out of this the prevalence of thrombocytopenia was 27.27% in males and 19.4% in females. From the total thrombocytopenic patients before ART, 73.8%, 23.0%, and 3.3% had mild, moderate, and severe thrombocytopenia cases

Table 1 Socio-Demographic and Clinical Characteristics of HIV-Positive Patients Taking ART at DBRH, North-East Ethiopia, 2020

Variables	Frequency	Percentage
Age in years		
18–29	34	12.5
30–39	97	35.9
40–49	82	30.0
≥50	59	21.6
Sex		
Male	110	40.4
Female	162	59.6
Marital status		
Single	23	49.8
Divorced	39	27.5
Married	135	8.4
Widowed	75	14.3
Educational status		
Illiterate	74	27.1
Primary school	92	33.7
High school	63	23.1
Certificate and above	44	16.1
WHO clinical stages		
Stage I	144	52.9
Stage II	36	13.2
Stage III	87	31.9
Stage IV	5	1.8
Types of ART regimen		
1c	14	5.1
1d	10	3.7
1e	75	27.6
1j	130	47.8
2f	19	7.0
2h	18	6.6
Not registered	6	2.2

Notes: 1c = AZT-3TC-NVP, 1d = AZT-3TC-EFV, 1e = TDF-3TC-EFV, 1f = TDF-3TC-NVP, 1j = TDF-3TC-DTG, 2f = AZT-3TC-ATV/r, 2h = TDF-3TC-ATV/r.

respectively (Figure 1). In the current study the majority of thrombocytopenia cases (28.81%) was observed in the age group ≥ 50 years, however, the difference was not statistically significant. HIV patients who had low CD4 counts (<200) were significantly associated with the highest prevalence of thrombocytopenia with a *P* <0.04 (Table 2).

Thrombocytopenia and Associated Factors in Post-ART Patients

The prevalence of thrombocytopenia was 40 (14.7% with 95% CI: 11.0–19.9) in post-ART patients. Of these patients, 80.0%, 17.5%, and 2.5% had mild, moderate, and severe thrombocytopenia (Figure 1). The prevalence of thrombocytopenia was decreased significantly after initiation of ART by 8.0% (*P* <0.0001). Out of the total thrombocytopenic patients 16.51% were males and 13.58% were females. The highest prevalence of thrombocytopenia was 16.33%, which was observed in the age groups between 30–39 years compared with the other age groups. The probability of having thrombocytopenia was high in patients on AZT-based therapy compared with TDF-based therapy. The prevalence of thrombocytopenia was 15.38% in patients whose CD4 counts were <200 cells/μL, while the prevalence of thrombocytopenia in patients whose CD4 counts were ≥350 cells/μL was 15.21% (Table 3).

Discussion

Hematological disorders are common among HIV-infected individuals.[1,4,11] Thrombocytopenia is a common hematological syndrome of HIV/AIDS-infected patients,[9,12] which is not affected by age group or gender difference.[2]

In the current study the prevalence of thrombocytopenia was 22.7% in pre-ART patients and 14.7% in post-ART patients. A similar study done in Uganda showed that the prevalence of thrombocytopenia was 17.8% in ART naive patients and 13.0% in patients on ART at least for six months.[7] Another study conducted in Addis Ababa, Ethiopia reported that the prevalence of thrombocytopenia before initiation of ART was 25.0% and it was 5.7% after six months of ART .[9] This variation may be due to differences in cut-off value platelet counts for the definition of thrombocytopenia, sample size, study design and study area.

In the current study the prevalence of thrombocytopenia in post-ART patients was decreased; this might be due to the possibility of opportunistic infections, hematologic and immune mediated related disorders being reversed in post–ART patients[9,13] In this study the prevalence of thrombocytopenia did not show any statistical significant differences among age groups, or gender difference. This was in agreement with a previous study.[1]
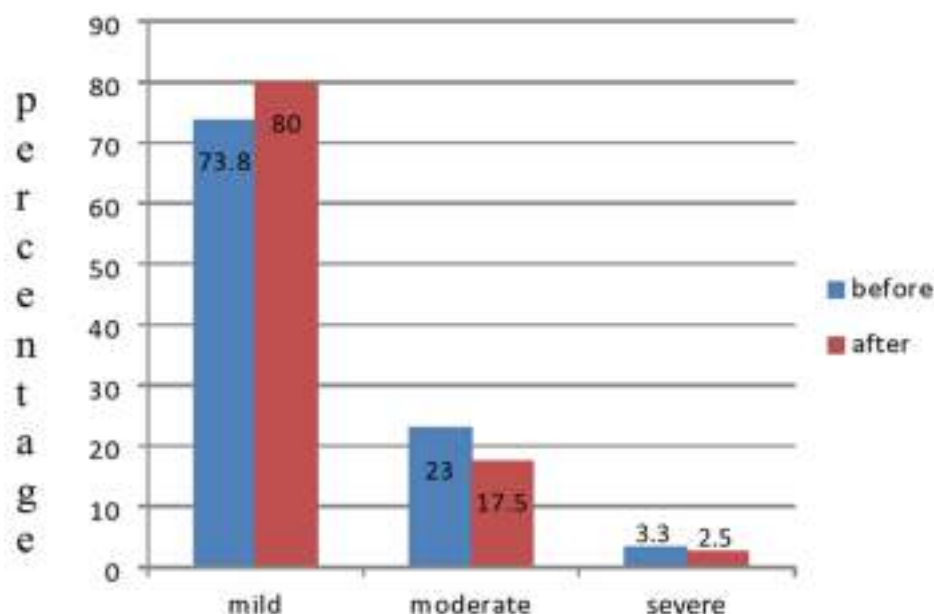
Figure 1 Degree of thrombocytopenia among HIV-infected patients on ART in DBRH, North-East Ethiopia.

This study showed that the prevalence of thrombocytopenia in pre-ART patients was significantly associated with low CD4 counts (<200) with a *P*<0.04. Similar to this result a study conducted in China reported that thrombocytopenia was significantly associated with low CD4 counts in pre-ART patients.[14] However, the prevalence of thrombocytopenia after the commencement of ART did not show a statistically significant difference after initiation of ART. This finding is supported by another study done in Gondar, Ethiopia which showed that the

Table 2 Thrombocytopenia and Associated Factors in Pre-ART HIV-Infected Patients Attending DBRH, North –East Ethiopia, 2020

Variables	Thrombocytopenia		COR	AOR	P-value
	Yes (%)	No (%)			
Age in groups					
18–29	8(23.5%)	26(76.5%)	1.32(0.49–3.48)	1.22(0.44–3.36)	0.28
30–39	15(15.3%)	83(84.7%)	2.24(1.02–4.92)	1.93(0.84–4.44)	
40–49	22(26.8%)	60(73.2%)	1.1(0.52–2.32)	0.94(0.43–2.07)	
≥50	17(28.8%)	42(71.2%)	I	I	
Sex					
Male	30(27.3%)	80(72.7%)	0.63(0.36–1.12)	0.74(0.4–1.38)	0.35
Female	31(19.1%)	131(80.9%)	I	I	
WHO clinical stages					
Stages I & II	34(18.8%)	147(81.2%)	1.89(1.1–3.38)	1.58(0.84–2.95)	0.15
Stages III & IV	28(30.4%)	64(69.6%)	I	I	
CD4 counts (cells/μL					
<200	41(35.0%)	76(65.0%)	0.33(0.16–0.69)	0.42(0.18–0.92)	0.04
200–349	10(12.0%)	73(88.0%)	1.29(0.52–3.25)	1.42(0.55–3.67)	
≥350	11(15.1%)	62(84.9%)	I	I	

Table 3 Thrombocytopenia and Associated Factors in HIV-Infected Patients on ART Attending DBRH, North –East Ethiopia, 2020

Variables	Thrombocytopenia		COR	AOR	P-value
	Yes (%)	No (%)			
Age in groups					
18–29	4(11.8%)	30(88.2%)	1.35(0.38–4.77)	1.35(0.38–4.79)	0.86
30–39	16(16.3%)	82(83.7%)	0.92(0.38–2.25)	0.87(0.36–2.13)	
40–49	11(13.6%)	70(86.4%)	1.15(0.44–2.97)	1.15(0.44–3.03)	
≥50	9(15.3%)	50(84.7%)	1	1	
Sex					
Male	18(16.5%)	91(83.5%)	0.79(0.4–1.56)	0.79(0.39–1.58)	0.49
Female	22(13.6%)	140(86.4%)	1	1	
ART regimen					
AZT based	7(16.3%)	36(83.7%)	0.89(0.37–2.12)	0.88(0.35–2.21)	0.79
TDF based	33(14.8%)	190(85.2%)	1	1	
CD4 counts (cells/µL					
<200	2(15.4%)	11(84.6%)	0.97(0.21–4.65)	0.96(0.19–4.78)	0.86
200–349	5(11.9%)	37(88.1%)	1.33(−0.49–3.63)	1.34(0.47–3.79)	
≥350	33(15.2%)	184(84.8%)	1	1	

prevalence of thrombocytopenia did not show a statistical significance with a P-value of 0.129.[3] This difference in results might be due to the increment of CD4 count after the initiation of ART.[15] In this study the risk of thrombocytopenia was higher in WHO clinical stages III and IV compared with WHO clinical stages I and II. This finding was in agreement with a previous study.[7]

The current study revealed that patients on an AZT-based ART regimen had a higher prevalence of of thrombocytopenia compared with those on a TDF-based regimen. A study conducted in Ethiopia showed the prevalence of thrombocytopenia was higher among HIV-infected patients after the initiation of ART.[9] However, there was no significant association between the presence of thrombocytopenia and types of ART-based regimen. The high prevalence of thrombocytopenia observed in zidovudine-based therapy could be due to destruction of both platelets and mega karyotypes by an immune-mediated reaction which can occurin a zidovudine containing ART-regimen.[16]

Conclusion

In the present study the prevalence of thrombocytopenia decreased significantly after the initiation of ART. HIV patients with low CD4 counts, advanced clinical stages and on AZT-based therapy had ahigh chance of developing thrombocytopenia. According to this study, the thrombocytopenic patients were observed even after the initiation of ART. In order to decrease thrombocytopenic associated mortality and morbidity there should be continuous screening for HIV-infected patients.

Abbreviations

AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; AZT, zidovudine; CD4, cluster of differentiation; HIV, human immunodeficiency virus; TDF, tenofovir; WHO, World Health Organization.

Data Sharing Statement

Data used and analyzed during the current study are available on a reasonable request from the main authors with acceptable reason.

Ethical Clearance and Consent Permission

This study was conducted after ethical letters obtained from the Institute of Research Ethics and Review Board (IRB), Institute of Medicine and Health Sciences of Debre Berhan University and complied with the Declaration of Helsinki. The institute of research and ethics board committee (IRB) had reviewed and looked at originality, feasibility; laboratory setting, and ethical aspects of the study. Following

746

Journal of Blood Medicine 2021:12

thorough discussion the committee approved the research proposal by authors with ethical approval using reference number med/220/2019. Then permission was taken from hospital higher management and data were collected after obtaining informed consent from the study participants. To keep confidentiality, codes were used and unauthorized persons did not have access to the data.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Kumar MB, Thippeswamy T, Shankar R, Prathima C. Hematological abnormalities in early and advanced HIV infection patients. *Int J Sci Study*. 2016;3(11):1–5.
2. Shen Y, Wang J, Wang Z, et al. A cross-sectional study of leukopenia and thrombocytopenia among Chinese adults with newly diagnosed HIV/AIDS. *Biosci Trends*. 2015;9(2):91–96. doi:10.5582/bst.2015.01024
3. Tamir Z, Seid A, Haileslassie H. Magnitude and associated factors of cytopenias among antiretroviral therapy naïve human immunodeficiency virus infected adults in Dessie, Northeast Ethiopia. *PLoS One*. 2019;14(2):e0211708. doi:10.1371/journal.pone.0211708
4. Enawgaw B, Alem M, Addis Z, Melku M. Determination of hematological and immunological parameters among HIV positive patients taking highly active antiretroviral treatment and treatment naïve in the antiretroviral therapy clinic of Gondar University Hospital, Gondar, Northwest Ethiopia: a Comparative Cross-Sectional Study. *BMC Hematol*. 2014;14(1):1–7.
5. Akinbami A, Oshinaike O, Adeyemo T, et al. Hematologic abnormalities in treatment-naive HIV patients. *Infect Dis*. 2010;3:IDRT.S6033.
6. Gebreweld A, Fiseha T, Girma N, Haileslasie H, Gebretsadik D. Prevalence of cytopenia and its associated factors among HIV infected adults on highly active antiretroviral therapy at Mehal Meda Hospital, North Shewa Zone, Ethiopia. *PLoS One*. 2020;15 (9):e0239215. doi:10.1371/journal.pone.0239215
7. Khayati S, Bahri R, El Ferssani F, Yahyaoui H, Ait M, Ameur MC. Biological haematological manifestations of HIV infection. *Hemoglobin*. 2020;20:80.
8. Taremwa IM, Muyindike WR, Muwanguzi E, Boum Y, Boum Y II. Prevalence of HIV-related thrombocytopenia among clients at Mbarara Regional Referral Hospital, Mbarara, southwestern Uganda. *J Blood Med*. 2015;6:109. doi:10.2147/JBM.S80857
9. Littleton N. Thrombocytopenia in HIV. *Contin Med Educ*. 2007;25 (6).
10. Durandt C, Potgieter J, Mellet J, et al. HIV and haematopoiesis. *S Afr Med J*. 2019;109(8 Supplement 1):S41–6. doi:10.7196/SAMJ.2019.v109i8b.13829
11. Ambler KLS, Vickars LM, Leger CS, et al. Clinical features, treatment, and outcome of HIV-associated immune thrombocytopenia in the HAART era. *Adv Hematol*. 2012;2012:910954. doi:10.1155/2012/910954
12. Woldeamanuel GG, Wondimu DH. Prevalence of thrombocytopenia before and after initiation of HAART among HIV infected patients at black lion specialized hospital, Addis Ababa, Ethiopia: a Cross Sectional Study. *BMC Hematol*. 2018;18(1):9. doi:10.1186/s12878-018-0103-6
13. Wondimeneh Y, Muluye D, Ferede G. Prevalence and associated factors of thrombocytopenia among HAART naive HIV positive patients at Gondar university hospital, northwest Ethiopia. *BMC Res Notes*. 2014;7(1):5. doi:10.1186/1756-0500-7-5
14. Ananworanich J, Phanuphak N, Nuesch R, et al. Recurring thrombocytopenia associated with structured treatment interruption in patients with human immunodeficiency virus infection. *Clin Infect Dis*. 2003;37(5):723–725. doi:10.1086/376989
15. Montaner JS, Le T, Fanning M, et al. The effect of zidovudine on platelet count in HIV-infected individuals. *J Acquir Immune Defic Syndr*. 1990;3(6):565–570.
16. Addis Z, Yitayew G, Tachebele B. Prevalence of some hematological abnormalities among HIV positive patients on their first visit to a tertiary health institution in ethiopia; a Cross Sectional Study. *Int Blood Res Rev*. 2014;2(6):270–278. doi:10.9734/IBRR/2014/11137
17. Hekimoğlu CH, Kaptan F, Vardar I, et al. Prevalence and associated factors of thrombocytopenia among human immunodeficiency virus-infected patients at a tertiary care hospital in Izmir, Turkey. *Turk J Med Sci*. 2017;47(1):69–75. doi:10.3906/sag-1510-140
18. Kulkarni MB, Bhalerao MM, Mokal SJ, Krishnan B. Thrombocytopenia among HIV infected patients attending a tertiary care hospital in India. *Indian J Clin Anat Physiol*. 2021;7 (4):363–366. doi:10.18231/j.ijcap.2020.076
19. Fan H-W, Guo F-P, Li Y-J, Li N, Li T-S. Prevalence of thrombocytopenia among Chinese adult antiretroviral-naive HIV-positive patients. *Chin Med J*. 2015;128(4):459. doi:10.4103/0366-6999.151078
20. Fan L, Li C, Zhao H. Prevalence and risk factors of cytopenia in HIV-infected patients before and after the initiation of HAART. *Biomed Res Int*. 2020;2020:3132589. doi:10.1155/2020/3132589
21. Abdulqadir I, Ahmed SG, Kuliya AG, Tukur J, Yusuf AA, Musa AU. Hematological parameters of human immunodeficiency virus positive pregnant women on antiretroviral therapy in Aminu Kano Teaching Hospital Kano, North Western Nigeria. *J Lab Physicians*. 2018;10 (1):60–63. doi:10.4103/JLP.JLP_80_17

The Correlation Between Iron Overload and Endocrine Function in Adult Transfusion-Dependent Beta-Thalassemia Patients with Growth Retardation

Tubagus Djumhana Atmakusuma, Faizal Drissa Hasibuan & Dyah Purnamasari

Submit your article to this journal

View related articles

View Crossmark data

ORIGINAL RESEARCH

The Correlation Between Iron Overload and Endocrine Function in Adult Transfusion-Dependent Beta-Thalassemia Patients with Growth Retardation

Tubagus Djumhana Atmakusuma [ID][1]
Faizal Drissa Hasibuan[2]
Dyah Purnamasari [ID][3]

[1]Division of Hematology-Medical Oncology, Department of Internal Medicine, Dr. Cipto Mangunkusumo General Hospital/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia; [2]Division of Hematology-Medical Oncology, Department of Internal Medicine, Yarsi Hospital, Jakarta, Indonesia; [3]Endocrine and Metabolic Division, Department of Internal Medicine, Dr. Cipto Mangunkusumo General Hospital/Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

Background: Iron overload is a major problem in patients with transfusion-dependent beta-thalassemia (TDT). Reports on the correlation between iron overload and endocrine function with growth retardation in such a population in Indonesia have not been established. Therefore, this study aims to obtain a profile of iron load and endocrine function of adult transfusion dependent beta-thalassemia patients and their correlation with growth retardation.

Methods: A cross-sectional study was performed, involving adult homozygous and HbE beta-thalassemia patients receiving blood transfusions at the Cipto Mangunkusumo Hospital, Jakarta. Iron overload was represented by serum ferritin (FS) and transferrin saturation (TS), while the endocrine function was examined by the Thyroid Stimulating Hormone-sensitive (TSHs), free T4 (fT4), and insulin-like growth factor-1 (IGF-1). The results were analyzed using bivariate analysis plus Pearson and Spearman correlation tests.

Results: In general, 58 subjects were selected from 224 adult transfusion dependent beta-thalassemia patients, consisting of 31 males (53.4%) and 27 females (46.6%). Furthermore, their median age was 21 (18–24) years, while the subclinical hypothyroid proportion was 32.7% and low IGF-1 levels were detected in 79.3% of the total population. There was a weak negative correlation between FS and fT4 (Spearman rho=−0.361; p=0.003), as well as IGF-1 (Spearman rho=−0.313; p=0.008), but FS and TSHs had no correlation (Spearman rho=0.074; p=0.29). Also, there was no correlation between ST with TSHs (Spearman rho=0.003; p=0.492), fT4 (Spearman rho=0.018; p=0.448), and IGF-1 (Spearman rho= −0.142; p=0.143).

Conclusion: Based on serum ferritin, iron overload is discovered to have a negative correlation with free T4 and insulin-like growth factor-1.

Keywords: IGF-1, TSHs, fT4, serum ferritin, transferrin saturation, transfusion-dependent thalassemia

Correspondence: Tubagus Djumhana Atmakusuma
Division of Hematology-Medical Oncology, Department of Internal Medicine, Dr. Cipto Mangunkusumo General Hospital/Faculty of Medicine Universitas Indonesia, Jalan Diponegoro No. 71, Jakarta Pusat, DKI Jakarta, Indonesia
Tel +6221-3162497
Email hom_fkui@yahoo.com

Background

Thalassemia is a hereditary disease caused by a defect in the synthesis of globin. Moreover, beta-thalassemia is the commonest type, affecting about 80–90 million people or 1.5% of the global population. Indonesia is one of the countries with a high thalassemia prevalence of the disease, where the beta-thalassemia trait carrier is estimated as about 3–10%.[1,2] With a population of approximately 250 million and a 20% birth rate, about 5000 babies are predicted to be born with beta-thalassemia.

Based on the data from Cipto Mangunkusumo Hospital (RSCM) in 2016, 9031 people were suffering from thalassemia major in Indonesia. Also, up to 441 adult patients (age over 18 years) were recorded in Kiara thalassemia and Hematology-oncology department of Internal Medicine clinics.[3]

Thalassemia major (TM) is one of the thalassemia phenotypes characterized by severe anemia. Patients with TM require regular red blood cell transfusions, which are mandatory for their survival, therefore this condition is called TDT. Repeated red blood cell transfusions lead to iron overload in tissues or organs, thereby causing an impairment called iron toxicity which has several targets including the endocrine system. In addition, endocrine disorders are often detected in those suffering from TDT. Some studies indicated approximately 40–50% of thalassemia patients that received adequate transfusion therapy remained to have endocrine disorders such as a decrease in hormone levels or substances produced by the endocrine organs.[4–8]

Many of the thalassemia patients in Cipto Mangunkusumo Hospital experience growth retardation which has an impact on their psychosocial development. Several factors contribute to the development of growth retardation such as inadequate transfusion causing chronic anemia, iron overload, malnutrition, and desferrioxamine toxicity. Iron overload in endocrine organs leads to hypogonadism, disorders of growth hormone-insulin-like growth factor 1 (GH-IGF-1) axis, and hypothyroidism.[9–12]

Iron toxicity is caused by an overload of the respective element, a condition related to non-transferrin-bound iron (NTBI). Furthermore, NTBI examination has not been standardized and is not available in Indonesia, but TS is used as a surrogate marker of this process.[13–15]

T2* MRI is the gold standard for assessing iron overload and toxicity.[16,17] However, in centers with limited resources, serum ferritin (SF) is often used to assess iron overload. Besides, the pancreas is an endocrine organ routinely measured using MRI T2*. The examination has become a standard procedure in developed countries, but it is still only used for study purposes in Indonesia. Since this sophisticated modality is not available nationwide, TS and SF are targeted to be used as surrogate markers of iron overload.

Soesanti et al[18] conducted a study on 67 children with thalassemia in 2012. The result showed the prevalence of endocrine disorders as follows: 64% growth retardation, 41% hypothyroidism, and 20% late puberty. There is no publication of endocrine function in adult TDT beta patients with growth retardation in Indonesia yet.

Therefore, this study aims to determine the correlation between iron overload and endocrine function.

Methods

This was a cross-sectional study that obtained a profile of iron load and endocrine function correlation in adult beta TDT patients with growth retardation in Cipto Mangunkusumo Hospital in December 2017. The endocrine function was observed by the thyroid hormone levels (Thyroid Stimulating Hormone-sensitive/TSHs and free T4/fT4) and insulin-like growth factor-1 (IGF-1).

Patients diagnosed with beta-thalassemia and beta-HbE (with high-performance liquid chromatography/HPLC or microcapillary) aged 18 years with growth retardation were included as subjects. The exclusion criteria were HbsAg or anti-HCV positive patients or when the subject did not agree to participate. This study was approved by the Ethical Committee of the Faculty of Medicine, Universitas Indonesia, as listed on the Ethical Approval Letter number 1133/UN.2.F1/ETIK/2017. All patients provided appropriate informed consent and were treated in accordance with the Declaration of Helsinki.

The subjects were recruited consecutively from Kiara thalassemia Polyclinic and Hematology-oncology department of Internal Medicine clinic. Each of them passed through venous blood sampling prior to the transfusion process. The blood samples collected were up to two tubes, namely 7 mL and 3 mL in size. The TSHs, FT4, Ferritin, SF, and TS examinations were performed at the clinical pathology laboratory of Cipto Mangunkusumo Hospital Jakarta, using chemiluminescence immunoassay (CMIA) method by Abbott™ Architect® i1000/i2000 device. Levels of SI and TIBC were measured using the direct colorimetric method by Abbott™ Architect® c4000/8000 device. Meanwhile, IGF-1 level was measured using the Solid-Phase method Electro-Chemiluminescence Immunoassay (ECLIA) by Siemens™ Immulite® 1000.

The data obtained were analyzed using Pearson's correlation test for those with normal distribution or Spearman correlation test for the ones with an abnormal (nonparametric) distribution. Since data distribution was abnormal, the nonparametric Spearman correlation was used.

Results

In December 2017, 68 people were diagnosed with growth retardation among 224 adult TDT beta patients in Kiara thalassemia Polyclinic and Hematology-oncology department of Internal Medicine clinic. Then, 10 of the 68 that

met the inclusion criteria were not willing to continue, therefore only 58 completed the study.

The subjects were 27 (46.6%) females and 31 (53.4%) males (Table 1). Furthermore, their median age was 21 years, with a range of 18 to 24 years. The number of homozygous beta-thalassemia patients was 31 (53.4%) and those with HbE beta-thalassemia were 27 (46.6%). The Mid Parental Height as a benchmark target for the patient's height was 161.25 cm. Meanwhile, the average height of the subjects was 149.05 cm.

The Proportion of Endocrine Disorders on Study Subjects

Table 2 shows the proportion of low IGF-1 was 79.3%, and subclinical hypothyroidism was 32.7%. The subclinical hypothyroidism was characterized by elevated TSHs (≥5 mu/L) with normal fT4 levels, and most patients had no complaints.

The Correlation Between Iron Overload and Endocrine Function in Adult TDT Beta Patients

The results from Spearman's test showed no significant correlation between transferrin saturation with TSHs, fT4, and IGF-1 (Table 3).

Table 1 Baseline Characteristics of Research Subject

Variables	N = 58
Gender, n (%)	
Male	31 (53.4)
Female	27 (46.6)
Diagnosis, n (%)	
Homozygous Beta Thalassemia	31 (53.4)
HbE Beta Thalassemia	27 (46.6)
Age (years), median (min-max)	21 (18–24)
Pre-transfusion hemoglobin, mean (SD)	8.98 (1.15)
Weight (kg), median (min-max)	40.9 (27–60)
BMI (kg/m^2), median (min-max)	18.79 (14.60–76.18)
Height (cm), mean (SD)	149.05 (8.47)
Mid Parental Height/MPH (cm), mean (SD)	161.25 (9.21)
Serum ferritin (ng/mL), median (min-max)	5,340 (355–22,352)
Transferrin saturation (%), median (min-max)	100 (9–107)
TSHs (mu/L), median (min-max)	3.36 (0.66–8.4)
fT4 (ng/dL), median (min-max))	1.11 (0.76–1.96)
IGF-I (ng/mL), median (min-max)	60.5 (24–206)
MRI T2 * Pancreas, median (min-max)	13.41 (3.96–57.4)

Abbreviations: SD, standard deviation; TSHs, thyroid stimulating hormone-sensitive; FT4, free T4; IGF-1, insulin-like growth factor-1; MRI, magnetic resonance imaging.

Table 2 Proportion of Endocrine Function in Adult Beta TDT Patients with Growth Retardation

Variables	n (%)
Euthyroid or normal	39 (67.3)
Hypothyroidism	0
Subclinical hypothyroidism	19 (32.7)
IGF-I	
Normal	12 (20.7)
Low	46 (79.3)

Abbreviations: TDT, transfusion dependent thalassemia; IGF-1, insulin-like growth factor-1.

Meanwhile, there was a weak significant correlation between serum ferritin with fT4 and IGF-1 (r = −0.361; p = 0.003 and r = −0.313; p = 0.008), but FS and TSHs had no correlation (Table 4)

It can be concluded that there is a negative correlation between serum ferritin with fT4 and IGF-1. However, FS and TSHs have no correlation, hence transferrin saturation does not correlate with TSHs, fT4, and IGF-1.

Discussion

This study is the first to report the proportion of endocrine function disorders in adult TDT patients with growth retardation and its correlation with iron overload in the Indonesian population, particularly in the RSCM Jakarta.

The average values of the subjects' TSHs and fT4 were in the normal range. But, there were 32.7% of patients with subclinical hypothyroidism, a condition that has an unclear mechanism in TDT beta occurrence. This is probably due to thyroid tissue damage causing iron overload which releases free radicals, while the pituitary has not been damaged. Low TSHs and fT4 levels were not detected, indicating there was no serious damage to the pituitary and thyroid glands due to iron overload.

In this study, the proportion of low IGF-1 was 79.3% with an average median value of 60.5 ng/mL. Poggi et al[8] did not report the proportion of low IGF-1, but only a mean value of

Table 3 Correlation Between Transferrin Saturation with TSH, fT4 and IGF-1

Variables	R	P
Transferrin saturation-TSHs	0.003	0.492
Transferrin saturation-FT4	0.018	0.448
Transferrin saturation-IGF1	−0.142	0.143

Abbreviations: TSHs, thyroid stimulating hormone-sensitive; FT4, free T4; IGF-1, insulin-like growth factor-1.

Table 4 The Correlation Between Serum Ferritin with TSH, fT4 and IGF-1

Variables	r	p
Serum ferritin-TSHs	0.074	0.290
Serum ferritin -FT4	−0.361	0.003*
Serum ferritin -IGFI	−0.313	0.008*

Abbreviations: TSHs, thyroid stimulating hormone-sensitive; FT4, free T4; IGF-1, insulin-like growth factor-1.**Note:** *p<0.05.

88.2±39.9 ng/mL. However, Rashid et al[20] obtained 51.43%, and Soliman et al[5] obtained 67%. Also, Scacchi et al[22] reported 46.8% and Moayeri, and Oolomi[25] discovered 42% in the group that was growth retarded. From the description above, the proportion of low IGF-1 in this study was greater than in previous ones possibly due to the higher iron overload.

Iron overload on the pituitary, thyroid, and liver causes iron toxicity that damages the cells playing a role in the synthesis of TSH, fT4, and IGF-1. Consequently, the levels of these hormones are reduced, leading to hypothyroidism or growth retardation.

In the test conducted, transferrin saturation had no significant correlation with TSH, fT4, and IGF-1. Supposedly as a surrogate marker of iron overload, TS correlated with endocrine variables. This was probably because transferrin saturation measurements were performed only once. Hence, the effect of iron overload for a long time was not envisaged. Similarly, Al-Hakeim et al[24] reported there was no transferrin saturation correlation with TSH and T4.

There was a weak negative correlation between serum ferritin with fT4 and IGF-1 (r = −0.361; p = 0.003 and r = −0.313; p = 0.008), but serum ferritin and TSHs had no significant correlation (r = 0.074; p = 0.29). The correlation between FS with fT4 and IGF-1 showed a decreased fT4 and low IGF-1 due to iron overload in the thyroid gland and liver. In contrast to Soliman et al[19] there is no significant correlation between FS and IGF-1. This is related to Eshragi et al[21] that did not obtain any correlation between FS and hypothyroidism as well as Zervas et al[23] that obtained no correlation between FS and thyroid disorders. In other Soliman et al[19] studies, a negative correlation was obtained between FS and fT4, however, FS and TSH had no correlation, which is similar to this current study.

Conclusions

In general, the proportion of IGF-1 is quite high, namely 79.3%, while that of subclinical hypothyroidism is 32.7% and MRI T2* pancreas is 87.5% in the group of adult TDT

beta patients that experienced growth retardation. Proportions of with low value of in adult patients who experienced a beta TDT beta growth retardation. As assessed by serum ferritin, there is a negative correlation between iron overload with fT4 and IGF-1. However, there is no correlation between FS and TSHs, hence transferrin saturation does not correlate with TSHs, fT4, and IGF-1.

Data Sharing Statement

Additional data can be requested by contacting the corresponding author on the email address provided.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Wahidayat I, Wahidayat PA. Genetic problems at present and their challenges in the future: as a model of thalassemia. *Paediatr Indonesiana*. 2006;46(5):189–194. doi:10.14238/pi46.5.2006.189-94
2. Sofro ASM. Molecular pathology of beta-thalassemia in Indonesia. *Southeast Asian J Trop Med Public Health*. 1995;26:221–224.
3. Thalassemia Center Cipto Mangunkusumo Hospital. *Data Thalassemia Major Patients in Indonesia in 2015–2016*. Jakarta; 2016.
4. Sanctis VD, Soliman AT, Skordis N. Endocrine disease. In: Cappellini CA, Porter J, editors. *Guidelines for the Management of Transfusion-Dependent Thalassemia (TDT)*. 3rd ed. Cyprus: Thalassemia International Federation; 2014:146–157.
5. Soliman A, Sanctis VD, Yassin M, Abdelrahman MO. Growth hormone – insulin-like growth factor-I axis and bone mineral density in adults with thalassemia major. Indian J Endocrinol Metab. 2014;18 (1):32c–8.
6. Abdelhadi MA. Endocrinopathies in beta-thalassemia major: prevalence, risk factors, and age at diagnosis in Northwest Saudi Arabia. *Saudi Med J*. 2013;34(1):67–73.
7. Perera NJ, Lau NS, Mathew S, Waite C, Ho PJ, Caterson ID. Overview of endocrinopathies associated with b-thalassemia major. *Intern Med J*. 2010;40(10):689–696. doi:10.1111/j.1445-5994.2010.02254.x
8. Poggi M, Pascucci C, Monti S, et al. Prevalence of adult growth hormone deficiency in β-thalassemia polytransfused Patients and Correlation with transfusional and chelation parameters. *J Endocrinol Invest*. 2010;33(8):534–538. doi:10.1007/BF03346643

9. Sanctis VD, Urso L, Scialpi V. Endocrine complications in β-thalassemia major. In: Maggio A, Hoffbrand AV, editors. *Clinical Aspects and Therapy of Hemoglobinopathies*. Italy: SEE-Firenze; 2014:293–315.

10. Olivieri NF, Weatherall DJ. Clinical aspects ofβ-thalassemia and related disorders. In: Steinberg MH, Forget BG, Higgs DR, editors. *Disorders of Hemoglobin: Genetics, Pathophysiology and Clinical Management*. UK: Cambridge University Press; 2009:357–369.

11. Kuskonmaz BC. Endocrinological problems in adult thalassemia patients. *Gazi J Med*. 2014;25:173–176.

12. Tzoulis P. Review of endocrine complications in adult patients with β-thalassemia major. *Thalassemia Rep*. 2014;4:51–56.

13. Kohgo Y, Ikuta K, Ohtake T, Torimoto Y, Kato J. Body of iron metabolism and pathophysiology of iron overload. *Int J Hematol*. 2008;88(1):7–15. doi:10.1007/s12185-008-0120-5

14. Crichton R. *Iron and Oxidative Stress. Iron Metabolism - from Molecular Mechanisms to Clinical Consequences*. 3rd ed. UK: Willey; 2009:346–367.

15. Barton JC, Edwards CQ, Phatak PD, Britton RS, Bacon BR. Normal Iron Metabolism and Iron Toxicity. In: *Handbook of Iron Overload Disorders*. 1st ed. UK: Cambridge University Press; 2010:10–32.

16. Argyropoulou MI, Kiortsis DN, Astrakas L, Metafratzi Z, Chalissos N, Efremidis SC. Liver, bone marrow, pancreas and pituitary gland iron overload in young and adult thalassemic patients: a T2 Relaxometry Study. *Eur Radiol*. 2007;17(12):3025–3030. doi:10.1007/s00330-007-0683-1

17. Chirico V, Rigoli L, Lacquniti A, et al. Endocrinopathies, metabolic disorders, and iron overload in thalassemia major and intermedia: serum ferritin as diagnostic and predictive markers associated with liver and cardiac T2 * MRI assessment. *Eur J Haematol*. 2014;1–9.

18. Soesanti F, Putriasih SA, Pulungan A, Wahidiyat PA. Endocrinopathies in thalassemia major patients in thalassemia center in Jakarta, Indonesia. *Int J Pediatr Endocrinol*. 2013;2013(S1):58. doi:10.1186/1687-9856-2013-S1-P58

19. Soliman AT, Al Yafei F, Al-Naimi L, Almarri N, Sabt A, Yassin M, et al. Longitudinal study on thyroid function in patients with thalassemia major: high incidence of central hypothyroidism by 18 years. Indian J Endocrinol Metab. 2013;17(6):1090–5.

20. Rashid HM, Shirodkar A, Ahmed J. Evaluation of growth, puberty and endocrine dysfunctions in relation to iron overload in thalassemia patients multitransfused Indian. *Indian J Pediatr*. 2011;78 (6):679–683. doi:10.1007/s12098-010-0351-3

21. Eshragi P, Tamaddoni A, Zarifi K, Mohammadhasani A, Aminzadeh M. Thyroid function in patients with thalassemia major: is it related to height and chelation therapy ? *Casp J Intern Med*. 2011;2(1):190–193.

22. Scacchi M, Danesi L, Cattaneo A, Valassi E, Giraldi FP, Argento C, et al. Growth hormone deficiency (GHD) in adult thalassaemic patients. Clin Endocrin. 2007;67(5):790–5.

23. Zervas A, Katopodi A, Protonotariou A, Livadas S, Karagiorga M, Politis C, et al. Assessment of thyroid function in two hundred patients with betathalassaemia major. Thyroid. 2002;12:151–4.

24. Al-Hakeim HK, Al-Hakany MF. The effect of iron overload on the function of some endocrine glands in β-thalassemia major patients. Magazin of Al-kufa University for Biology. 2013;5:1–22.

25. Moayeri H, Oolomi Z. Prevalence of growth and puberty failure with respect to growth hormone and gonadotropins secretions in beta-thalassemia major. *Arch Iran Med*. 2006;9(4):329–334.

26. Hasibuan FD, Atmakusuma TD, Purnamasari D, Rumende CM. *Korelasi antara muatan besi berlebih dengan fungsi endokrin pada pasien dewasa thalassemia beta bergantung transfusi yang mengalami retardasi pertumbuhan [The Correlation Between Iron Overload and Endocrine Function in Adult Transfusion Dependent Beta Thalassemia Patients with Growth Retardation]* [Thesis]. Indonesia; 2018.

Taylor & Francis

Why is Misdiagnosis of von Willebrand Disease Still Prevalent and How Can We Overcome It? A Focus on Clinical Considerations and Recommendations

Chanukya K Colonne, Benjamin Reardon, Jennifer Curnow & Emmanuel J Favaloro

Published online: 17 Aug 2021.

Submit your article to this journal ⬚

Article views: 371

View related articles ⬚

View Crossmark data ⬚

Citing articles: 26 View citing articles ⬚

REVIEW

Why is Misdiagnosis of von Willebrand Disease Still Prevalent and How Can We Overcome It? A Focus on Clinical Considerations and Recommendations

Chanukya K Colonne [1,*]
Benjamin Reardon [1,*]
Jennifer Curnow [2–4]
Emmanuel J Favaloro [1,4,5]

[1]Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), NSW Health Pathology, Westmead Hospital, Sydney, NSW, Australia; [2]Department of Clinical Haematology, Westmead Hospital, Sydney, NSW, Australia; [3]Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia; [4]Sydney Centres for Thrombosis and Haemostasis, Sydney, NSW, Australia; [5]School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia

*These authors contributed equally to this work

Correspondence: Emmanuel J Favaloro
Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead, Sydney, NSW, 2145, Australia
Tel +612 8890 6618
Fax +612 9689 2331
Email Emmanuel.Favaloro@health.nsw.gov.au

Abstract: Despite von Willebrand disease (VWD) being the most common inherited bleeding disorder, its accurate diagnosis is frequently shrouded by diagnostic pitfalls. VWD is frequently under-diagnosed, over-diagnosed and misdiagnosed, leading to significant avoidable patient morbidity and health care system burden. At the heart of this dilemma lies the heterogeneity and complexity of von Willebrand factor (VWF) and associated defects, and the necessity of coalescing clinical and laboratory features to obtain an accurate diagnosis. Common pitfalls include poor clinical and scientific understanding and familiarity with VWD, incomplete clinical history and lack of routine use of standardised bleeding assessment tools (BAT), difficulty in accessing a comprehensive repertoire of laboratory tests, significant pre-analytical, analytical and post-analytical issues, and lack of expertise in laboratory testing and interpretation. Errors, resulting in under-diagnosis, over-diagnosis, and misdiagnosis of VWD, are presented and discussed. Strategies to minimise errors include better education of clinicians and laboratory staff on VWD, routine use of validated BAT, utilising a comprehensive gamut of laboratory investigations according to a standardised algorithm, and repeating testing to minimise pre-analytical errors. Recommendations on appropriate patient selection for VWD testing, how VWD should be investigated in the laboratory, and how to ensure test results are accurately interpreted in the correct clinical context are detailed.

Keywords: von Willebrand disease, VWD, diagnosis

Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder.[1,2] Despite this, VWD is one of the most commonly misdiagnosed or overlooked entities in everyday clinical practice. Of interest, VWD may be both over- and under-diagnosed, as well as misdiagnosed, either as another entity or as a different subtype of the disorder. This review will detail the causes underlying the diagnostic uncertainties overshadowing VWD and provide solutions on how to overcome these.

von Willebrand Disease

VWD was first described by Finnish physician Erik Adolf von Willebrand in 1926, following the presentation of a young girl with recurrent episodes of bleeding,

which were clinically distinct from haemophilia.[3] VWD is caused by quantitative or qualitative deficiencies in a plasma protein now called von Willebrand factor (VWF). VWF is a large, complex protein that has essential roles in primary and secondary hemostasis.[4] High-molecular-weight VWF multimers mediate platelet adhesion at sites of vascular injury in primary hemostasis by binding to connective tissue and platelets.[4] VWF also plays a key role in secondary hemostasis, acting as a chaperone to clotting factor VIII (FVIII) by binding to and stabilizing it in the circulation.[4] The multifunctional nature of VWF explains the heterogeneity in clinical symptoms and bleeding risk seen in VWD, as well as the resulting diagnostic challenges.[5]

VWD is autosomally inherited and arises due to mutations in *VWF*, mapped at 12p13.3 and comprised of 52 exons.[6] Approximate exon locations have been mapped to binding locations on the VWF protein with corresponding

defects. The majority of mutations in VWD are missense, with some having variable penetrance, particularly in Type 1 VWD.[4]

VWD is classified into three major types (summarised in Table 1). Type 1 reflects a mild to moderate reduction in functionally normal VWF; Type 2 involves the expression of functionally abnormal VWF; and, Type 3 is the (near) complete absence of VWF.[4] 65–80% of identified VWD cases are Type 1, whereas Type 2 and 3 account for 20–35% and <1% of cases, respectively.[7] A potential subtype of Type 1 called VWD Type 1C has been proposed. In contrast to classic VWD type 1, where there is a reduction in the production of VWF and/or a mild increased clearance, VWD Type 1C is characterised by a significant increase in clearance of VWF resulting in low VWF levels and an exaggerated but short-lived response to desmopressin.[8] Type 2 VWD is separated into four 'subtypes' (2A, 2B, 2M, 2N), according to the functional

Table 1 Classification Scheme for von Willebrand Disease, Inheritance, Genetic Defect and Phenotypic Presentation

Type	Mechanism	Inheritance	Genetic Defect	Clinical Phenotype
1	Partial quantitative deficiency VWF	Autosomal dominant	Missense mutation (85–90%), null-alleles (10–15%), variable penetrance	Severity of bleeding associated with level of VWF (the lower the level, the more severe the bleeding presentation)
2A	Decreased VWF-dependent platelet adhesion due to deficiency of HMW VWF multimers	Autosomal dominant and recessive	Missense mutations, mainly in D3, A2 and CK domains Missense mutations in propeptide Exons 5, 28 and 52	Severity of bleeding associated with level and functionality of VWF.
2B	Increased affinity of VWF for platelet GPIb	Autosomal dominant	Missense mutations in A1 domain Exon 28	Generally, presents with moderate or moderately severe bleeding.
2M	Decreased VWF-dependent platelet adhesion without a selective deficiency of MHW VWF multimers	Autosomal dominant	Missense mutations in A1 domain Exons 28, 45	Severity of bleeding associated with level and functionality of VWF.
2N	Decreased binding affinity of VWF for Factor VIII	Autosomal recessive	Missense mutations in D' and D3 domains Exons 5, 10, 18	Generally present with moderate or moderately severe bleeding and may have a phenotype more typically seen in mild or moderate hemophilia A (due to factor VIII deficiency): soft tissue, joint, and urinary bleeding, and bleeding after invasive procedures.
3	Complete deficiency of VWF	Autosomal recessive	Null-alleles, often consanguinity	Most severe form of VWD, and patients show bleeding symptoms similar to moderate or severe hemophilia A.

defect. A separate defect affecting the platelet receptor for VWF, namely glycoprotein Ib (GPIb) is called platelet type (PT-) VWD.

VWD diagnosis requires the presence of both clinical features, such as a personal (typically lifelong) history of primarily mucocutaneous bleeding, and laboratory evidence of absence, deficiency, or defect in VWF. This is usually accompanied by a family history of the disease.

VWD has a variable clinical presentation due to the heterogeneity of the disease.[9] VWD is mainly associated with mucocutaneous bleeding, although there are more severe bleeding phenotypes, which carry significant morbidity and mortality.[10] Mucocutaneous bleeding may present as spontaneous or minimally provoked bruising, excessive bleeding from minor wounds, gum bleeding, epistaxis, menorrhagia and bleeding from the gastrointestinal tract.[11] Site of bleeding may also change with patient age, with higher rates of gastrointestinal bleeding reported in older age groups.[12] Other than spontaneous bleeding, patients with VWD also experience bleeding after invasive procedures, such as surgery or tooth extraction and during hemostatic challenges, such as childbirth or trauma.[6] The severity of VWD ranges from very mild, with bleeding only after major procedures, up to spontaneous bleeding, including muscle and joint bleeding, in the most severe cases.

Clinical presentation and bleeding history can also assist with diagnosis (see Table 1). Type 1 VWD has a range of clinical presentations, from mild to severe, with bleeding phenotype broadly corresponding to the level of plasma VWF. Type 3 presents as the most severe phenotype of VWD, and is sometimes likened to moderate to severe hemophilia A. Type 2A and 2M presentations correspond to the quantity and functionality of VWF. Types 2B and 2N VWD generally present as a moderate to severe bleeding phenotype. Type 2N may also present with joint bleeding or urinary bleeding and presents as phenotypically similar to mild-to-moderate hemophilia A, but instead is due to the inability of VWF to appropriately bind to (and thus protect) Factor VIII.[6]

The assessment of bleeding history is the most important initial step in the analysis of a suspected bleeding tendency, as laboratory evaluation will only be initiated after the appropriate clinical suspicion.[9] The severity of bleeding diathesis can be judged by the frequency of bleeding events as well as the age of onset.[11] A patient's bleeding phenotype can be identified and quantified with the use of various tools including the International Society

on Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) and should be used to aid in clinical suspicion of a diagnosis of VWD (Table 2).[13] A lack of understanding of the difference between "normal" and "abnormal" bleeding symptoms is a common reason for misdiagnosis of VWD.[13]

Bleeding frequency and severity are influenced by many factors, which must be carefully evaluated to avoid diagnostic oversight. Important considerations for diagnosis are the age and gender of the patient, their comorbidities, their family history of bleeding diathesis and diet (for relative vitamin C intake). It is essential to ascertain whether the patient is using any anticoagulant or antiplatelet medications, as well as otherwise undisclosed over-the-counter or herbal remedies, such as glucosamine, ginkgo, garlic, ginseng, fish oil, primrose oil, echinacea, dong quai or feverfew, all of which can dampen hemostasis and thus increase bleeding risk.[14] Concurrent diagnoses such as joint hypermobility may also exaggerate joint-based symptoms.

Differential diagnoses for an individual who presents with symptoms consistent with VWD include mild haemophilia A or mild haemophilia B, vitamin C deficiency, platelet function disorder, or deficiency of other coagulation factors (II, VII, X or XI). In addition, should VWD be identified, the type of VWD should also be characterised. Optimal management of patients depends on an accurate diagnosis, and an error in diagnosis will potentially lead to inappropriate testing and compromise patient management, including over-diagnosis and unnecessary treatment.

Laboratory Testing

While thorough clinical evaluation is essential to guide appropriate investigations in the workup of VWD, a lack of expertise in laboratory testing and interpretation remains a common reason for diagnostic inaccuracies.

The first step to a correct laboratory diagnosis is ensuring the most appropriate tests are performed for the correct indication. Testing for VWD should be considered when there is a bleeding history, a positive family history, a mild unexplained thrombocytopenia, a mildly prolonged activated partial thromboplastin time (APTT), or an apparent hemophilia A in a female. Even males "diagnosed" with hemophilia A should be considered for VWD testing, as Type 3 or Type 2N VWD can sometimes be misdiagnosed as hemophilia A.

An approach to testing is outlined in Table 3.[6,15] Laboratory assessment should commence with a review

Table 2 ISTH Bleeding Assessment Tool (BAT)

Symptoms (Up to Time of Diagnosis)	Score				
	0§	1§	2	3	4
Epistaxis	No/trivial	>5/year or >10 minutes	Consultation only*	Packing/cauterization or antifibrinolytic	Blood transfusion, replacement therapy^^ or desmopressin
Cutaneous	No/trivial	≥5 bruises of >1 cm in exposed areas	Consultation only*	Extensive	Spontaneous haematoma requiring blood transfusion
Bleeding from minor wounds	No/trivial	>5/year or >10 minutes	Consultation only*	Surgical hemostasis	Blood transfusion, replacement therapy^^ or desmopressin
Oral cavity	No/trivial	Present	Consultation only*	Surgical hemostasis or antifibrinolytic	Blood transfusion, replacement therapy^^ or desmopressin
Gastro intestinal bleeding	No/trivial	Present (not associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia)	Consultation only*	Surgical hemostasis or antifibrinolytic	Blood transfusion, replacement therapy^^ or desmopressin
Hematura	No/trivial	Present (macroscopic)	Consultation only*	Surgical hemostasis, iron therapy	Blood transfusion, replacement therapy^^ or desmopressin
Tooth extraction	No/trivial or none done	≤25% of all procedures, no intervention**	>25% of all procedures, no intervention**	Resuturing or packing	Blood transfusion, replacement therapy^^ or desmopressin
Surgery	No/trivial or none done	≤25% of all procedures, no intervention**	>25% of all procedures, no intervention**	Surgical hemostasis or antifibrinolytic	Blood transfusion, replacement therapy^^ or desmopressin
Menorrhagia	No/trivial	Consultation only* or Changing pads > every 2 hours or Clot and flooding or PBAC score >100#	Time off work/school >2/year or Antifibrinolytics/hormonal/iron therapy	Combined treatment with antifibrinolytics and hormonal therapy or Present since menarche and >12 months	Acute menorrhagia requiring hospital admission and emergency treatment or Blood transfusion, replacement therapy^^ or desmopressin or Dilatation and curettage or endometrial ablation or hysterectomy
Post-partum hemorrhage	No/trivial or no deliveries	Consultation only* or Use of syntocin or Lochia >6 weeks	Iron therapy or Antifibrinolytics	Blood transfusion, replacement therapy^^ or desmopressin or Examination under anaesthesia and/or uterine balloon/package to tamponade uterus	Any procedure requiring critical care or surgical intervention (eg hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures)
Muscle haematomas	Never	Post trauma, no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy^^	Spontaneous or traumatic, requiring surgical intervention or blood transfusion

(*Continued*)

Table 2 (Continued).

Symptoms (Up to Time of Diagnosis)	Score				
	0[§]	1[§]	2	3	4
Hemarthrosis	Never	Post trauma, no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy^^	Spontaneous or traumatic, requiring surgical intervention or blood transfusion
CNS bleeding	Never			Subdural, any intervention	Intracerebral, any intervention
Other bleeding^	No/trivial	Present	Consultation only*	Surgical hemostasis, antifibrinolytics	Blood transfusion or replacement therapy^^ or desmopressin

Notes: In addition to the guidance offered by the table, it is mandatory to refer to the text for more detailed instructions. [§]Distinction between 0 and 1 is of critical importance. Score 1 means that the symptom is judged as present in the patient's history by the interviewer but does not qualify for a score 2 or more. *Consultation only: the patient sought medical evaluation and was either referred to a specialist or offered detailed laboratory investigation. **Example: 1 extraction/surgery resulting in bleeding (100%): the score to be assigned is 2; 2 extractions/surgeries, 1 resulting in bleeding (50%): the score to be assigned is 2; 3 extractions/surgeries, 1 resulting in bleeding (33%): the score to be assigned is 2; 4 extractions/surgeries, 1 resulting in bleeding (25%): the score to be assigned is 1. #If already available at the time of collection. ^Include: umbilical stump bleeding, cephalohematoma, cheek hematoma caused by sucking during breast/bottle feeding, conjunctival hemorrhage or excessive bleeding following circumcision or venipuncture. Their presence in infancy requires detailed investigation independently from the overall score. ^^Use of hemostatic blood components and rFVIIa. Reproduced from Rodeghiero F, Tosetto A, Abshire T, Arnold DM, Coller B, James P, Neunert C, Lillicrap D; ISTH/SSC joint VWF and Perinatal/Pediatric Hemostasis Subcommittees Working Group. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063–2065. © 2010 International Society on Thrombosis and Haemostasis.[13]

of the full blood count and blood film to investigate any platelet deficiencies or clumping. Blood group assessment is also important, given group O individuals are known to have a lower baseline VWF level.[15] Routine coagulation tests, including prothrombin time (PT) and APTT are often useful as a baseline screen.

Most importantly, a group of tests useful for evaluating VWD should also be performed. At a bare minimum, the VWD screening tests should include a factor VIII activity assay, a VWF protein level assessment (VWF:Ag; "antigen"), and sufficient tests to properly investigate the activity of VWF. Most laboratories utilise ELISA (enzyme-

Table 3 Laboratory Work Up of a Patient with Suspected VWD

Investigation	Purpose
FBC + blood film	Platelet deficiencies Morphological changes
PT/APTT/fibrinogen	Screen of factor deficiencies
Blood group	To check for group O
FVIII VWF:Ag VWF:RCo or VWF:GPIbR (or VWF:GPIbM) VWF:CB	Initial screen for VWD
DDAVP Challenge VWF:FVIIIB Platelet aggregometry - RIPA Multimer assays	Extended testing for VWD: Type 1C VWD, Type 2N VWD 2A vs 2B vs PT-VWD
Genetic studies	Confirmatory testing: Type 2A, 2B, 2M, 2N, PT-VWD, Type 3 VWD
1-hour and 4-hour desmopressin trial	Type 1C VWD

linked immunosorbent assay) or LIA (latex immunoassay) based methods to test for VWF:Ag, both offering low variability, high sensitivity, and full automation capabilities.[6] ELISA assays may predominate in research laboratories due to the common use of ELISA methodology for other research analysis. LIA assays tend to dominate in diagnostic laboratories. The newest addition of CLIA (chemiluminescent immunoassays)-based testing methods is less readily available, although they offer the best low-level sensitivity and lowest assay variation in VWF:Ag testing.[6] Importantly, no VWF:Ag assay identifies any functional parameter for VWF.

The activity assays for VWF should incorporate both an assessment of the ability of VWF to bind platelets and collagen, and where indicated also to FVIII. Thus, a ristocetin cofactor (VWF:RCo) or platelet GPIb recombinant surrogate to ristocetin cofactor assay (VWF:GPIbR) is required to assess for VWF platelet binding and a collagen binding assay (VWF:CB) is required to assess for VWF collagen binding; in our view, this pair of assays is required for all VWD investigations. An alternate gain of function "mutant" GPIb assay (VWF:GPIbM) is used in some laboratories instead of VWF:RCo or VWF:GPIbR.[6] In general, VWF:RCo, VWF:GPIbR and VWF:GPIbM all represent platelet GPIb binding assays, and in diagnostic laboratories, they are usually performed as agglutination assays, including LIA-based.[6] VWF:GPIbR is also available using CLIA. VWF:CB is usually performed by ELISA or increasingly by CLIA. Again, research labs may also perform some VWF activity assays, including VWF:GPIbM, by ELISA.

In cases of suspected 2N VWD, an additional assay, the factor VIII binding assay (VWF:FVIIIB) is also recommended. These tests will allow identification of the two quantitative deficiencies (Type 1 and Type 3 VWD), as well as all the qualitative defects.

In brief, a low level of VWF:Ag with a similar level of activity (VWF:RCo, VWF:GPIbR, VWF:GPIbM, VWF: CB), suggests Type 1 VWD; a level of VWF:Ag and activity of <5% likely indicates Type 3 VWD; a disproportion of VWF activity and VWF:Ag, suggests a qualitative (Type 2) VWD. If Type 1 VWD is suspected, a VWF propeptide assay may be considered in those who do not have a sustained response to DDAVP to confirm Type 1C subtype, indicative of reduced survival of VWF in plasma.[16] A provisional exclusion or diagnosis of VWD may require further testing for confirmation. This may include repeat testing of the same panel of tests or

extending the test panel. For example, while a discordant low ratio of RCo/Ag and CB/Ag provides a likely indication of Type 2A, 2B, or PT-VWD, separating out these disorders, or diagnosis of difficult cases, requires an extended test repertoire, such as utilisation of the ristocetin induced platelet agglutination assay (RIPA), genetic testing or VWF multimer analysis. An appropriate testing algorithm is shown in Figure 1. Genetic testing is sometimes also useful in Type 3 VWD cases.

Though not as well recognised, it is important to consider Type 1C VWD when subtyping VWD. Accurate identification of Type 1C VWD has significant therapeutic implications, as management of bleeding or prophylaxis against bleeding will likely require treating with VWF concentrates rather than desmopressin, given the short half-life of native VWF in these patients. Type 1C VWD can be identified by the abnormally high ratio of VWF pro-peptide to VWF:Ag associated with this condition due to the increased clearance of the mature VWF molecule compared with the pro-peptide.[17,18] Pro-peptide measurement is not readily available in clinical practice, and in some cases, the ratio can be normal despite rapid clearance of VWF.[19] Another method for detection of Type 1C VWF is via a desmopressin trial with a 1- and 4-hour post-infusion VWF level measurement, with Type 1C VWD patients expected to show a >30% decrease of VWF levels from peak levels at 4 hours post desmopressin infusion.[16,19]

The current definition of VWD is not restricted to those with *VWF* gene mutations.[4] Underscoring this is the complexity, limited availability, high cost, and low clinical utility of genetic testing generally associated with VWD.[5] A significant proportion of patients have no identifiable VWF mutations on readily available genetic methods.[4] In particular, quantitative defects, such as seen in Type 1 VWD, often bear unsuccessful genetic analyses, despite undertaking evaluations of the entire *VWF* gene, and therefore also proving very costly.[4,5] A positive result is more likely attainable for qualitative VWF defects or Type 2 VWD, as often a more directed analysis of the VWF gene can be performed, thereby making this more cost-effective.[5] The diagnostic uncertainty that generally accompanies Type 2 VWD further increases the utility of genetic testing in these scenarios. In general, it is recommended to only perform genetic testing in select investigations, such as for confirmatory testing of Type 2A, 2B, 2M, 2N, PT-VWD given their diagnostic difficulty, and selected cases of Type 3 VWD, for example, to assist with pre-natal diagnoses (Table 3).[5]

760

Journal of Blood Medicine 2021:12
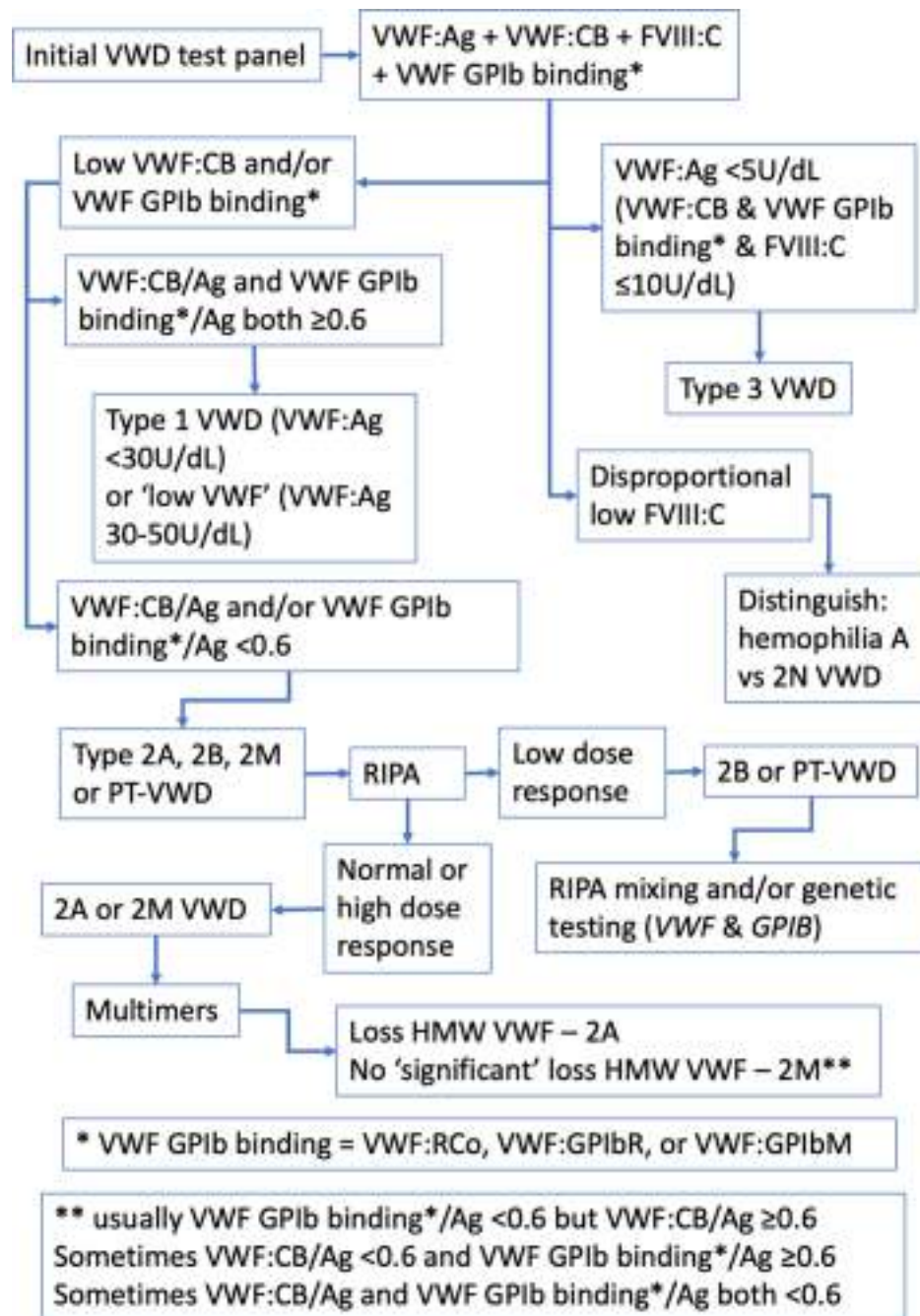
Figure 1 A simplified algorithm that describes the VWD diagnostic process using laboratory testing. This considers the differential utility of different VWF methods, as well as VWF multimers, and potentially genetic testing.

Abbreviations: Ag, antigen; CB, collagen binding; FVIII, factor VIIII; GPIb, glycoprotein Ib (the platelet VWF receptor); GPIbM, GPIb mutation-based assay; GPIbR, recombinant GPIb-based assay; HMW, high molecular weight (VWF); RCo, ristocetin cofactor; RIPA, ristocetin induced platelet aggregation; VWF, von Willebrand factor; VWD, von Willebrand disease.

Causes of Misdiagnosis

The complexity and breadth of tests required for the proper diagnosis of VWD leads itself to being an entity that is commonly under-diagnosed, misdiagnosed, or over-diagnosed.[5,6,15,20–22] Both clinical and laboratory-related issues contribute to this. This is summarized in Table 4.

Table 4 Summary of Diagnostic Errors Encountered in von Willebrand Disease and How to Avoid Them

	Examples	Overcoming Errors
Over diagnosis	Mild bleeding symptoms with no confirmatory laboratory investigations Inadequate clinical correlation of laboratory results	Correlation of laboratory and clinical data Accurate clinical history taking
	Extraneous influences to plasma VWF levels (group O blood group, exercise, pregnancy)	Correlation of laboratory and clinical data Accurate clinical history taking Repeating VWD laboratory testing prior clinical decisions are made
	Incorrect laboratory reference ranges	Establishing laboratory-specific reference ranges that are clinically relevant
	Pre-analytical sample issues – overfilled collection tubes, contamination with anticoagulants, high haematocrits, EDTA plasma/serum samples, excessive heating of samples, delay in laboratory processing, pneumatic tube systems	Minimising pre-analytical sample issues Repeating VWD laboratory testing prior clinical decisions are made
	A lack of expertise in laboratory testing and interpretation	Utilising a laboratory with experience in VWD testing with well-studied reference ranges Improved education of laboratory scientists and medical practitioners on VWD and its laboratory investigation
Under diagnosis	Lack of awareness and familiarity with VWD clinical phenotype	Improved education of medical practitioners on VWD
	Extraneous influences to plasma VWF levels (Non-group O blood groups, exercise, pregnancy, African ancestry, older age) High biological variability of VWF levels and activity (acute phase reactant, adrenalin associated with physiological stressors, sex hormone changes in pregnancy/oral contraception/menstruation, nicotine/caffeine exposure, medications such as NSAIDs, previous/current thromboembolic disease)	Thorough clinical evaluation of patients with bleeding complaints
	Pre-analytical sample issues – overfilled coagulation tubes with inadequate mixing and partial clotting, excessive delays in sample testing causing sample stasis, improper collection tubes	Blood collection is undertaken appropriately in a citrate tube filled to the correct level Samples processed and transported in a timely manner without overheating Repeating VWD laboratory testing prior clinical decisions are made
	Utilising inappropriate screening tests/test panels or a panel that is too restricted	Utilising the best methodologies available and a broad gamut of tests
	Analytical issues secondary to acquired antibodies (eg rheumatoid factor, paraproteins, human anti-mouse antibodies or HAMA)	Recognizing affected patients Recognizing effect of specific acquired antibodies on specific diagnostic assay Improved education to assist laboratories to consider these issues when performing diagnostic testing and reporting results
	A lack of expertise in laboratory testing and interpretation	Improved education of laboratory scientists and medical practitioners on VWD and its laboratory investigation

(*Continued*)

762

Journal of Blood Medicine 2021:12

Table 4 (Continued).

	Examples	Overcoming Errors
Incorrect diagnosis	Lack of awareness and familiarity with VWD clinical phenotype and what abnormal bleeding symptoms are	Improved education of medical practitioners on VWD and what constitutes an abnormal bleeding phenotype
	Misinterpretation of laboratory results	Complete clinical assessment of patients and correlation with laboratory results
	Use of inappropriate or restrictive test panels	Utilising the best methodologies available and a broad gamut of tests
	Pre-analytical errors including use of poorly mixed thawed plasma, filtered plasma or serum	Blood collection is undertaken appropriately in a citrate tube filled to the correct level Samples processed and transported in a timely manner without overheating Repeating VWD laboratory testing prior clinical decisions are made
	Analytical issues secondary to acquired antibodies (eg rheumatoid factor, paraproteins, human anti-mouse antibodies or HAMA)	Recognizing affected patients Recognizing effect of specific acquired antibodies on specific diagnostic assay Improved education to assist laboratories to consider these issues when performing diagnostic testing and reporting results
	A lack of expertise in laboratory testing and interpretation	Improved education of laboratory scientists and medical practitioners on VWD and its laboratory investigation

Overdiagnosis of von Willebrand Disease

Overdiagnosis has been extensively described in the medical literature, and commonly identifies "problems" that were never destined to cause morbidity, or results in over-medicalisation of normal physiology through expanded definitions of disease.[23] Mild bleeding symptoms may be a common feature of normal life and are thus often identified by the general public, particularly with regard to mucosal bleeding from dental procedures, epistaxis or menorrhagia.[7] Such symptoms may lead to an overdiagnosis of VWD, particularly when VWD has been described in as much as 1% of the population, whereas only 0.05% actually present for investigation.[6] In many of these patients, a true haemostatic challenge may never be obtained, and they may be labelled as von Willebrand-like bleeding without confirmatory laboratory investigations. Asking questions to distinguish normal from abnormal bleeding, together with the use of an appropriate bleeding assessment tool, is critical. This is particularly important in patients who have a family history of VWD, but have a normal bleeding phenotype, therefore not meeting diagnostic criteria for VWD.

Among those having laboratory investigations, there are several factors that may lead to an overdiagnosis of VWD, such as laboratory reference ranges, extraneous influences on plasma VWF levels (such as the ABO system) and current high availability of laboratory testing.[6] Laboratories, which are not performing the full repertoire of tests, sometimes incorrectly interpret results, especially given recent changes in test methods used.[6] Patients with an O-type blood group have up to 25% less plasma VWF than non-O type, and are therefore more likely to be falsely diagnosed as having VWD.[15]

Pre-analytical factors may also contribute to overdiagnosis, including inadequate patient history. Accurate history taking is considered one of the best screening tests for the risk of bleeding.[13] Overfilled collection tubes, samples contaminated with anticoagulants, samples with high haematocrit, EDTA plasma or serum samples can all result in falsely low levels of VWF and/or its activity. Excessive heating of collected samples (eg, due to poor sample transport), delay in time taken to reach a laboratory, and use of pneumatic tube systems causing platelet activation and VWF adhesion may also affect VWF testing and are recommended to be avoided.[24]

Overdiagnosis of VWD can lead to increased patient morbidity and health care system burden. Overdiagnosis can result in unnecessary intervention for patients who require urgent or elective surgery by restricting the locations of appropriate interventions (such as dedicated bleeding centre facilities). Overdiagnosis can lead to unnecessary medical anxiety and extended familial testing, in addition to potentially exposing patients to an increased thrombotic risk with therapies.

Underdiagnosis of von Willebrand Disease

Underdiagnosis of VWD can also occur, and, for example, result from under-recognition of the VWD clinical phenotype and/or a lack of awareness of VWD, both resulting in appropriate VWD diagnostic investigations not being performed. A mild bleeding phenotype, such as with Type 1 VWD in a male, may not be clinically apparent, especially if there have been limited haemostatic challenges, such as surgery, dental work, or trauma. Females presenting with excessive uterine bleeding are often not tested for VWD, as menorrhagia may not be recognised as a "bleeding disorder" symptom.

High biological variability of VWF levels and activity also contributes to diagnostic uncertainty of VWD (especially in Type 1 and Type 2). VWF levels can increase transiently as an acute-phase reactant in inflammatory conditions, with increased adrenalin associated with exercise or other physiological stressors, recent caffeine exposure, and with changes in sex hormones as seen with pregnancy, the oral contraceptive pill, and menstruation.[25,26] VWF levels hit a nadir on days 1 to 4 of the menstrual cycle, and testing at this phase of menses, when VWF levels are at their lowest and least likely to be elevated due to hormonal effects, is recommended to avoid underdiagnosis of VWD.[27] Furthermore, there is an increase in VWF levels with increasing age, with African ancestry, and non-group O blood groups.[22,28] These factors, if unappreciated, can result in underdiagnosis of VWD, especially if laboratory testing is not repeated or appropriately timed, and correlated with clinical bleeding history.

Pre-analytical issues related to test sample integrity are also an important factor leading to missed diagnoses of VWD. Using an overfilled coagulation tube may lead to inadequate mixing with partial clotting that can falsely elevate FVIII levels.[22] Excessive delays in testing with sample stasis can result in potential shortening of routine

APTT and factor activation that may result in false high levels.[22] Ensuring blood collection is undertaken appropriately in a citrate tube filled to the correct level, with samples being processed and transported in a timely manner will assist with avoiding issues related to sample integrity.

Laboratory-related analytical issues can also result in an underdiagnosis of VWD. Using inappropriate screening tests or an inappropriate test panel or a test panel that is too restricted is a commonly recognised issue.[6,22] For example, performing only a VWF:Ag test as the initial screening test, has the potential of missing Type 2 VWD, as many Type 2 VWD have VWF:Ag within the normal range.[22] Performing only the VWF:Ag and the highly variable classical VWF:RCo as screening tests with exclusion of VWF:CB, as is commonly practiced worldwide, can also result in missed VWD diagnosis, especially Type 2 VWD.[29] A study of North American specialised coagulation laboratories found that only one third of laboratories correctly identified Type 2 VWD when using either VWF:Ag and VWF:RCo tests.[30] The presence of various acquired antibodies, such as rheumatoid factor, paraproteins, and human anti-mouse antibodies or HAMA, can result in a false normal laboratory test result with some assay methods, especially in immune assays such as latex-based immunoturbidimetric assays (LIA) which are commonly utilised in the investigation of VWD.[22] Another issue is not utilising the best methodologies available. Utilising VWF:Ag assays that lack a high degree of sensitivity to VWF can result in the underdiagnosis of Type 3 VWD, since falsely high VWF levels may be reported. The newer chemiluminescence latex immunoassay technology (CLIA) for ristocetin cofactor activity measurement using non-platelet recombinant GPIb receptor (VWF:GPIbR) has been shown to have superior sensitivity and reduced variability to other methods, and is recommended in preference to traditional VWF:RCo testing methods in current bleeding disorder guidelines.[31–33] As another example, testing only for FVIII, and not testing for VWF parameters, may miss patients with Type 3 and Type 2N VWD, and falsely identify haemophilia A.

Underdiagnosis of VWD can lead to increased patient morbidity and mortality. Underdiagnosis may result in an increased uncorrected bleeding risk in patients when subjected to haemostatic challenges, such as surgery. Under-recognised VWD can cause ongoing preventable morbidity in patients, such as those with excessive uterine bleeding. Underdiagnosis of VWD can also result in missed

identification of similarly affected family members given the variable expressivity and penetrance of genes encoding VWF, as well as lost opportunities for pre-pregnancy counselling of patients.[7] Under-recognition of VWD also precipitates misdiagnosis or incorrect diagnoses of other bleeding disorders, which may result in patients receiving incorrect treatment.

Incorrect Diagnosis of von Willebrand Disease

Pre-analytical and analytical issues that potentiate under-diagnosis of VWD can also result in misdiagnosis of bleeding disorders. Lack of familiarity with the different VWD types, misinterpretation of laboratory results, or use of inappropriate or restrictive test panels, can lead to qualitative types of VWD (Type 2 VWD) being misdiagnosed as Type 1 VWD.[6] A common issue is pre-analytical errors, such as poorly mixed thawed plasma, filtered plasma or serum, leading to identification of a false VWD phenotype. This is especially a problem that results in misdiagnosis of Type 1 VWD as Type 2 VWD.[6] Analytical issues stemming from acquired antibodies, which can result in a false normal VWF:Ag on LIA test, in combination with a low activity assay, can also lead to a false Type 2 VWD diagnosis in a Type 1 VWD.

VWD may also be misdiagnosed as an entirely different bleeding disorder. Whilst haemophilia is well ingrained in the clinician's mind, VWD, despite its higher prevalence, remains an esoteric entity to many, resulting in missed ordering of specific VWD tests. This can result in certain types of VWD being incorrectly diagnosed as haemophilia A. If VWD testing is completely disregarded, a patient with severe Type 3 VWD may be inadvertently diagnosed with haemophilia A, due to associated low FVIII activity found in both conditions. This potential pitfall should especially be considered when there is no family history of severe haemophilia A. Even if a select panel of screening VWD tests are performed, not performing an FVIII binding assay when a low FVIII activity is detected can result in the misdiagnosis of Type 2N VWD as haemophilia A. The correct diagnosis of Type 2N VWD can only be achieved by performing a VWF:FVIII binding assay or by genetic testing. Misdiagnosis of VWD as haemophilia A has significant negative clinical implications, as treating severe VWD patients with recombinant FVIII products devoid of any VWF will not alleviate their bleeding diathesis. Such a misdiagnosis can also result in

significant issues when it comes to genetic and pre-pregnancy counselling, given the autosomal inheritance of VWD and X-linked inheritance of haemophilia A.

VWD may also be misdiagnosed as immune thrombocytopenic purpura (ITP) or an inherited platelet disorder, particularly in cases of Type 2B VWD associated with thrombocytopenia. It is important to consider VWD in any patient that presents with a thrombocytopenia, or with abnormal platelet function, especially if there is a family history or prolonged personal history of a bleeding phenotype. Accurate diagnosis is important to avoid unnecessary and potentially harmful treatments, such as immunosuppression, splenectomy and platelet transfusions. Alternatively, Type 2B VWD and PT-VWD may be misdiagnosed for the other. Here, RIPA testing with mixing studies will point to the correct type; and, genetic testing of *VWF* and *GPIb* may be required (Table 3).

Acquired von Willebrand Syndrome

Acquired von Willebrand syndrome (AVWS) is a rare, potentially underdiagnosed bleeding disorder that may also be misdiagnosed as congenital VWD (Table 5).[34,35] Diagnosis of AVWS is challenged by the same problems already mentioned for congenital VWD. In addition, diagnosis of AVWS is further hampered by the lack of a single diagnostic test that can rule in or rule out this disorder.[34] The main distinguishing features from inherited VWD are lack of family history, late onset bleeding phenotype, and presence of a suspicious underlying condition.[34] In difficult cases, testing family members, genetic analysis, and specialised assays such VWF pro-peptide levels and VWF mixing studies may be helpful. Differentiating the two conditions, one acquired and the other congenital, is important, as the disease course, management principles, and prognosis may differ.[34]

It is noted that several primary conditions can lead to AVWS, and there are several potential mechanisms involved in AVWS (Table 5). First, AVWS can be due to decreased protein production, such as in hypothyroidism, leading to a Type 1 deficiency of VWF. Second, VWF can be absorbed onto aberrant cells, such as in blood cancers, leading to clearance of plasma VWF. This may lead to either a Type 1 or 2 AVWS, depending on whether or not high molecular weight (HMW) is preferentially absorbed. Third, structural blood vessel deformities or the presence of artificial surfaces can lead to loss of VWF, typically HMW forms, again leading to a Type 2 AVWS. Absorption of VWF can also occur during extracorporeal

Table 5 Pathogenesis of Acquired von Willebrand Disease[34,35]

Mechanism	Examples
Reduced protein production, leading to reduced production of VWF	Hypothyroidism
Adsorption to surface of transformed cells or platelets	Myeloproliferative neoplasms Multiple myeloma Lymphoma
Mechanical destruction of VWF under high shear stress and increased proteolysis	Aortic stenosis Paravalvular leak Hypertrophic obstructive cardiomyopathy Congenital heart disease Left ventricular assist device use Extracorporeal membrane oxygenation devices
Autoantibody-mediated clearance or functional interference	Myeloma Lymphoma Autoimmune conditions – eg systemic lupus erythematosus

membrane oxygenation. Fourth, antibodies against VWF may be formed in autoimmune disease and certain blood disorders, and thus also leading to clearance of VWF, or interference to its function.

In addition to potentially being underdiagnosed or misdiagnosed as congenital VWD, AVWS can also be overdiagnosed in certain conditions. For example, during extracorporeal membrane oxygenation, additional confounders may be present leading to an increased bleeding phenotype, such as anticoagulation, to provide blood compatibility with foreign surfaces.

Recommendations

In order to avoid the underdiagnosis, overdiagnosis and misdiagnosis of VWD, it is important to consider three central questions.

Who Should Have VWD Testing?

It is important to have VWD readily present in the repertoire of differential diagnoses one considers in order to avoid underdiagnosing and misdiagnosing this common inherited disorder. It is, however, equally important not to cast a mindless wide net of VWD testing without first considering the pre-test probability of the diagnosis. Testing in situations of low pre-test probability, especially if paired with an inadequate test panel and pre-analytical sample issues, sets the stage for inaccurate diagnoses including overdiagnosis and misdiagnosis that can lead to significant patient morbidity. VWD testing should be considered in patients with a significant rather than trivial bleeding history, a family history of significant bleeding or VWD, or in patients with laboratory abnormalities, such as a mild unexplained thrombocytopenia, a mild prolonged APTT or an apparent factor VIII deficiency.

How Should VWD Testing Be Performed?

The importance of a comprehensive test panel that utilises the best methodologies cannot be overstated. Having a basic understanding of the type of tests and methodologies utilised by local laboratories will help understand the potential pitfalls and rigor of the diagnostic process. A suitable diagnostic algorithm is provided (Figure 1).

How to Ensure VWD Test Results are Correctly Interpreted?

It is important to always repeat VWD studies on a fresh sample for confirmation of initial test results. VWD testing should always be supplemented by a full blood count, coagulation studies, and blood group analysis (Table 3). The results should be interpreted in context with any anticoagulant use, hormone therapy, pregnancy and potential physiological stressors, or testing timed to minimise the impact of these factors. Utilising the results of a desmopressin trial can also be helpful in providing supporting evidence of the diagnostic possibilities.[6] Correlation between clinical phenotype and bleeding history is essential for an accurate diagnosis. Finally, family studies can be a valuable adjunct, especially when trying

to disentangle differential diagnoses such as acquired bleeding disorders and X-linked hemophilias.

Conclusions

VWD is a complex, heterogenous condition, which lends itself to significant diagnostic dilemmas and inaccuracies, and in turn leads to significant avoidable patient harm including inappropriate testing, overdiagnosis, underdiagnosis and inadequate treatment. Utilising a structured approach, the careful consideration of differential diagnoses, the integration of accurate clinical and laboratory features, as well as ensuring tests are repeated to minimise pre-analytical errors, are all important aspects in ensuring the accuracy of the diagnosis of VWD.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

References

1. Bowman M, Hopman WM, Rapson D, Lillicrap D, James P. The prevalence of symptomatic von Willebrand disease in primary care practice. *J Thromb Haemost*. 2010;8(1):213–216. Epub 2009 Oct 23. PMID: 19874468. doi:10.1111/j.1538-7836.2009.03661

2. Lavin M, O'Donnell JS. How I treat low von Willebrand factor levels. *Blood*. 2019;133(8):795–804. Epub 2018 Dec 21. PMID: 30578256. doi:10.1182/blood-2018-10-844936

3. von Willebrand EA. Hereditar pseudohemofili. *Fin Laekaresaellsk Hand*. 1926;68:87–112.

4. Sadler JE, Budde U, Eikenboom JC, et al.; Working Party on von Willebrand Disease Classification. Update on the pathophysiology and classification of von Willebrand disease: a report of the subcommittee on von Willebrand factor. *J Thromb Haemost*. 2006;4(10):2103–2114. Epub 2006 Aug 2. PMID: 16889557. doi:10.1111/j.1538-7836.2006.02146.x

5. Favaloro EJ. Diagnosing von Willebrand disease: a short history of laboratory milestones and innovations, plus current status, challenges, and solutions. *Semin Thromb Hemost*. 2014;40(5):551–570. Epub 2014 Jun 30. PMID: 24978322. doi:10.1055/s-0034-1383546

6. Favaloro EJ, Pasalic L, Curnow J. Laboratory tests used to help diagnose von Willebrand disease: an update. *Pathology*. 2016;48(4):303–318. Epub 2016 May 4. PMID: 27131932. doi:10.1016/j.pathol.2016.03.001

7. Lillicrap D. von Willebrand disease: advances in pathogenetic understanding, diagnosis, and therapy. *Blood*. 2013;122(23):3735–3740. Epub 2013 Sep 24. PMID: 24065240; PMCID: PMC3952678. doi:10.1182/blood-2013-06-498303

8. Ng C, Motto DG, Di Paola J. Diagnostic approach to von Willebrand disease. *Blood*. 2015;125(13):2029–2037. Epub 2015 Feb 23. PMID: 25712990; PMCID: PMC4375103. doi:10.1182/blood-2014-08-528398

9. De Jong A, Eikenboom J. Developments in the diagnostic procedures for von Willebrand disease. *J Thromb Haemost*. 2016;14(3):449–460. Epub 2016 Feb 12. PMID: 26714181. doi:10.1111/jth.13243

10. Bolton-Maggs PH, Lillicrap D, Goudemand J, Berntorp E. von Willebrand disease update: diagnostic and treatment dilemmas. *Haemophilia*. 2008;14(Suppl s3):56–61. PMID: 18510523. doi:10.1111/j.1365-2516.2008.01713.x

11. Siboni S, Biguzzi E, Caiani V, Mistretta C, Bucciarelli P, Peyvandi F. Baseline factor VIII plasma levels and age at first bleeding in patients with severe forms of von Willebrand disease. *Haemophilia*. 2016;22(4):564–569. doi:10.1111/hae.12900

12. Sanders YV, Giezenaar MA, Laros-van Gorkom BA, et al.; WiN study group. von Willebrand disease and aging: an evolving phenotype. *J Thromb Haemost*. 2014;12(7):1066–1075. PMID: 24750783. doi:10.1111/jth.12586

13. Rodeghiero F, Tosetto A, Abshire T, et al.; ISTH/SSC joint VWF and Perinatal/Pediatric Hemostasis Subcommittees Working Group. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063–2065. PMID: 20626619. doi:10.1111/j.1538-7836.2010.03975.x

14. Abebe W. Review of herbal medications with the potential to cause bleeding: dental implications, and risk prediction and prevention avenues. *EPMA J*. 2019;10(1):51–64. PMID: 30984314; PMCID: PMC6459456. doi:10.1007/s13167-018-0158-2

15. Castaman G, Montgomery RR, Meschengieser SS, Haberichter SL, Woods AI, Lazzari MA. von Willebrand's disease diagnosis and laboratory issues. *Haemophilia*. 2010;16 Suppl 5(5):67–73. PMID: 20590859; PMCID: PMC4313748. doi:10.1111/j.1365-2516.2010.02296.x

16. James PD, Connell NT, Ameer B, et al. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. *Blood Adv*. 2021;5(1):280–300. PMID: 33570651; PMCID: PMC7805340. doi:10.1182/bloodadvances.2020003265

17. Sharma R, Haberichter SL. New advances in the diagnosis of von Willebrand disease. *Hematology Am Soc Hematol Educ Program*. 2019;2019(1):596–600. PMID: 31808831; PMCID: PMC6913428. doi:10.1182/hematology.2019000064

18. Sztukowska M, Gallinaro L, Cattini MG, et al. Von Willebrand factor propeptide makes it easy to identify the shorter Von Willebrand factor survival in patients with type 1 and type Vicenza von Willebrand disease. *Br J Haematol*. 2008;143(1):107–114. Epub 2008 Aug 7. PMID: 18691167. doi:10.1111/j.1365-2141.2008.07311.x

19. Haberichter SL, Castaman G, Budde U, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood*. 2008;111(10):4979–4985. Epub 2008 Mar 14. PMID: 18344424; PMCID: PMC2384129. doi:10.1182/blood-2007-09-110940

20. Koutts J. A short history of diagnostic tests for von Willebrand disease: in memory of Barry Firkin (1930 to 2001) and Ted Zimmerman (1937 to 1988). *Semin Thromb Hemost*. 2006;32(5):445–455. PMID: 16862517. doi:10.1055/s-2006-947858

21. Sadler JE. Von Willebrand disease type 1: a diagnosis in search of a disease. *Blood*. 2003;101(6):2089–2093. Epub 2002 Oct 31. PMID: 12411289. doi:10.1182/blood-2002-09-2892

22. Favaloro EJ, Lippi G. Preanalytical issues that may cause misdiagnosis in haemophilia and von Willebrand disease. *Haemophilia*. 2018;24(2):198–210. Epub 2017 Dec 22. PMID: 29271545. doi:10.1111/hae.13396

23. Brodersen J, Schwartz LM, Heneghan C, O'Sullivan JW, Aronson JK, Woloshin S. Overdiagnosis: what it is and what it isn't. *BMJ Evid Based Med*. 2018;23(1):1–3. PMID: 29367314. doi:10.1136/ebmed-2017-110886

24. Magnette A, Chatelain M, Chatelain B, Ten Cate H, Mullier F. Pre-analytical issues in the haemostasis laboratory: guidance for the clinical laboratories. *Thromb J*. 2016;14(1):49. PMID: 27999475; PMCID: PMC5154122. doi:10.1186/s12959-016-0123-z

25. Lippi G, Maffulli N. Biological influence of physical exercise on hemostasis. *Semin Thromb Hemost*. 2009;35(3):269–276. Epub 2009 May 18. PMID: 19452402. doi:10.1055/s-0029-1222605

26. Brenner B. Haemostatic changes in pregnancy. *Thromb Res*. 2004;114(5–6):409–414. PMID: 15507271. doi:10.1016/j.thromres.2004.08.004

27. Kouides PA, Conard J, Peyvandi F, Lukes A, Kadir R. Hemostasis and menstruation: appropriate investigation for underlying disorders of hemostasis in women with excessive menstrual bleeding. *Fertil Steril*. 2005;84(5):1345–1351. PMID: 16275228. doi:10.1016/j.fertnstert.2005.05.035

28. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L, Favaloro JW. Reassessment of ABO blood group, sex, and age on laboratory parameters used to diagnose von Willebrand disorder: potential influence on the diagnosis vs the potential association with risk of thrombosis. *Am J Clin Pathol*. 2005;124(6):910–917. Erratum in: Am J Clin Pathol. 2006 May;125(5):796.PMID: 16416741. doi:10.1309/W76QF806CE80CL2T

29. Favaloro EJ, Bonar RA, Meiring M, et al. Evaluating errors in the laboratory identification of von Willebrand disease in the real world. *Thromb Res*. 2014;134(2):393–403. Epub 2014 May 21. PMID: 24913998. doi:10.1016/j.thromres.2014.05.020

30. Chandler WL, Peerschke EI, Castellone DD, Meijer P; NASCOLA Proficiency Testing Committee. Von Willebrand factor assay proficiency testing. The North American specialized coagulation laboratory association experience. *Am J Clin Pathol*. 2011;135(6):862–869. PMID: 21571959. doi:10.1309/AJCPH5JK4ONENPAE

31. Verfaillie CJ, De Witte E, Devreese KM. Validation of a new panel of automated chemiluminescence assays for von Willebrand factor antigen and activity in the screening for von Willebrand disease. *Int J Lab Hematol*. 2013;35(5):555–565. Epub 2013 Apr 3. PMID: 23551532. doi:10.1111/ijlh.12087

32. Cabrera N, Moret A, Caunedo P, et al. Comparison of a new chemiluminescent immunoassay for von Willebrand factor activity with the ristocetin cofactor-induced platelet agglutination method. *Haemophilia*. 2013;19(6):920–925. Epub 2013 Jun 4. PMID: 23730809. doi:10.1111/hae.12203

33. de Maistre E, Volot F, Mourey G, et al. Performance of two new automated assays for measuring von Willebrand activity: hemosIL AcuStar and innovance. *Thromb Haemost*. 2014;112(4):825–830. Epub 2014 Aug 7. PMID: 25103956. doi:10.1160/TH14-02-0108

34. Tiede A, Rand JH, Budde U, Ganser A, Federici AB. How I treat the acquired von Willebrand syndrome. *Blood*. 2011;117(25):6777–6785. Epub 2011 May 3. PMID: 21540459. doi:10.1182/blood-2010-11-297580

35. Shetty S, Kasatkar P, Ghosh K. Pathophysiology of acquired von Willebrand disease: a concise review. *Eur J Haematol*. 2011;87 (2):99–106. PMID: 21535159. doi:10.1111/j.1600-0609.2011.01636.x

External-Beam Radiotherapy Alone Management of Primary CNS Lymphoplasmacytic Lymphoma: A Vietnamese Case Report and Literature Review

Dang Nguyen Van, Nghia Duong Van, Quang Le Van, Tung Ngo Thanh & To Ta Van

Published online: 21 Aug 2021.

Submit your article to this journal ☑

Article views: 143

View related articles ☑

View Crossmark data ☑

CASE REPORT

External-Beam Radiotherapy Alone Management of Primary CNS Lymphoplasmacytic Lymphoma: A Vietnamese Case Report and Literature Review

Dang Nguyen Van [ID][1,2]
Nghia Duong Van[1]
Quang Le Van[1]
Tung Ngo Thanh[2]
To Ta Van[3]

[1]Department of Oncology, Hanoi Medical University, Hanoi, Vietnam; [2]Department of Head and Neck Radiation Oncology, Vietnam National Cancer Hospital, Hanoi, Vietnam; [3]Center of Pathology and Molecular Biology, Vietnam National Cancer Hospital, Hanoi, Vietnam

Background: Primary central nervous system (CNS) lymphoma is an uncommon non-Hodgkin disease limited to the CNS, and most cases are diffuse large B-cell lymphomas. Other pathologies, including lymphoplasmacytic lymphoma (LPL), are exceedingly rare and poorly understood. The clinical presentation of primary CNS LPL is diverse. It depends on the original site and the tumor's extension. There is currently no consensus on a treatment strategy for this uncommon manifestation. To our knowledge, no previously published case was successfully treated with radiation therapy alone.

Case Presentation: We present here a case of primary CNS LPL. A 46-year-old, previously healthy woman was presented with a worsening headache and lower extremity numbness. Multifocal enhanced masses were detected in an MRI with biopsy results consistent with LPL. A complete staging workup was performed with no evidence of systemic disease. The patient received external-beam radiotherapy alone and had a complete remission. After 2 years of follow-up, she remains disease-free.

Conclusion: Radiation alone is a promising treatment option for primary CNS lymphoplasmacytic lymphoma.

Keywords: lymphoplasmacytic lymphoma, primary CNS lymphoma, radiation therapy

Introduction

Lymphoplasmacytic lymphoma is a low-grade, B-cell neoplasm composed of small lymphocytes, plasmacytoid lymphocytes, and plasma cells that typically involve the bone marrow and it is associated with an immunoglobulin M (IgM) gammopathy.[1] The diagnosis of LPL is mainly based on the histological evaluation of involved tissue, usually bone marrow or lymph nodes. Although the vast majority of LPL cases are Waldenström macroglobulinemia (WM), there are some exceptions not satisfied with the diagnosis of WM.[1] They include tumors producing other immunoglobulins, combined immunoglobulins, mixed cryoglobulins, gamma heavy chains, or non-gammopathy disease.[2] Primary CNS lymphoma is an uncommon variant of non-Hodgkin lymphoma that involves the brain, leptomeninges, spinal cord, or eyes without systemic involvement. In literature, only a few cases diagnosed with LPL in the setting of primary CNS lymphoma have been reported. The current treatment strategy is not consistent due to the rarity and limited knowledge of this entity.

Correspondence: Dang Nguyen Van
Department of Oncology, Hanoi Medical University, 01 Ton That Tung Street, Dong Da District, Hanoi, 10000, Vietnam
Tel +84 94 2727 899
Email nguyenvandang@hmu.edu.vn; drdangnguyen@gmail.com

Case Presentation

A forty-six-year-old female was presented to the emergency department of our hospital with a two-month-headache and progressive numbness of her right leg. She also complained of nausea and dizziness 2 weeks before admission. The patient's medical and family history revealed no significance. On admission, the patient seemed excitable with a Glasgow Coma Scale (GCS) of 14 (E:4; V:4; M:6); other vital signs were in their normal ranges. In a general examination, no lymphadenopathy or organomegaly was found. A neurological examination revealed numbness in her right lower limb with no motor dysfunction, cranial neuropathy, or signs of increased intracranial pressure. However, magnetic resonance imaging (MRI) of her brain showed multifocal lesions scattered across the left occipital and parietal lobes; the largest one measured 16 mm in its transverse dimension. These lesions demonstrated a hypointense signal on the T1W sequence, a hyperintense signal on FLAIR, and no abnormal diffusion restriction on DWI images (Figure 1). They also revealed rim enhancement following gadolinium contrast injection.

Concern for brain metastases prompted further diagnostic procedures, including thoracic and abdominal contrast CT, lumbar and thoracic spine MRI, endoscopy of the gastrointestinal tract and ENT, but they identified no abnormalities. The patient then underwent a surgical biopsy with two samples resected from the two largest masses in the left occipital lobe. Microscopically, the lesions were composed of small lymphocytes and neoplasm cells with plasma cell morphological features. The immunohistochemical (IMH) test showed tumor cells are positive for CD79a, CD138, CD38, and MUM-1, but negative for CD3 and CD20 (Figure 2). These microscopic and IMH findings were consistent with lymphoplasmacytic lymphoma. Quantitative determination of the serum globulin test showed an increase in IgE at 740 IU/mL; other globulin levels were in their normal ranges (IgG 1206 mg/dL, IgM 155 mg/dL, IgA 220 mg/dL, Free Kappa 12.2 mg/L, Free Lambda 12.5 mg/L). Her serum protein electrophoresis and serum immunoelectrophoresis tests identified no monoclonal gammopathy. Bone marrow biopsy and cerebrospinal fluid (CSF) analysis detected no cancerous involvement. Pieces of the evidence above confirmed the diagnosis of stage I primary CNS LPL, according to the Arbor-Cotswold staging system for lymphomas.

Since the disease was confined to her brain and she denied every chemotherapy approach, we decided to treat her with whole-brain radiation at a 32 Gy dose (20 fractions of 1,6 Gy per faction). She also received a combination of dexamethasone during radiation therapy. Her headache was relieved after 2 weeks, and the left-foot numbness occasionally only after treatment. Two years after irradiation, clinical examination and imaging workup revealed no neurological deficits or recurrences.
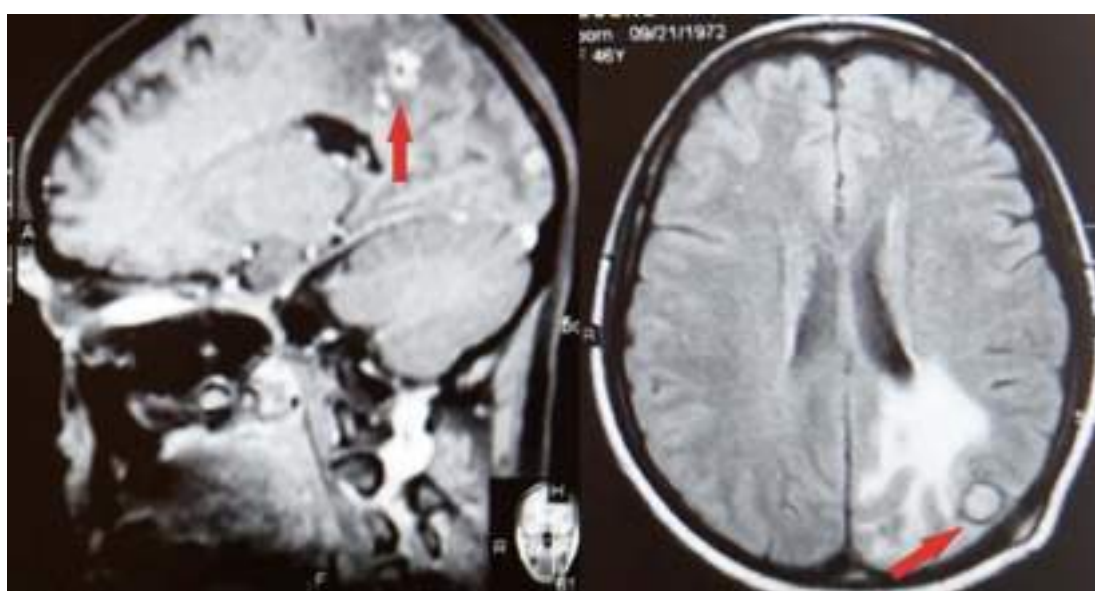
Figure 1 Contrasted brain MRI revealed enhanced masses in the occipital and parietal lobes (arrow).
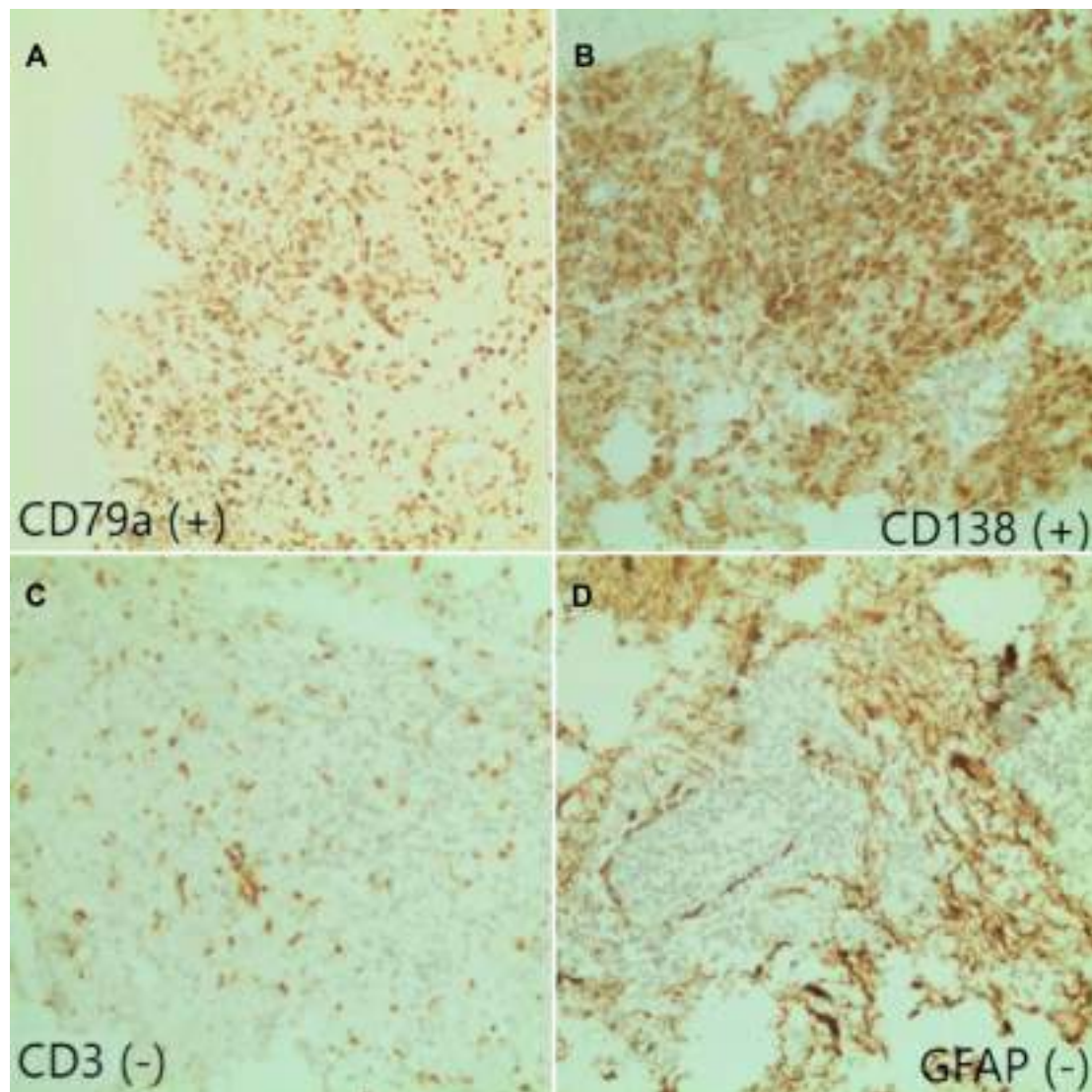
Figure 2 Immunohistochemistry results: (**A**) CD79a (+); (**B**) CD138 (+); (**C**) CD3 (-); (**D**) GFAP (-).

Discussion

Lymphoplasmacytic lymphoma is an uncommon variety of non-Hodgkin lymphoma composed of small lymphocytes, plasmacytoid lymphocytes, and plasma cells. It accounts for about less than 1% of hematologic malignancies and is even rarer in Asia, with an incidence of 0.31–0.43 cases per million.[3] Waldenström macroglobulinemia (WM), the most common subset of LPL, is featured by an elevated serum level of monoclonal immunoglobulin M and bone marrow involvement.[4]

Primary CNS lymphoma is a very rare subgroup of extranodal non-Hodgkin lymphoma that is confined to the brain parenchyma, leptomeninges, the eye or spinal cord and has no evidence of extra-CNS involvement. Its clinical expression are not specific and vary by site of involvement, including focal neurologic deficits, neuropsychiatric symptoms, signs of increased intracranial pressure, and seizures. Due to the lack of specific symptoms, a definitive diagnosis of primary CNS lymphoma requires a pathological assessment of the involved tissue. Some cases can be diagnosed through the histopathological evaluation of a vitreous biopsy or through a CSF analysis. Diffuse large B-cell lymphoma causes most cases of primary CNS lymphoma, while in rare cases, other entities such as T-cell, lymphoplasmacytic lymphoma and Burkitt's lymphoma are encountered. Primary CNS LPL

clinical features and optimal treatment remain poorly understood due to the rarity of cases and the difficulty of diagnosis.

In the literature, we found no more than 30 cases of primary CNS LPL published (via a PubMed search for "primary CNS" and "lymphoplasmacytic lymphoma" in case reports). We presented here nine cases with detailed patient information (Table 1); the other cases reported are listed in the references.[5–7]

LPL, like other primary CNS lymphomas, can manifest in the brain, meninges, or spinal cord, but no eye involvement has been reported. However, brain parenchyma is the most common primary site (Table 1). Its clinical expression are atypical and vary by site of involvement, including focal neurologic deficits, neuropsychiatric symptoms, signs of increased intracranial pressure, or seizures. Intracranial masses are usually detected by a head and neck MRI.[9] In addition, other laboratory assessment tools such as Positron Emission Tomography Scan (PET-CT), blood immunoglobulin tests and bone marrow biopsy are performed to detect other site involvement and exclude WM criteria. Pathological evaluation of tissue, which is essential for confirming LPL diagnosis, typically demonstrates infiltration by small lymphocytes, plasmacytoid lymphocytes, and plasma cells with intranuclear inclusion (Russell bodies) in some reported cases.[8] The typical immunophenotype of LPL

includes expression of CD19, CD22, CD20, CD38, and CD79a and the negative T-cell marker CD3, BCL. Because of limited features clinically and pathologically, LPL needs to be approached as a diagnosis of exclusion.

A major differential diagnosis of primary CNS LPL is Bing-Neel syndrome (BNS). Fintelman et al proposed restricting the definition of BNS to patients with previously confirmed WM and CNS signs not due to hyperviscosity or lymphocyte transformation.[16] Minnema et al 2017 have provided guidelines for the diagnosis, treatment, and response criteria for Bing-Neel syndrome. It emphasizes the role of cerebral spinal fluid analysis with multiparameter flow cytometry in establishing B-cell clonality, protein electrophoresis and MRI of the brain and spinal cord.[17] However, bone marrow involvement and IgM monoclonal paraprotein are essential criteria for WM, but they were omitted in our case, so the diagnosis of primary CNS LPL was established. It is also noted that Bing-Neel syndrome can be diagnosed without fulfilling WM diagnostic criteria.[18]

There is still no consensus guideline for optimal treatment for patients with primary CNS LPL. The chosen therapies in reported cases included tumor resection, chemotherapy, radiation therapy, and targeted therapy. The reviewed case with the best outcome is a 50-year-old patient diagnosed with a mass at the T4 level of the spinal cord. She was treated by complete surgical resection only

Table 1 Comparison of Clinical Features of Documented Case Reports

Reference	Gender/ Age	Primary Site	Treatment	Follow-Up
Abbi KK et al Case 1[8]	F/53	Left parietal lobe	Therapeutic Surgery/Tumor bed RT	1 year with no recurrence
Yan et al Case 2[9]	F/43	Cerebellopontine angle	Total tumor resection/CT	6 months with no recurrence
Layden et al Case 3 2011[10]	M/50	Hypothalamic region	Tumor biopsy/CT	6 months of stable disease
Ikeda et al Case 4[11]	F/43	Hypothalamus and optic chiasm		
Lim T et al Case 5[12]	M/50	T4 spinal cord	Total tumor resection	Alive in remission after 51 months
Lim T et al Case 6[12]	M/38	Corpus callosum	Tumor biopsy/CT	2.6 months
Braks et al Case 7[13]	F/42	Right centrum semiovale	Tumor biopsy/ CT + intrathecal	6 months with no recurrence
Carrasco et al Case 8[14]	F/49	Sellar and suprasellar region	Total tumor resection	4 years with no recurrence
Kanavaros et al Case 9[15]	F/59	Corpus callosum	Tumor biopsy	

(corpectomy) and lived without neurological sequelae and had no relapse after 51 months. Interestingly, in the treatment of primary CNS lymphoma, the majority of which is DLBCL, surgery has a limited role because the primary CNS lymphoma nature tends to be systemic rather than localized. Up to now, high-dose intravenous methotrexate (MTX) is the most effective agent against primary CNS lymphoma. In contrast, other combined regimens used in treating systemic lymphoma are either ineffective or intolerable.

Radiation therapy used to be used as a single-modality regimen to treat primary CNS lymphoma in the '70s.[19] Nowadays, radiation therapy plays a restricted role in primary CNS lymphoma and is only used as a consolidation modality after induction of MTX or as a salvage treatment after disease recurrence or persistence. However, primary CNS lymphoma is a radiosensitive disease, with over 90% of patients achieving a response and symptoms relieved in 84%.[20] For patients who are unsuitable or unwilling to engage in chemotherapy, whole-brain radiation is an acceptable alternative, despite the higher risk of disease progression and late neurotoxicity. Since low-grade B-cell lymphomas, including LPL, have a better prognosis than diffuse large B-cell lymphoma, radiation therapy alone is also a promising pathway, besides chemotherapy and surgical resection. One of the late toxicity of whole-brain radiation therapy is damage to the hippocampus. However, with the advancement of radiotherapy techniques today, it is possible to minimize the late toxicity of whole-brain radiation therapy.[21] Further investigation and randomized trials should be executed to confirm the effect and validate the optimal radiotherapy dose.

Conclusion

Primary CNS lymphoplasmacytic lymphoma is an uncommon variety of non-Hodgkin lymphoma very similar to the Bing – Neel syndrome. The definitive diagnosis is mainly based on pathology and immunohistochemistry. Currently, the treatment strategy is not uniform but individualized depending on each case. We report a clinical case of a patient with primary CNS lymphoplasmacytic lymphoma, and was treated with radiation therapy alone. We suggest that radiotherapy may be an encouraging strategy for this disease.

Abbreviations

CNS, central nervous system; LPL, lymphoplasmacytic lymphoma; WM, Waldenström macroglobulinemia; IMH, immunohistochemical; CSF, cerebrospinal fluid; BNS, Bing-Neel syndrome; MTX, methotrexate.

Data Sharing Statement

The datasets used in the current study are available upon reasonable request from the corresponding author.

Ethics Approval and Informed Consent

The study was approved by our research committee, Hanoi Medical University, Hanoi, Vietnam and Vietnam National Cancer Hospital, Hanoi, Vietnam.

Consent for Publication

The publication of this study has been consented to by the patient.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, agreed to the submitted journal, and agree to be accountable for all aspects of the work.

Funding

There is no funding to report.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Naderi N, Yang DT. Lymphoplasmacytic lymphoma and Waldenström macroglobulinemia. *Arch Pathol Lab Med*. 2013;137(4):580–585. doi:10.5858/arpa.2012-0034-RS
2. Marzia Varettoni, Emanuela Boveri, Silvia Zibellini, et al. Clinical and molecular characteristics of lymphoplasmacytic lymphoma not associated with an IgM monoclonal protein: A multicentric study of the Rete Ematologica Lombarda (REL) network. *Am J Hematol*. 2019;94 (11):1193-1199. doi: 10.1002/ajh.25600
3. Iwanaga M, Chiang C-J, Soda M, et al. Incidence of lymphoplasmacytic lymphoma/Waldenström's macroglobulinaemia in Japan and Taiwan population-based cancer registries, 1996–2003. *Int J Cancer*. 2014;134(1):174–180. doi:10.1002/ijc.28343
4. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon, France: International agency for research on cancer; 2008.
5. Jahnke K, Korfel A, O'Neill BP, et al. International study on low-grade primary central nervous system lymphoma. *Ann Neurol*. 2006;59(5):755–762. doi:10.1002/ana.20804
6. Bogdahn U, Bogdahn S, Mertens HG, et al. Primary non-Hodgkin's lymphomas of the CNS. *Acta Neurol Scand*. 1986;73(6):602–614. doi:10.1111/j.1600-0404.1986.tb04607.x

7. Shenkier TN. Unusual variants of primary central nervous system lymphoma. *Hematol Oncol Clin North Am*. 2005;19(4):651–664. doi:10.1016/j.hoc.2005.05.001

8. Abbi KKS, Muzaffar M, Gaudin D, et al. Primary CNS lymphoplasmacytic lymphoma: a case report and review of literature. *Hematol Oncol Stem Cell Ther*. 2013;6(2):76–78. doi:10.1016/j.hemonc.2013.06.002

9. Yan C, Kong X, Yang L, et al. An uncommon case of lymphoplasmacytic lymphoma in cerebellopontine angle region. *Medicine (Baltimore)*. 2016;95(34):e4627. doi:10.1097/MD.0000000000004627

10. Layden BT, Dubner S, Toft DJ, et al. Primary CNS lymphoma with bilateral symmetric hypothalamic lesions presenting with panhypopituitarism and diabetes insipidus. *Pituitary*. 2011;14(2):194–197. doi:10.1007/s11102-008-0166-7

11. Ikeda T, Hara K, Yamanaka T, et al. [A case of primary central nervous system malignant lymphoma developing from the optic chiasma and hypothalamus]. *Rinsho Shinkeigaku*. 2006;46(7):475–479. Japanese.

12. Lim T, Kim SJ, Kim K, et al. Primary CNS lymphoma other than DLBCL: a descriptive analysis of clinical features and treatment outcomes. *Ann Hematol*. 2011;90(12):1391–1398. doi:10.1007/s00277-011-1225-0

13. Braks E, Urbach H, Pels H, et al. Primary central nervous system immunocytoma: MRI and spectroscopy. *Neuroradiology*. 2000;42:738–741. doi:10.1007/s002340000392

14. Carrasco CA, Rojas ZD, Chiorino R, et al. Primary pituitary lymphoma in immunocompetent patient: diagnostic problems and prolonged follow-up. *Pituitary*. 2012;15(1):93–96. doi:10.1007/s11102-010-0219-6

15. Kanavaros P, Mikol J, Nemeth J, et al. Stereotactic biopsy diagnosis of primary non-Hodgkin's lymphoma of the central nervous system: a histological and immunohistochemical study. *Pathol Res Pract*. 1990;186(4):459–466. doi:10.1016/S0344-0338(11)80464-4

16. Fintelmann F, Forghani R, Schaefer PW, et al. Bing-Neel syndrome revisited. *Clin Lymphoma Myeloma*. 2009;9(1):104–106. doi:10.3816/CLM.2009.n.028

17. Minnema MC, Kimby E, D'Sa S. Guideline for the diagnosis, treatment and response criteria for Bing-Neel syndrome. *Haematologica*. 2017;102(1):43–51. doi:10.3324/haematol.2016.147728

18. Grainger BT, Issa S. Bing-Neel syndrome presenting as isolated CNS lymphoplasmacytic lymphoma: a case report and review of the literature. *J Clin Neurosci*. 2019;71:277–280.

19. Henry JM, Heffner RR, Dillard SH, et al. Primary malignant lymphomas of the central nervous system. *Cancer*. 1974;34(4):1293–1302. doi:10.1002/1097-0142(197410)34:4<1293::AID-CNCR2820340441>3.0.CO;2-P

20. Kwak Y-K, Choi B-O, Choi KH, et al. Radiotherapy as an alternative treatment option for primary central nervous system lymphoma patients who are noncandidates for chemotherapy. *Oncotarget*. 2017;8(63):106858–106865. doi:10.18632/oncotarget.22427

21. Kazda T, Pospíšil P, Doleželová H, et al. Whole brain radiotherapy: consequences for personalized medicine. *Rep Pract Oncol Radiother*. 2013;18(3):133–138. doi:10.1016/j.rpor.2013.03.002

Assessing and Management of Neurotoxicity After CAR-T Therapy in Diffuse Large B-Cell Lymphoma

Omar Castaneda-Puglianini & Julio C Chavez

REVIEW

Assessing and Management of Neurotoxicity After CAR-T Therapy in Diffuse Large B-Cell Lymphoma

Omar Castaneda-Puglianini [iD] [1]
Julio C Chavez [2]

[1]Virginia Commonwealth University, Massey Cancer Center, Cellular Immunotherapies and Transplant Program, Richmond, VA, USA;
[2]Department of Malignant Hematology, Moffitt Cancer Center, Tampa, FL, USA

Abstract: Chimeric antigen receptor T-cell (CAR-T) therapy represents the most important advances in cancer immunotherapy, especially in hematological malignancies such as B-cell lymphomas. CAR-T cell therapy has significant activity in poor risk B-cell lymphomas. CAR-T cell therapy is associated with potentially life-threatening toxicities such as cytokine release syndrome (CRS) and neurotoxicity (NT). While CRS pathophysiology and management are well established, the understanding and treatment of NT continues to develop. All current CAR-T products approved for DLBCL have been associated with NT with some differences in their severity. As cell therapies continue to advance and its access broadening, it will be imperative for clinicians to be aware of the signs and symptoms of NT, its stratification and basic management.

Keywords: CAR-T, neurotoxicity, ICANS, encephalopathy

Introduction

Immunotherapy with chimeric antigen receptor T-cell (CAR-T) therapy is one of the most important advances in the treatment of cancer and, particularly, hematologic malignancies. In lymphoma, CAR-T cells targeting CD19 have been widely developed resulting in three commercially available products and several ongoing clinical trials.[1] In diffuse large B-cell lymphoma (DLBCL), there are currently three CAR-T cell products approved (after at least 2 lines of therapy): axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel) and lisocabtagene ciloleucel (liso-cel). Axi-cel, tisa-cel and liso-cel are genetically modified anti-CD19 autologous T-cells that are designed to target CD19 in B-cell malignancies. Based on the ZUMA-1, JULIET and TRANSCEND-NHL-001 studies, all three showed prominent activity in poor risk relapsed/refractory DLBCL with an overall response rate (ORR) between 50% and 82% with many patients having a durable response.[2–5]

The efficacy of CAR-T cell therapy is offset by potential class-effect toxicities that could be life-threatening and will require the implementation of appropriate measures to mitigate these side effects. CAR-T cell therapy is associated with high rates of unique toxicities, namely cytokine release syndrome (CRS) and neurotoxicity (NT). CRS is a well-known syndrome driven by a rapid release and expansion of inflammatory cytokines that leads to several systemic symptoms and clinical findings such as fevers, hypoxemia, constitutional symptoms, hypotension, tachycardia and organ dysfunction.[6,7] The pathophysiology and management of CRS is well described and standardized, and involves the use of anti-cytokine therapy (ie, tocilizumab) with/without steroids. This treatment strategy leads to a rapid

Correspondence: Julio C Chavez
Department of Malignant Hematology, Moffitt Cancer Center, 12902 Magnolia Drive FOB, Tampa, FL, 33612, USA
Email julio.c.chavez@moffitt.org

resolution of symptoms.[6–8] Unlike CRS, the understanding of NT pathophysiology and management is less established and continues to evolve.[9]

Pathophysiology

The pathophysiology of NT, currently known as immune effector cell-associated neurotoxicity syndrome (ICANS), continues to develop. Current knowledge highlights the disruption of the blood brain barrier (BBB) caused by inflammation and increased cytokines as a key initiating factors.[10] BBB disruption is triggered by endothelial cell activation as early event, which subsequently leads to BBB breakdown, increased permeability and coagulopathy as demonstrated in a mouse model.[11] In healthy states, angiopoietin-1 (ANG1) and −2 (ANG2) remain in normal ratios, hence preventing endothelial activation. During severe ICANS, levels of ANG2 and ANG2: ANG1 ratio are significantly increased representing a disruption of the BBB and capillary leak.[11] In addition, patients with severe ICANS had increased levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-a), interferon gamma (IFN-g) and interleukin (IL)-6 that are likely released by CD14+ myeloid derived suppressor cells (MDSCs) which are also increased during NT.[11,12] The presence of high levels of Von Willebrand Factor (WWF), high molecular weight WWF multimers and depleted ADAMTS 13 levels also supported the endothelial activated and coagulopathic state during severe ICANS.[11]

Other mouse models demonstrated the role of MDSCs and monocytes in NT pathophysiology and their key role as main sources of the above-mentioned pro-inflammatory cytokines including IL-1.[13,14] Additionally, intratumoral interactions between CAR-T and macrophages/monocytes mediated by the CD40 ligand seems to be associated with CRS (and perhaps NT).[13] Administration of anakinra (an IL-1 antagonist) prevented the development of CRS and NT in mouse models, without affecting the CAR-T cell efficacy.[14]

Clinical Predictors and Biomarkers
Clinical Predictors

In general, neurological events have been reported more frequently and with more severity with axi-cel than tisa-cel and liso-cel, likely related to the type of costimulatory domain (CD28 vs 4–1BB). The pivotal ZUMA-1 study of axi-cel in R/R DLBCL, that included 111 patients, reported grade ≥3 NT in 28% of patients. High tumor burden and CAR-T peak/expansion were associated with grade ≥3 ICANS.[2,15] The frequency of grade 3/4 NT in the JULIET

trial (tisa-cel for R/R DLBCL) was 12%, majority presenting with concurrent CRS.[3] There were no specific factors associated with NT but appears that high tumor burden and elevated lactate dehydrogenase (LDH) were associated with severe NT.[16] The TRANSCEND trial of liso-cel for R/R DLBCL reported grade ≥3 NT in 10% of cases. Similar to the findings of ZUMA-1 and JULIET, patients with higher tumor burden (16%), elevated LDH (19%) and higher CAR expansion/peak were more likely to develop grade ≥3 NT.[5]

In the off-trial setting, axi-cel had an incidence of grade >3 NT between 30% and 35%. Bulky disease (>10 cm), higher day 0 and peak C-reactive protein (CRP) and peak ferritin levels were associated with severe NT.[17,18] Similarly, higher baseline LDH levels and development of CRS were associated with NT severity.[19] In another retrospective experience, the Massachusetts General Hospital reported that high CRP, LDH ≥ 400 and thrombocytopenia were associated with grade ≥3 NT.[20] Again, development of CRS was predictive of increased frequency and severity of NT, as previously seen in leukemias.[20,21]

In one of the largest datasets of neurotoxicity in lymphoma, the Dana Farber Cancer Institute developed a potential scoring system that could predict NT incidence and severity. This included age, LDH, baseline CRP, CRS (severity and timing), lymphoma histologic type, ferritin, leukocytes, number of doses of tocilizumab. A score of 6 or higher was predictive of grade 2 or higher NT.[22]

Biomarkers and Cytokines

Several cytokines have been implicated with the development and severity of ICANS in clinical studies. In patients with B-cell lymphoblastic leukemia treated with CAR-T cell therapy, the Memorial Sloan Kettering demonstrated that IL-1a, IL-2, IL-3, IL-5, IL-6, IL-10, IL-15, IFN-g, G-CSF, GM-CSF, and MCP1 were elevated and correlated with severe NT.[23] The Fred Hutchinson Cancer and Research Center showed that increased levels of IL-6, IL-8, IFN-g, TNF-g and monocyte chemoattractant protein 1 (MCP-1) were associated with incidence and severity of neurotoxicity.[24] The ZUMA-1 study showed that increased levels of IL-1Ra, IL-2Ra, IL-6, IL-8, IL-10, IL-15, IFN-g, granzyme B, GM-CSF and ferritin were seen in patients with grade > NT.[2] The role of GM-CSF supporting inflammatory macrophages and monocytes generating CRS and NT has been established.[12,25]

In 75 patients who received axi-cel as standard of care for R/R LBCL at the Moffitt Cancer Center, point of care cytokine profiling was performed baseline (at the conditioning chemotherapy starting day and daily until

discharge or up to 60 days post CAR-T cell infusion).[26] Baseline levels of IL-6, ANG2, ANG2/ANG1 ratio and ferritin were associated with severe NT. Peak levels of IL-6, IL-15, IFN-g, GM-CSF and ANG2/ANG1 were also seen in patients with grade ≥3 NT.[26] Gene expression profiling by Nanostring in lymphoma tissue samples showed that low T-cell signature and high macrophage signature score were associated with NT severity, reflecting the role of the lymphoma microenvironment in the development of CAR-T related toxicities.[26]

Clinical Findings

ICANS occurs with a variable frequency and severity after CAR T cell therapy in DLBCL. It usually starts after the onset of CRS and, many times, after its resolution. Symptoms of ICANS can occur concomitantly with CRS, especially with severe grades of toxicities.[22,27] Based on the published data of the 3 FDA approved CAR T-cell products for DLBCL, the median time to onset of neurotoxicity ranges from 5 to 9 days following CAR-T cell infusion; however, the incidence and clinical findings differ between patients receiving different products.[2,3,5]

Initial symptoms of ICANS do not have a specific pattern and can be vague. In most patients, early symptoms of ICANS include word-finding difficulty, reduced attention, calculation defect, tremors, impaired handwriting and lethargy.[6,7] A clinical finding in the early phase of ICANS that seems to be very specific is expressive aphasia which can progress into global aphasia at later stages.[24]

Overall, neurotoxicity is, in general, self-limited. A summary of neurological events noted with approved CAR T-cell products for the treatment of DLBCL is shown in Table 1. Some symptoms and signs have been excluded from the definition of ICANS even though they may be associated with CAR T-cell therapy including headaches, hallucinations, asterixis, tremors and

Table 1 Neurological Events Noted with Approved CAR T-Cell Products for Large B-Cell Lymphoma

Axicabtagene Ciloleucel	Lisocabtagene Maraleucel	Tisagenlecleucel
Encephalopathy: Includes encephalopathy, cognitive disorder, confusional state, depressed level of consciousness, disturbed attention, hypersomnia, leukoencephalopathy, memory impairment, mental status changes, paranoia, somnolence, and stupor	**Encephalopathy:** Includes encephalopathy, confusional state, encephalopathy, mental status changes, somnolence, lethargy, amnesia, cognitive disorder, depressed level of consciousness, memory impairment, flat affect, depersonalization, disturbance in attention, incoherent, and hypersomnia	**Encephalopathy:** Includes encephalopathy, cognitive disorder, confusional state, depressed level of consciousness, disturbance in attention, lethargy, mental status changes, somnolence, and automatism
Delirium: Includes agitation, delirium, disorientation, hallucination, hyperactivity, irritability, restlessness, and delusion	**Delirium:** Includes delirium, agitation, disorientation, delusion, hallucination, visual hallucination, and irritability	**Delirium:** Includes delirium, agitation, hallucination, hallucination visual, irritability, and restlessness
Headache	**Headache:** Includes headache and migraine	**Headache:** Includes headache and migraine
Dizziness: Includes dizziness, presyncope, syncope	**Dizziness:** Includes dizziness and syncope	**Sleep disorder:** Includes sleep disorder, nightmares, and insomnia
Aphasia: Includes aphasia, dysphasia	**Aphasia:** Includes aphasia, dysarthria, dysphemia, dysphonia, slow speech, and speech disorder	**Anxiety**
Tremor	**Tremor:** Includes tremor and essential tremor	
Ataxia	**Ataxia:** Includes ataxia and gait disturbance	
Motor dysfunction: Includes muscle spasms, muscular weakness	**Cerebellar:** Includes cerebellar syndrome, dysmetria, balance disorder, dyskinesia and impaired hand-eye coordination	
Seizure		
Dyscalculia		
Myoclonus		

Note: Data from references.[3–6]

myoclonus because they are usually treated symptomatically without triggering specific interventions such as corticosteroids. Also, headaches have been deemed not an appropriate indicator of ICANS leading to the exclusion from the definition as this is a non-specific symptom often seen in patients receiving chemotherapy or during febrile episodes in the absence of focal neurological deficits.[7] Electroencephalographic typically shows diffuse generalized slowing with or without triphasic waves suggesting diffuse encephalopathy.[28]

Severe cases of ICANS with death have been described. Rapidly evolving cerebral edema and death from cerebral toxicity have been seen with anti-CD19 CAR T-cell and it was first described in the ROCKET study (JCAR015) where five patients developed fatal cerebral edema and death, which lead closure of the trial.[28–30] The incidence of fatal neurotoxicity following anti-CD19 CAR T-cell therapy has been reported to be 3%.[11] As mentioned above, in LBCL, rates of severe NT in the pivotal trials ranged from 10% to 28%.[2,3,5] It appears that the difference in the incidence of neurotoxicity may be accredited to the different costimulatory domains of the CAR T-cell constructs (CD28 or 4–1BB); however, no randomized trials have compared CAR T-cell constructs with different costimulatory domains in LBCL.

Radiological findings have been noted, specially, in severe NT, aside from the well-known cerebral edema, reported with JCAR015.[30] Patients with severe NT had T2-FLAIR hyperintensities involving the brain stem, thalamus and corpus callosum that resolves with improvement of NT.[24] Some acute changes with leptomeningeal enhancement, vasogenic edema and microscopic bleeding have been reported as well.[11]

Grading and Stratification

There were not well-developed and consensual systems to grade neurotoxicity that were available during the early phase of clinical trials with CAR-T cells in R/R DLBCL. Subsequent pivotal studies that lead to the approval of the currently commercially available CAR T-cell constructs adopted the National Institutes of Health (NIH) Common Terminology Criteria for Adverse Events (CTCAE).

In order to improve grading and surveillance of neurotoxicity, the CAR-T-cell-therapy-associated TOXicity (CARTOX) Working Group was formed, comprising investigators from different disciplines and multiple institutions to establish recommendations for monitoring, grading and management of CAR T-cell associated toxicities, this was called CARTOX-10 and included an assessment of the patient's orientation, naming, writing, and orientation (Table 2).[28] Additionally, the CARTOX system developed the term CAR T-cell-related encephalopathy syndrome (CRES) that included the evaluation of motor symptoms, seizures, and signs of elevated intracranial pressure (ICP) (Table 3). The ICP was evaluated by determination of cerebrospinal fluid (CSF) opening pressure and papilledema grading, according to the modified Frisén scale.[28,31] CARTOX-10 was further refined giving birth to what is widely known as the immune effector cell-associated encephalopathy (ICE) score. The ICE score includes testing of simple commands, orientation, naming, writing, attention while removing the cumbersome, time consuming and potentially inaccurate evaluation of the ICP which may be not practical during daily practice.

Evidently, with the growing number of patients receiving adoptive cell therapy in particular CAR T-cell, there is a need to improve patient care and outcomes by allowing

Table 2 Encephalopathy Assessment Tools for Neurotoxicity Grading

CARTOX-10	ICE
Orientation: Time (year, month), place (city, hospital), president/prime minister of country of residence: 5 points **Naming:** Ability to name three objects (eg, point to clock, pen, button): 3 points **Writing:** Ability to write a standard sentence (eg, "our national bird is the bald eagle"): 1 point **Attention:** Ability to count backwards from 100 by 10: 1 point	**Orientation:** Time (year, month), place (city, hospital): 4 points **Naming:** Ability to name 3 objects (eg, point to clock, pen, chair): 3 points **Writing:** Ability to write a standard sentence (eg, "our national bird is the bald eagle"): 1 point **Attention:** Ability to count backwards from 100 by 10: 1 point **Following commands:** Ability to follow simple commands (eg, "Show me 2 fingers" or "Close your eyes and stick out your tongue"): 1 point
Total: 10 points	**Total: 10 points**

Notes: Data from references.[6,8] Scoring: 10, no impairment; 7–9, grade 1 ICANS; 3–6, grade 2 ICANS; 0–2, grade 3 ICANS; 0 due to patient unarousable and unable to perform ICE assessment, grade 4 ICANS.
Abbreviations: CARTOX-10, CAR-T-cell-therapy-associated toxicity 10-point neurological assessment; ICE, immune effector cell-associated encephalopathy.

Table 3 Grading of CAR T-Cell-Related Encephalopathy Syndrome (CRES)

Symptom or Sign	Grade 1	Grade 2	Grade 3	Grade 4
Neurological assessment score (by CARTOX-10*)	7–9 (mild impairment)	3–6 (moderate impairment)	0–2 (severe impairment)	Patient in critical condition, and/or obtunded and cannot perform assessment of tasks
Raised intracranial pressure	N/A	N/A	Stage 1–2 papilledema**, or CSF opening pressure <20mmHg	Stage 3–5 papilledema**, or CSF opening pressure ≥20mmHg, or cerebral edema
Seizures or motor weakness	N/A	N/A	Partial seizure, or non-convulsive seizures on EEG with response to BZD	Generalized seizures, or convulsive or non-convulsive status epilepticus, or new motor weakness

Notes: Data from reference.[8] *As noted on Table 2; **Papilledema grading according to modified Frisén scale.[12]
Abbreviations: CAR, chimeric antigen receptor; CARTOX-10, CAR-T-cell-therapy-associated toxicity 10-point neurological assessment; N/A, non-applicable; CSF, cerebrospinal fluid; EEG, electroencephalogram; BZD, benzodiazepine.

the standardized comparison of toxicities profiles and the efficacy of therapeutic interventions across studies and institutional practices under a common criterion. On that line, the American Society for Transplantation and Cellular Therapy (ASTCT) published a consensus grading system for CRS and ICANS which has been adopted as a systematic approach for reporting of CAR T-cell related toxicities.[7] ICANS was defined as

> A disorder characterized by a pathologic process involving the central nervous system following any immune therapy that results in the activation or engagement of endogenous or

infused T cells and/or other immune effector cells. Symptoms or signs can be progressive and may include aphasia, altered level of consciousness, impairment of cognitive skills, motor weakness, seizures, and cerebral edema.[7]

In addition to the ICE score, ICANS grading also requires assessment of other neurological domains including level of consciousness, motor symptoms, seizures and signs of elevated ICP/cerebral edema with or in the absence of encephalopathy (Table 4).

It is clear that CTCAE is suboptimal for grading and assessment of neurotoxicity. As opposed to CTCAE v4.03,

Table 4 ASTCT Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS) Consensus Grading for Adults

Neurotoxicity Domain	Grade 1	Grade 2	Grade 3	Grade 4
ICE score*/**	7–9	3–6	0–2	0 (patient is unarousable and unable to perform ICE)
Depressed level of consciousness†	Awakens spontaneously	Awakens to voice	Awakens only to tactile stimulus	Patient is unarousable or requires vigorous or repetitive tactile stimuli to arouse. Stupor or coma
Seizure	N/A	N/A	Any clinical seizure focal or generalized that resolves rapidly or nonconvulsive seizures on EEG that resolve with intervention	Life-threatening prolonged seizure (>5 min); or Repetitive clinical or electrical seizures without return to baseline in between
Motor findings‡	N/A	N/A	N/A	Deep focal motor weakness such as hemiparesis or paraparesis
Elevated ICP/Cerebral edema	N/A	N/A	Focal/local edema on neuroimaging¥	Diffuse cerebral edema on neuroimaging; decerebrate or decorticate posturing; or cranial nerve VI palsy; or papilledema; or Cushing's triad

Notes: Data from reference.[8] ICANS grade is determined by the most severe event (ICE score, level of consciousness, seizure, motor findings, raised ICP/cerebral edema) not attributable to any other cause; for example, a patient with an ICE score of 3 who has generalized seizure is classified as grade 3 ICANS. *As noted on Table 2. **A patient with an ICE score of 0 may be classified as grade 3 ICANS if awake with global aphasia, but a patient with an ICE score of 0 may be classified as grade 4 ICANS if unarousable. †Depressed level of consciousness should be attributable to no other cause (eg, no sedating medication). ‡Tremors and myoclonus associated with immune effector cell therapies may be graded according to CTCAE v5.0, but they do not influence ICANS grading. ¥Intracranial hemorrhage with or without associated edema is not considered a neurotoxicity feature and is excluded from ICANS grading. It may be graded according to CTCAE v5.0.
Abbreviation: N/A, not applicable.

CAR T-Cell-Related Encephalopathy Syndrome (CRES) and American Society for Transplantation and Cellular Therapy (ASTCT) grading systems provide a more specific and accurate assessment of the occurrence and severity of neurotoxicity after CAR T-cell therapy as demonstrated by retrospective application of the three grading systems to the same patient data set from the JULIET trial.[27,28]

Management

The management of neurotoxicity post CAR T-cell therapy continues to evolve and constitutes an area of ongoing research. Different management algorithms have been published including consensus programs and institution-specific interventions.[2,3,28,32] Despite considerable variation in practice in terms of when and how to treat neurotoxicity, there are basic steps that guide most interventions. The CARTOX working group published one of the first general guidelines based on their extensive experience using axicabtagene ciloleucel for the management of CRS and neurotoxicity.[28] The Society of Immunotherapy of Cancer (SITC) has recently released a clinical guideline for the management of CAR-T cell related complications including ICANS.[8]

In general, patients should have baseline neurological assessment prior to the infusion of CAR T-cell followed by close monitoring of new neurological signs/symptoms after CAR T-cell infusion. In most institutions, the baseline assessment includes a full neurological history and examination and a brain MRI to rule out any structural neurological abnormalities or possible central nervous involvement by lymphoma. Following CAR T-cell infusion, serial monitoring of ICE score, at least daily, is recommended (Table 3).[6] Patients with presumed ICANS should undergo a detailed neurological examination and ICE scoring conducted by a medical provider experienced in the management of CAR-T cell patients. Evaluation by neurologist is encouraged, specially for severe cases. Neuroimaging is recommended, being a brain MRI with and without contrast the preferred modality if the clinical status of the patient allows it.[28,33] For unstable and/or agitated patients, CT brain may be preferred. Neuroimaging of patients with ICANS usually show unremarkable findings for any structural pathology that explain neurotoxicity symptoms, even in patients with severe ICANS; however, it is needed to rule out other causes, such as intracranial hemorrhage or lymphoma involvement of the CNS.[11,20,24,28] In some cases, patients with moderate to severe neurotoxicity might show patchy T2 hyperintensities throughout the white matter or T2 hyperintensities in the bilateral thalami, dorsal pons, and cerebral edema have been reported.[2,11,24,28,30]

Work up for patients with ICANS should also include some basic laboratory evaluation including CRP, CBC, CMP, fibrinogen, prothrombin time test, and international normalized ratio (PT/INR).[24] The CSF analysis as well as opening pressure should be pursued unless there is a contraindication. CSF should be assessed for chemistry, cytology, microbiology and virology.[11,24,28,33] EEG is recommended even with low-grade ICANS and in patients with seizures or suspected non-convulsive status epilepticus. Findings on EEG in patients with neurotoxicity, include diffuse slowing and frontal intermittent rhythmic delta activity, generalized background slowing and generalized periodic discharges which are both non-specific signs of diffuse cerebral dysfunction also seen in metabolic encephalopathy, infections, or centrally acting medications.[11,24,34] Febrile patients require infectious workup with blood cultures, urine analysis, urine culture, chest X-ray, etc. per institutional guidelines.

The therapeutic strategy to follow is based on the grade of neurotoxicity and the coexistence of cytokine release syndrome. The treatment of neurotoxicity involves supportive care, frequent neurological monitoring and neuroimaging.[7,8,35] Many institutions start prophylactic antiseizure therapy; however, the clinical evidence of benefit is not clear. The preferred antiseizure drug is levetiracetam, due to its minimal drug interactions.[8] Previously, the negative impact of high-dose corticosteroids on the in vivo expansion of CAR T-cells and the presumed deleterious effect on the antileukemia control was described in a small cohort of patients with relapsed/refractory B cell acute lymphoblastic leukemia treated with autologous T cells expressing the 19–28z CAR specific to the CD19 antigen.[36] Most recently, in a retrospective analysis a higher cumulative dose and prolonged and early steroid use is associated with early progression as well as shorter overall survival after anti-CD19 CAR T-cell therapy in DLBCL patients.[37] Regarding cytokine-directed therapy, tocilizumab is not usually recommended for treatment of neurotoxicity in the absence of concurrent CRS, because its inability to cross the blood-brain barrier and may paradoxically increase the concentration of IL-6 in the central nervous system.[24] In the ZUMA-1 study, patients who received prophylactic tocilizumab on day 2 following axicabtagene ciloleucel infusion had a higher incidence of

grade ≥3 neurotoxicity per CTCAE v4.03 compared to patients that did not receive prophylactic tocilizumab.[12] Data from preclinical models suggesting that anti-IL-1-based therapies might provide a new therapeutic target for the management of neurotoxicity has led to the development of a significant number of clinical trials (NCT03430011, NCT04359784, NCT04148430, etc.).[14] Since IL-1 seems a key mediator of neurotoxicity, the use of anakinra (an IL-1 receptor antagonist) could be a therapeutic strategy with potential efficacy.[38] The group at MD Anderson Cancer Center has also reported the outcomes of a small cohort of patients receiving anakinra for the management of high-grade ICANS and HLH. In this series, 4 of 6 patients that received IL-1 blockade with anakinra for the management of high-grade ICANS

experienced clinical benefit.[39] Our approach for the management of ICANS is noted in Table 5.

Future Strategies

As the use of CAR-T therapy continues to expand for other conditions (multiple myeloma, acute leukemias, other B-cell malignancies, etc.), NT or ICANS will remain as one of the most important challenges. It also has the potential to add to the cost of care and physical burden for patient candidates for CAR-T cell therapy.[19,40]

A potential best strategy is the development of strategies to prevent NT. Given the preliminary efficacy of IL-1 blockade in improving NT in CAR-T treated patients, several trials using anakinra for NT prevention are ongoing (NCT04205838, NCT04359784, NCT04148430). In

Table 5 Management of ICANS

ASTCT ICANS Grade	Management
Grade 1	-Consider seizure prophylaxis with levetiracetam if not already started. -Review of medications, avoid medications that can cause CNS depression. -Swallowing assessment and aspiration precautions. -Neurocognitive assessment Q6hrs using ICE scoring system. -Neurology consult. -Consider EEG. -Consider lumbar puncture with opening pressure and samples for chemistry, cytology, virology, & culture. -Brain imaging (MRI preferred if no contraindication). Spinal MRI based on neurological findings. -For febrile patients, infectious workup per institutional guidelines. -Consider tocilizumab if concurrent CRS.
Grade 2	-Supportive care and workup per Grade 1. -Consider dexamethasone 10mg IV every 6hrs or methylprednisolone equivalent. -Tocilizumab if concurrent CRS. -Consider transfer to intensive care unit.
Grade 3	-Supportive care and workup per Grade 1. -Transfer to intensive care unit. -Dexamethasone 10–20mg IV every 6 hours or methylprednisolone equivalent. -High-dose methylprednisolone (1000mg/day) for focal/local edema. -Seizure control with benzodiazepines (for short-term control) and levetiracetam ± lacosamide. -If evidence of increased ICP (stage 1–2 by fundoscopy or opening pressure >20 mmHg), urgent neurology consultation to guide management. -Repeat neuroimaging if persistent grade ≥3 ICANS.
Grade 4	-Supportive care and workup per Grade 1. -Transfer to intensive care unit, may need mechanical ventilation for airway protection. -High-dose methylprednisolone 1000mg/day for 3 days followed by taper. -Seizure control per Grade 3. -Management of raised ICP per neurology/neurosurgery intensive care recommendations. May use hyperosmolar therapy (mannitol/hypertonic saline), hyperventilation strategy.

Note: Data from references.[6,8,17,18]
Abbreviations: ASBMT, American Society for Transplantation and Cellular Therapy; ICANS, immune effector cell-associated neurotoxicity syndrome; CRS, cytokine release syndrome; EEG, electroencephalogram; ICE, Immune effector Cell-associated Encephalopathy; ICP, intracranial pressure; IV, intravenous.

xenograft models of acute leukemia, the use of lenzilumab has shown that blocking GM-CSF, may reduce the incidence of CRS and NT without affecting CAR-T cell efficacy.[41] This is being investigated in the ZUMA-19 trial which combines lenzilumab with axi-cel for patients with R/R LBCL (NCT04314843). Finally, short course of steroids prophylactically may reduce the incidence of CRS and NT. The cohort 6 of the ZUMA-1, patients received dexamethasone 10 mg on days 0 (prior to the infusion), 1 and 2.[42] While the rate of grade ≥3CRS was significantly decreased, grade ≥3 NT rates did not change significantly but the incidence of NT within 72 hours from CAR-T cell infusion was lower.[42] It is unclear whether this approach will be adopted widely; however, it may become attractive given its low cost and toxicity.

Funding

Disclosure

References

1. Brudno JN, Kochenderfer JN. Chimeric antigen receptor T-cell therapies for lymphoma. *Nat Rev Clin Oncol.* 2018;15(1):31–46. doi:10.1038/nrclinonc.2017.128

2. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med.* 2017;377(26):2531–2544. doi:10.1056/NEJMoa1707447

3. Schuster SJ, Bishop MR, Tam CS, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med.* 2019;380(1):45–56. doi:10.1056/NEJMoa1804980

4. Locke Fl, Ghobadi A, Jacobson CA, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1–2 trial. *Lancet Oncol.* 2019;20(1):31–42. doi:10.1016/S1470-2045(18)30864-7

5. Abramson JS, Palomba ML, Gordon LI, et al. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. *Lancet.* 2020;396(10254):839–852. doi:10.1016/S0140-6736(20)31366-0

6. Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood.* 2014;124 (2):188–195. doi:10.1182/blood-2014-05-552729

7. Lee DW, Santomasso BD, Locke FL, et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transplant.* 2019;25 (4):625–638. doi:10.1016/j.bbmt.2018.12.758

8. Maus MV, Alexander S, Bishop MR, et al. Society for Immunotherapy of Cancer (SITC) clinical practice guideline on immune effector cell-related adverse events. *J Immunother Cancer.* 2020;8(2):e001511. doi:10.1136/jitc-2020-001511

9. Acharya UH, Dhawale T, Yun S, et al. Management of cytokine release syndrome and neurotoxicity in chimeric antigen receptor (CAR) T cell therapy. *Expert Rev Hematol.* 2019;12(3):195–205. doi:10.1080/17474086.2019.1585238

10. Mackall CL, Miklos DB. CNS endothelial cell activation emerges as a driver of CAR T cell-associated neurotoxicity. *Cancer Discov.* 2017;7(12):1371–1373. doi:10.1158/2159-8290.CD-17-1084

11. Gust J, Hay KA, Hanafi LA, et al. Endothelial activation and blood-brain barrier disruption in neurotoxicity after adoptive immunotherapy with CD19 CAR-T cells. *Cancer Discov.* 2017;7(12):1404–1419. doi:10.1158/2159-8290.CD-17-0698

12. Locke FL, Neelapu SS, Bartlett NL, et al. Preliminary results of prophylactic tocilizumab after axicabtageneciloleucel (axi-cel; KTE-C19) treatment for patients with refractory, aggressive Non-Hodgkin Lymphoma (NHL). *Blood.* 2017;130(Supplement 1):1547.

13. Giavridis T, Van Der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med.* 2018;24(6):731–738. doi:10.1038/s41591-018-0041-7

14. Norelli M, Camisa B, Barbiera G, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med.* 2018;24(6):739–748. doi:10.1038/s41591-018-0036-4

15. Locke FL, Rossi JM, Neelapu SS, et al. Tumor burden, inflammation, and product attributes determine outcomes of axicabtagene ciloleucel in large B-cell lymphoma. *Blood Adv.* 2020;4(19):4898–4911. doi:10.1182/bloodadvances.2020002394

16. Maziarz RT, Schuster SJ, Ericson SG, et al. Cytokine release syndrome and neurotoxicity by baseline tumor burden in adults with relapsed or refractory diffuse large B-cell lymphoma treated with tisagenlecleucel. *Hematol Oncol.* 2019;37(S2):307. doi:10.1002/hon.117_2630

17. Jacobson CA, Hunter BD, Redd R, et al. Axicabtagene ciloleucel in the non-trial setting: outcomes and correlates of response, resistance, and toxicity. *J Clin Oncol.* 2020;38(27):3095–3106. doi:10.1200/JCO.19.02103

18. Nastoupil LJ, Jain MD, Feng L, et al. Standard-of-care axicabtagene ciloleucel for relapsed or refractory large B-cell lymphoma: results from the US lymphoma CAR T consortium. *J Clin Oncol.* 2020;38 (27):3119–3128. doi:10.1200/JCO.19.02104

19. Wudhikarn K, Pennisi M, Garcia-Recio M, et al. DLBCL patients treated with CD19 CAR T cells experience a high burden of organ toxicities but low nonrelapse mortality. *Blood Adv.* 2020;4(13):3024–3033. doi:10.1182/bloodadvances.2020001972

20. Karschnia P, Jordan JT, Forst DA, et al. Clinical presentation, management, and biomarkers of neurotoxicity after adoptive immunotherapy with CAR T cells. *Blood.* 2019;133(20):2212–2221. doi:10.1182/blood-2018-12-893396

21. Gofshteyn JS, Shaw PA, Teachey DT, et al. Neurotoxicity after CTL019 in a pediatric and young adult cohort. *Ann Neurol.* 2018;84(4):537–546. doi:10.1002/ana.25315

22. Rubin DB, Al Jarrah A, Li K, et al. Clinical predictors of neurotoxicity after chimeric antigen receptor T-cell therapy. *JAMA Neurol.* 2020;77(12):1536–1542. doi:10.1001/jamaneurol.2020.2703

23. Hay KA, Hanafi LA, Li D, et al. Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor-modified T-cell therapy. *Blood.* 2017;130(21):2295–2306. doi:10.1182/blood-2017-06-793141

24. Santomasso BD, Park JH, Salloum D, et al. Clinical and biological correlates of neurotoxicity associated with CAR T-cell therapy in patients with B-cell acute lymphoblastic leukemia. *Cancer Discov.* 2018;8(8):958–971. doi:10.1158/2159-8290.CD-17-1319

25. Singh N, Hofmann TJ, Gershenson Z, et al. Monocyte lineage-derived IL-6 does not affect chimeric antigen receptor T-cell function. *Cytotherapy.* 2017;19(7):867–880. doi:10.1016/j.jcyt.2017.04.001

26. Faramand R, Jain M, Staedtke V, et al. Tumor microenvironment composition and severe Cytokine Release Syndrome (CRS) influence toxicity in patients with large B-cell lymphoma treated with axicabtagene ciloleucel. *Clin Cancer Res*. 2020;26(18):4823–4831. doi:10.1158/1078-0432.CCR-20-1434

27. Maziarz RT, Schuster SJ, Romanov VV, et al. Grading of neurological toxicity in patients treated with tisagenlecleucel in the JULIET trial. *Blood Adv.* 2020;4(7):1440–1447. doi:10.1182/bloodadvances.2019001305

28. Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. *Nat Rev Clin Oncol*. 2018;15(1):47–62. doi:10.1038/nrclinonc.2017.148

29. Turtle CJ, Hay KA, Gust J, et al. Cytokine release syndrome (CRS) and neurotoxicity (NT) after CD19-specific chimeric antigen receptor- (CAR-) modified T cells. *J Clin Oncol*. 2017;35(15_suppl):3020. doi:10.1200/JCO.2017.35.15_suppl.3020

30. Poh A. JCAR015 in ALL: a root-cause investigation. *Cancer Discov.* 2018;8(1):4–5.

31. Frisen L. Swelling of the optic nerve head: a staging scheme. *J Neurol Neurosurg Psychiatry.* 1982;45(1):13–18. doi:10.1136/jnnp.45.1.13

32. Brudno JN, Kochenderfer JN. Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood*. 2016;127(26):3321–3330. doi:10.1182/blood-2016-04-703751

33. Rubin DB, Danish HH, Ali AB, et al. Neurological toxicities associated with chimeric antigen receptor T-cell therapy. *Brain*. 2019;142(5):1334–1348. doi:10.1093/brain/awz053

34. Herlopian A, Dietrich J, Abramson JS, Cole AJ, Westover MB. EEG findings in CAR T-cell therapy-related encephalopathy. *Neurology.* 2018;91(5):227–229. doi:10.1212/WNL.0000000000005910

35. Neelapu SS. Managing the toxicities of CAR T-cell therapy. *Hematol Oncol*. 2019;37(Suppl 1):48–52. doi:10.1002/hon.2595

36. Davila ML, Riviere I, Wang X, et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med*. 2014;6(224):224ra225. doi:10.1126/scitranslmed.3008226

37. Strati P, Ahmed S, Furqan F, et al. Prognostic impact of corticosteroids on efficacy of chimeric antigen receptor T-cell therapy in large B-cell lymphoma. *Blood*. 2021;137(23):3272–3276. doi:10.1182/blood.2020008865

38. Tummala S. Anakinara to mitigate CAR T cell therapy associated toxicity (4838); 2021.

39. Strati P, Ahmed S, Kebriaei P, et al. Clinical efficacy of anakinra to mitigate CAR T-cell therapy-associated toxicity in large B-cell lymphoma. *Blood Adv.* 2020;4(13):3123–3127. doi:10.1182/bloodadvances.2020002328

40. Hernandez I, Prasad V, Gellad WF. Accounting for All costs in the total cost of chimeric antigen receptor T-cell immunotherapy-reply. *JAMA Oncol*. 2018;4(12):1785–1786. doi:10.1001/jamaoncol.2018.4657

41. Sterner RM, Sakemura R, Cox MJ, et al. GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function in xenografts. *Blood*. 2019;133(7):697–709. doi:10.1182/blood-2018-10-881722

42. Oluwole OO, Bouabdallah K, Muñoz J, et al. Prophylactic steroid use with Axicabtagene Ciloleucel (Axi-Cel) in Patients (Pts) with Relapsed/Refractory Large B Cell Lymphoma (R/R LBCL). Presented at: 2021 TCT| Transplantation & Cellular Therapy Meetings of ASTCT and CIBMTR.
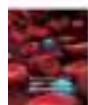
Thrombotic and Hypercoagulability Complications of COVID-19: An Update

Sapha Shibeeb & Muneera Naseer Ahmad

REVIEW

Thrombotic and Hypercoagulability Complications of COVID-19: An Update

Sapha Shibeeb[1,2]
Muneera Naseer Ahmad[1]

[1]Department of Biomedical Science, College of Health Sciences, QU Health, Qatar University, Doha, Qatar; [2]Biomedical and Pharmaceutical Research Unit, QU Health, Qatar University, Doha, Qatar

Abstract: The current COVID-19 pandemic emerged in December 2019, in China, affecting millions of people worldwide. COVID-19 is mainly a disease of the respiratory system, yet systematic complications have also been reported among SARS-CoV-2 infected patients. Thrombotic complications are one of the severe clinical outcomes of COVID-19, especially among critically ill patients, and are associated with poor prognosis. To date, many studies have concluded that COVID-19 increases the incidence of thrombotic events and coagulopathies; however, the exact mechanism behind such a disease outcome is not well known. Various pathophysiological mechanisms for thrombotic events in COVID-19 have been proposed, these include virus-induced endothelial cell damage, inflammation, and excess production of pro-inflammatory cytokines. As a result, most critically diseased COVID-19 patients are managed with prophylactic anticoagulant, yet some still develop thrombotic episodes. Therefore, better understanding of the mechanisms behind the thrombotic complications is needed to develop treatments that specifically target such pathways, which may aid in better disease management and improve the prognosis.

Keywords: COVID-19, SARS-CoV-2, thrombosis, thromboembolism

Introduction

In December 2019, the Chinese Center for Disease Control and Prevention identified the presence of a novel coronavirus in throat swab samples from a series of pneumonia cases of unknown etiology, that presented with dry cough, dyspnea, and fever.[1] The newly-identified virus was named as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), since it resembled SARS-CoV that emerged in 2002–2003 and resulted in high morbidities and mortalities. By March 2020, the World Health Organization declared a global pandemic, and the disease caused by the virus was named Coronavirus disease 2019 (COVID-19).[2] Subsequently, many countries around the world implemented various preventive strategies to minimize the rate of the virus spread. Yet, SARS-CoV-2 affected millions of different age groups worldwide. The latest statistics show the infected cases exceeded 158 million with more than 3 million deaths worldwide.

The novel coronavirus, SARS-CoV-2, belongs to the coronaviridae family, which are positive-sense single-stranded enveloped RNA viruses. There are four different genera for coronaviruses, and SARS-CoV-2 belongs to the β coronaviruses.[3] SARS-CoV-2 and coronaviruses in general have four essential structural proteins (Figure 1), namely spike (S) glycoprotein, envelope (E) glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N) protein. These proteins are necessary for successful viral attachment and penetration of the host cells,

Correspondence: Sapha Shibeeb
Department of Biomedical Science, College of Health Sciences, QU Health, Qatar University, P.O. Box 2713, Doha, Qatar
Tel +974 4403 6551
Email sapha@qu.edu.qa

785
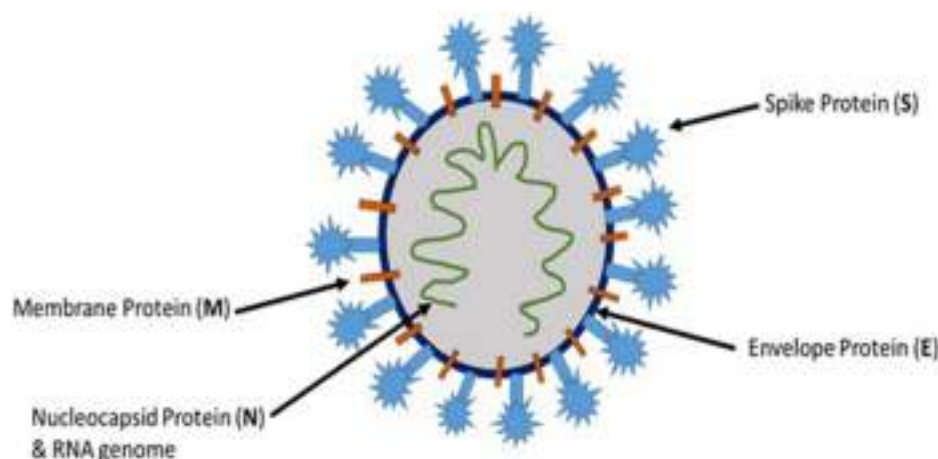
Figure 1 Schematic representation of SARS-CoV-2 structure.

synthesis of viral proteins, maturation, and release of the viral progeny.[4] The viral S protein, a transmembrane protein that protrudes from the viral surface, has been considered as an important factor in the pathogenesis of coronaviruses including SARS-CoV-2. S glycoprotein aids in the viral attachment and fusion to the host cell membrane.[5]

Thrombotic episodes are one of the severe complications of COVID-19, particularly in critically ill patients, which can be associated with poor prognosis. A growing body of evidence suggests that COVID-19 increases the incidence of thrombotic complications; however, the exact mechanisms are not fully elucidated. Therefore, the current review aims to provide an overview of the available literature to outline the current knowledge of the risks, pathogenesis, and the therapeutic interventions for thrombotic complications that are associated with the COVID-19 pandemic.

Pathogenesis of COVID-19 Infection

SARS-CoV-2 is a zoonotic virus that evolved in a way to be able to be transmitted from human-to-human.[6] SARS-CoV-2 can transmit from one individual to another either by direct transfer through aerosols or indirectly via contaminated surfaces. SARS-CoV-2 contains large RNA genomes flanked by 5′ and 3′ untranslated regions containing cis-acting secondary RNA structures necessary for RNA synthesis and replication. At the 5′ end, the viral RNA possesses two large open reading frames (ORFs; ORF1a and ORF1b) that occupy two-thirds of the nonstructural genome.[7] The currently proposed pathogenesis of SARS-

CoV-2 is derived from the knowledge that is available on SARS-CoV, where the viral spike protein binds to host's angiotensin-converting enzyme 2 (ACE2) receptor, which is differentially expressed on various tissues, including respiratory tract cells, gastrointestinal tract cells, cardiac muscle cells, and endothelial cells.[8]

A successful binding of the spike protein to ACE2 and the internalization of the virus requires the presence of the specific protease transmembrane serine protease 2 (TMPRSS2) that cleaves and activates ACE2.[9] With the successful viral entry into the cells, host replication machinery is used, and viral progenies are produced, which can further infect adjacent cells (Figure 2). Indeed, recent evidence shows TMPRSS2 is expressed in human endothelial cells obtained from the lungs which may
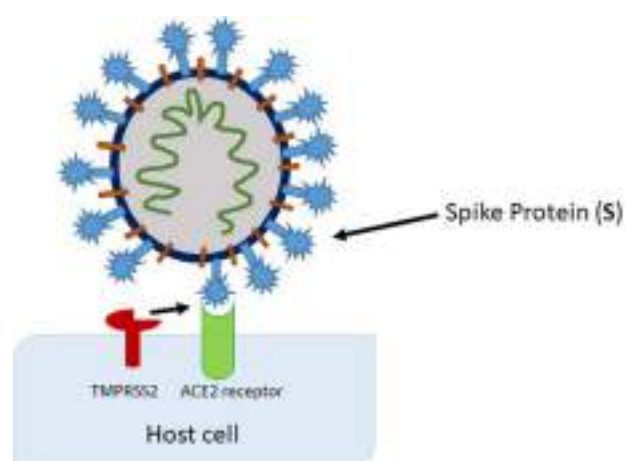
Figure 2 Activation of the spike protein by TMPRSS2 at (or close to) the cell surface, leading to fusion of the viral membrane with the plasma membrane.

explain the pulmonary complications of COVID-19.[10] Furthermore, smokers and patients with moderate-to-severe COPD had been found to have higher levels of ACE2 mRNA and protein levels in lung tissue. Furthermore, hypertensive patients were found to have the more severe form of the disease, suggesting antihypertensive therapy may play a role in developing serious COVID-19 infection due to enhanced ACE2 and SARS-CoV-2 interactions.[11]

Upon entry, the viral genome is released into the cytosol, this leads to the immediate translation of ORF1a and ORF1b which encode for 15–16 non-structural proteins. The majority of these non-structural proteins make up the viral replication and transcription complex (TRC). In turn, the TRC houses RNA-processing and modifying enzymes as well as a RNA proofreading mechanism that is essential for maintaining the integrity of the viral genome. Translation of ORF1a and ORF1b results in polyproteins that are co-translationally and post-translationally processed into the single non-structural proteins, which leads to the formation of the viral replication and transcription system. As non-structural proteins are formed and expressed, the synthesis of viral replication machinery also takes place, which consists of perinuclear double-membrane vesicles (DMVs), convoluted membranes (CMs), and small open double-membrane spherules (DMSs) forming a protective and suitable microenvironment for viral genomic RNA replication and transcription of subgenomic mRNAs. The newly synthesized viral genomic RNA results in budding into the lumen of secretory vesicular compartments, which are then secreted from the infected cell by exocytosis.[7,12]

The clinical presentations of COVID-19 are highly variable from one individual to another. COVID-19 ranges from asymptomatic to severe disease that could result in death. However, mild-to-moderate flu-like symptoms are the most common presentations among COVID-19 patients, including fever, dry cough, sore throat, runny nose, and, in some cases, involvement of the lower respiratory tract that may lead to acute respiratory distress syndrome (ARDS).[13] Other general symptoms such as weakness, headache, and gastrointestinal symptoms including diarrhea and vomiting have also been reported.[14] Moreover, to a lower extent some of the COVID-19 patients experienced loss of smell and taste.[15] Some affected individuals could further progress to severe forms of the disease, where they develop severe pneumonia, pulmonary edema, septic shock, and organ failure that

would result in death.[16] Thromboembolic events and acute kidney injury have also been reported as COVID-19 complications.[13] Indeed, laboratory findings such as thrombocytopenia, increased prothrombin time, and activated partial thromboplastin time along with elevated D-dimer were reported among critically ill patients, which further supports the thrombotic and coagulopathy complications in COVID-19 patients. Together these findings indicate that COVID-19 is a systematic disease with serious thrombotic complications that require more attention.

Thrombotic Complications of SARS-CoV-2 Infection

A number of studies have reported thrombotic and hypercoagulability complications among COVID-19 cases. These complications represent a serious worsening of the disease since it is associated with adverse outcomes. A recent systematic review reported that among 2,928 COVID-19 severely diseased patients, 56.3% developed thrombosis, and 34% of ICU admitted patients were found to have thrombotic complications, where 16.1% were reported with deep vein thrombosis and 12.6% with pulmonary embolism.[17] Many other independent studies have reported different rates of thrombotic complications among COVID-19 patients (Table 1). It is now established that critically ill COVID-19 patients were associated with a higher incidence of thrombotic events than other COVID-19 patients. This could be associated with higher levels of pro-inflammatory and dysregulated fibrinolytic states among severe COVID-19 cases.

Indeed, it was reported in a cohort of 54 COVID-19 patients who died from the infection, it was found those patients had higher levels of D-dimer, troponin, and interleukin-6 (IL-6).[18] Furthermore, Wang et al[19] reported that, among 199 COVID-19 patients, elevated levels of D-dimer, fibrinogen degradation products, prolonged prothrombin time, and thrombin time were found to be higher among patients with severe disease manifestations. These findings indicate that COVID-19 is associated with abnormal coagulation and subsequently leads to thrombotic complications and severe disease outcome. Moreover, Harenberg et al[20] reported there was an initial increase in the prothrombin time and activated partial thromboplastin time, but later both are decreased due to consumption of coagulation factors. These findings were also associated with an increase in the platelet count, fibrinogen, and

Table 1 Incidence of Thrombotic Complications Among COVID-19 Patients

Author	Number of Subjects, N	Incidence of Thrombotic Event	Type of Thrombotic Event
Jenner et al[17]	2,928	90.3% experienced thrombotic complications (34% of ICU patients 56.3% non-ICU patients)	Deep vein thrombosis (DVT) reported (16.1% of ICU patients) Pulmonary embolism (12.6% of ICU patients) Venous thrombosis (56.3% routine screening)
Chen et al[56]	88	46%	Deep vein thrombosis
Shah et al[57]	187	N=81 (43.3%)	Pulmonary embolism (22.5%) Deep vein thrombosis (11.8%) Arterial complications (13.4%)
Gibson et al[58]	72	N=12 (16.7%)	Lower extremity of deep vein thrombosis
Monfardini et al[59]	34	N=26 (76%)	Pulmonary thromboembolism
Avruscio et al[60]	85 (41 critical cases)	N=43 (50.6%)	Pulmonary embolism (9.8%) Deep vein thrombosis (42.4%) Superficial vein thrombosis (3.5%)
Al-Samkari et al[61]	400 (144 critically ill)	N=38	Venous thromboembolism Arterial thrombosis
Demelo-Rodriguez et al[62]	156	N=23	Deep vein thrombosis
Piazza et al[63]	1,114	N=66	Venous thromboembolic event (4.6%) Deep vein thrombosis (3.5%) Pulmonary embolism (0.7%) Disseminated intravascular coagulation (1.3%)

D-dimer. In addition, in a cohort of 248 COVID-19 cases, Yao et al[21] reported that D-dimer levels were higher among 185 patients, and it was significantly increasing with an increase in disease severity.

Interestingly, the ABO blood group system has also been suggested to contribute to susceptibility and severity of COVID-19 infection. Indeed, a study by Marcos et al[22] concluded that blood group O was associated with lower ICU admission and thrombotic complications when compared to other blood groups. Furthermore, blood group B had significantly higher rates of thrombotic complications and was associated with more ICU. A possible mechanism for this is that plasma levels of Von Willebrand factor ((VWF) a glycoprotein essential for thrombus formation) levels are lower in group O patients when compared to non-O blood group patients.

Pathogenesis of Thrombotic Complications of COVID-19

Various factors have been reported to be associated with increased incidence of coagulopathies among COVID-19 patients. These include patients' age, and coexistence of chronic disease such as cardiovascular disease, diabetes, and hypertension.[23] Indeed, COVID-19 patents with chronic disease such as diabetes have been shown to have increased pro-inflammatory mediators IL-6, suggesting these patients are at higher risk of developing more serious complications of COVID-19.[24,25] In addition, males have been shown to have been more at risk of developing thrombotic complications. Indeed, various COVID-19 reports have so far shown that elderly patients are severely affected by the infection and they account for most of the severe cases that requires ICU admission, with a higher incidence of thrombosis.[26]

The exact mechanism responsible for COVID-19 induced thrombosis is not fully understood. However, several mechanisms have been put forward in an attempt to explain these complications among COVID-19 patients. Such mechanisms include increased platelet activation and activation coagulation cascade and/or decreased fibrinolysis, in addition to immune activation. However, a central mechanism involves virus-induced endothelial damage.

Endothelial cells express the ACE2 receptor which is a SARS-CoV-2 target receptor. Ut has been suggested that viral penetration of endothelial cells inflicts tissue damage and subsequently exposure of collagen, a potent platelet agonist, leading to platelet adhesion and activation, furthermore, injured endothelial cells release tissue factor.[27,28] Initially this leads to the activation and the recruitment of platelets, and further release of platelet granules content that aid in the platelet plug formation,[29] whilst tissue factor activates the extrinsic pathway of the coagulation cascade. Subsequently this leads to the activation of the intrinsic pathway of coagulation cascade, formation of thrombin that converts fibrinogen to fibrin which further stabilizes platelet plug, and forms thrombus.[28,30] Evidence of increased ACE2 expression on the endothelial cells of SARS-CoV-2 infected patients has been reported, which suggest a higher probability of endothelial cells damage that leads to thrombotic outcome.[31]

Abnormal fibrinolysis has also been suggested to contribute to thrombotic events during COVID-19 infection. Wright et al[32] reported that among 44 ICU admitted patients, 57% experienced a complete lack in the lysis of the clots, suggesting defective fibrinolysis. This was later supported by the increased levels of plasminogen activator inhibitor 1 among COVID-19 patients, which inhibits the plasminogen activator and leads to a decrease in the fibrin degradation.[33]

The immune system and homeostasis complement each other in order to defend against a pathogen and prevent further dissemination of the pathogen, by a process known as immuno-thrombosis. This physiological mechanism could be dysregulated, leading to the formation of excess thrombus formation.[34] When endothelial cells are infected with SARS-CoV-2, they undergo pyroptosis, a form of cell death.[35] This process is associated with the release of cellular content such as pathogen associated molecular pattern (PAMPs), and damage associated molecular patterns (DAMPs) causing inflammation. DAMPs and PAMPs interact with pattern recognition receptor and Toll-like receptor on innate immune cells triggering immune response and the release of pro-inflammatory cytokines and this enhances the expression of tissue factor.[35,36] Therefore, some patients develop an induced immune response to SARS-CoV-2 that is defined as cytokine storm.[37] COVID-19 patients are found to have increased levels of specific chemokines and cytokines, which include IL-6, interferon-gamma, and IL-2.[38] IL-6 is reported to increase the production of platelets in COVID-19 patients,

enhances the expression of tissue factor on monocytes and endothelial cells, and by itself can lead to further endothelial damage.[38] To a similar extent it has been reported that interferon-gamma also increases the production of platelets and causes endothelial dysfunction, creating a prothrombotic status. On the other hand, IL-2 in COVID-19 patients is reported to increase the production of plasminogen activator inhibitor-1, which reduces fibrinolysis[39] (Figure 3).

In addition to these proposed mechanisms, the complement system has also been implicated in the development of thrombotic complications in COVID-19 patients. Indeed, in a study conducted by Magro et al,[40] it was found that there is a continuous activation of the alternative and the lectin pathways of the complement system, that was supported by the presence of terminal C5b-9 complement complex on the vessels of the affected individual's lungs. The membrane attack complex, C5b-9, has the ability to activate platelets, endothelial damage, and enhance the release of VWF. Individual complement components, including C5a, have the ability to induce the production of tissue factor, plasminogen activator inhibitor-1 and IL-6, which further stimulate thrombus formation.[41] Furthermore, since SARS-CoV-2 invades the cells through an ACE2 receptor, this causes downregulation of the receptor and subsequent upregulation of angiotensin II, which is normally degraded by ACE2. High levels of angiotensin II cause vasocontraction,[42] increased levels of tissue factor, and plasminogen activator inhibitor, therefore favoring a hypercoagulable state.[43]

Clinical Manifestation of Thrombotic Events in COVID-19 Patients

Thrombotic complications of COVID-19 have been reported from the start of this pandemic and are highly reported among severely ill patients.[17] Severe infection with SARS-CoV-2 has been reported to be associated with the development of microvascular and macrovascular thrombosis that eventually leads to an increase in the mortality rates. Macrovascular and microvascular thrombosis results from platelet activation and the activation of the coagulation cascade and subsequent thrombus formation in blood vessels of varying sizes that could cause partial or complete occlusion of the blood vessels.[44,45] Venous thromboembolism and arterial thrombosis are among the macrovascular thrombotic complications of COVID-19. Both forms of venous thromboembolism, pulmonary embolism, and deep vein
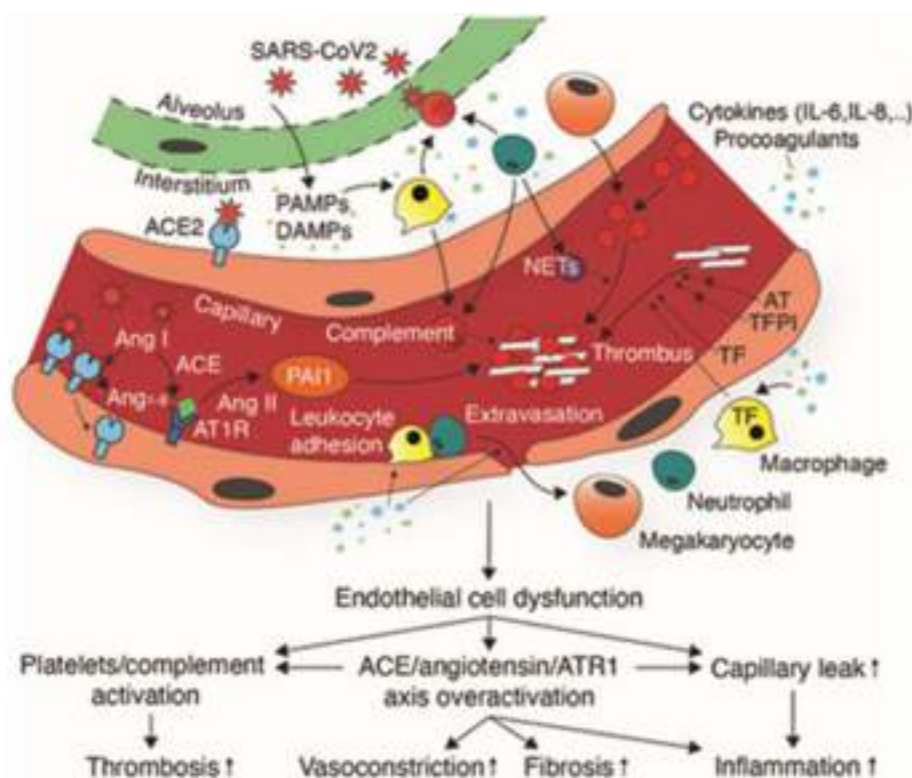
Figure 3 Pathogenesis of thrombotic complications of COVID-19. SARS-CoV2 infection induces endothelial damage triggering endothelial release of cytokines, increasing capillary permeability. PAMPs and DAMPs induced activation of neutrophils, and macrophages results in localized production of cytokines, procoagulants, and complement activation, leading to further endothelial damage and tissue factor release. Endothelial damage exposes collagen and other prothrombotic mediators leading to thrombus formation.
Notes: Adapted from Brosnahan SB, Jonkman AH, Kugler MC, Munger JS, Kaufman DA. COVID-19 and Respiratory System Disorders: Current Knowledge, Future Clinical and Translational Research Questions. Arterioscler Thromb Vasc Biol. 2020;40(11):2586–2597.[64] © 2020 The Authors. Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/legalcode). https://www.ahajournals.org/doi/pdf/10.1161/ATVBAHA.120.314515?download=true.

thrombosis have been reported among COVID-19 patients. It was reported that among 1,765 patients the rate of venous pulmonary embolism in COVID-19 patients was around 22%, with higher incidence rates among ICU admitted patients.[46] Whilst some patients experienced deep vein thrombosis. Zhang et al[47] reported that among 143 hospitalized COVID-19 patients, 66 (46.2%) developed deep vein thrombosis, and it was common in elderly patients and was associated with poor disease outcome and higher mortality rates. These rates could be even higher than the reported data, since not all COVID-19 patients undergo computerized tomography, mainly because of the risk of spreading the infection. Moreover, a meta-analysis involved 46,348 COVID-19 cases reported that around 7% had increased levels of troponin which is associated with myocardial injury is also reported among COVID-19 patients.[48] Identifying the risks and incidence rate of these complications among COVID-19 is essential for determining the best procedures

for diagnosing such outcomes for best patient's management. Furthermore, COVID-19 patients also experience coagulation abnormalities that are similar but not identical to thrombotic thrombocytopenic purpura, or hemolytic uremic syndrome or disseminated intravascular coagulation (DIC). Higher levels of D-dimer, thrombocytopenia and prolonged prothrombin time in COVID-19 patients suggest the occurrence of DIC, yet in DIC the levels of D-dimer is higher than that of COVID-19 and thrombocytopenia is more predominant. In addition, COVID-19 patients with thrombotic complications have high levels of ferritin and lactate dehydrogenase along with platelet rich plaques in the lungs and other organs, yet there is no schistocytes that are normally present in the case of thrombotic microangiopathies.[49] This indicates that thrombotic manifestation in COVID-19 have unique features and the mechanism behind this variation need to be investigated for better disease diagnosis and management.

Management of COVID-19 Associated Thrombotic Complications

Due to the rapidly evolving literature, there are no unified guidelines on how best to diagnose and manage thrombotic and hypercoagulability in COVID-19 patients. This is partly due to the variability in incidence among studies which can be attributed to differences in population. However, most of the published guidelines recommend using prophylactic anticoagulant for all hospitalized COVID-19 patients, to avoid such severe disease outcomes.[50] Most of the guidelines suggest the use of daily low-molecular-weight heparins or subcutaneous unfractionated heparin. Low-molecular-weight heparin is considered more beneficial than other prophylactic regimens, particularly in COVID-19 cases, as it has been previously reported that it has a longer half-life than unfractionated heparin,[51] acts as an inhibitor for viral attachment by binding to SARS-CoV-2 spike protein,[52] and it has immunomodulatory and anti-inflammatory effects.[53] Despite using thromboprophylaxis, some critically ill patients still developed thrombotic complications. This could be due to heparin resistance and lower levels of anti-activated factor X in these patients, which could be attributed to high levels of fibrinogen and reduced antithrombin levels in COVID-19 patients, yet the exact mechanism for heparin resistance among COVID-19 patients is not known.[54] Therefore, it is recommended to use higher doses of prophylactic anticoagulant, as it was reported that with higher doses the levels of anti-activated factor X are higher, which could aid in preventing thrombosis. Yet, this finding cannot be generalized since it is not known if all COVID-19 hospitalized subjects would benefit from higher doses of thromboprophylaxis without leading to additional complications.

Venous thromboembolism is mainly managed by the use of therapeutic anticoagulants. The best choice of the anticoagulant as a treatment depends on patients' renal and hepatic functions, gastrointestinal function, and thrombocytopenia. Furthermore, for patients admitted to hospital and receiving direct care it is preferred to use low-molecular weight heparin, unfractionated heparin (UFH), or fondaparinux (particularly for patients with heparin-induced thrombocytopenia), but for out-patients it is better to use direct oral anticoagulants. In addition, a number of drugs have been studied to be used as a treatment for sepsis-induced thrombotic complications, and it is suggested that they might have a therapeutic role in

COVID-19 and these include; Danaparoid, that weakens thrombin production, Sulodexide, which potentiates anti-proteolytic activity of anti-thrombin and heparin co-factor, antithrombin, that inactivates coagulation enzymes, and thrombomodulin, which acts as a co-factor for thrombin.[55]

Conclusion

SARS-CoV-2 infection rates are still increasing with an increase in the morbidity and mortality rates. There is accumulating evidence that COVID-19 is associated with various systematic complications, including thrombotic and hypercoagulability. Yet, the exact mechanism of such complication is not fully understood. However, a central mechanism that has been postulated is SARS-CoV-2 induced endothelial damage via ACE2 receptor. Endothelial injury and inflammation result in a series of reactions leading to the activation of the coagulation pathways and platelet. Moreover, increased levels of pro-inflammatory mediators complement cascade activation and shut down of anti-thrombotic pathways have been shown to play a role in the pathogenesis of thrombotic complications. However, the available data are limited, and further investigations are required for better understanding of the disease mechanisms.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Lu H, Stratton CW, Tang Y-W. Outbreak of pneumonia of unknown etiology in Wuhan, China: the mystery and the miracle. *J Med Virol*. 2020;92(4):401–402. doi:10.1002/jmv.25678
2. Sohrabi C, Alsafi Z, O'Neill N, et al. World health organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). *Int J Surg*. 2020;76:71–76. doi:10.1016/j. ijsu.2020.02.034
3. Phan T. Novel coronavirus: from discovery to clinical diagnostics. *Infect Genet Evol*. 2020;79:104211. doi:10.1016/j.meegid.20 20.104211
4. Satarker S, Nampoothiri M. Structural proteins in severe acute respiratory syndrome coronavirus-2. *Arch Med Res*. 2020;51(6):482–491. doi:10.1016/j.arcmed.2020.05.012
5. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. *Methods Mol Biol*. 2015;1282:1–23. doi:10.1007/ 978-1-4939-2438-7_1
6. Wong ACP, Li X, Lau SKP, Woo PCY. Global epidemiology of bat coronaviruses. *Viruses*. 2019;11(2):174. doi:10.3390/v11020174
7. V'kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. *Nat Rev Microbiol*. 2021;19(3):155–170. doi:10.1038/s41579-020-00468-6

8. Zou X, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. *Front Med*. 2020;14(2):185–192. doi:10.1007/s11684-020-0754-0

9. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. 2020;181(2):271–80.e8. doi:10.1016/j.cell.2020.02.052

10. Matarese A, Gambardella J, Sardu C, Santulli G. miR-98 regulates TMPRSS2 expression in human endothelial cells: key implications for COVID-19. *Biomedicines*. 2020;8(11):462. doi:10.3390/biomedicines8110462

11. Sardu C, Maggi P, Messina V, et al. Could anti-hypertensive drug therapy affect the clinical prognosis of hypertensive patients with COVID-19 infection? data from centers of southern Italy. *J Am Heart Assoc*. 2020;9(17):e016948. doi:10.1161/JAHA.120.016948

12. Sanyal S. How SARS-CoV-2 (COVID-19) spreads within infected hosts—what we know so far. *Emerging Topics Life Sci*. 2020;4(4):383–390. doi:10.1042/ETLS20200165

13. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;395(10223):497–506. doi:10.1016/s0140-6736(20)30183-5

14. Shi H, Han X, Jiang N, et al. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. *Lancet Infect Dis*. 2020;20(4):425–434. doi:10.1016/S1473-3099(20)30086-4

15. Agyeman AA, Chin KL, Landersdorfer CB, Liew D, Ofori-Asenso R. Smell and taste dysfunction in patients with COVID-19: a systematic review and meta-analysis. *Mayo Clin Proc*. 2020;95(8):1621–1631. doi:10.1016/j.mayocp.2020.05.030

16. Tabata S, Imai K, Kawano S, et al. Clinical characteristics of COVID-19 in 104 people with SARS-CoV-2 infection on the diamond princess cruise ship: a retrospective analysis. *Lancet Infect Dis*. 2020;20(9):1043–1050. doi:10.1016/S1473-3099(20)30482-5

17. Jenner WJ, Kanji R, Mirsadraee S, et al. Thrombotic complications in 2928 patients with COVID-19 treated in intensive care: a systematic review. *J Thromb Thrombolysis*. 2021;51(3):595–607. doi:10.1007/s11239-021-02394-7

18. Zhou F, Yu T, Du R, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet (London, England)*. 2020;395(10229):1054–1062. doi:10.1016/S0140-6736(20)30566-3

19. Wang J, Zhang H, Qiao R, et al. Thrombo-inflammatory features predicting mortality in patients with COVID-19: the FAD-85 score. *J Int Med Res*. 2020;48(9):300060520955037. doi:10.1177/0300060520955037

20. Harenberg J, Favaloro E. COVID-19: progression of disease and intravascular coagulation - present status and future perspectives. *Clin Chem Lab Med*. 2020;58(7):1029–1036. doi:10.1515/cclm-2020-0502

21. Yao Y, Cao J, Wang Q, et al. D-dimer as a biomarker for disease severity and mortality in COVID-19 patients: a case control study. *J Intensive Care*. 2020;8(1):49. doi:10.1186/s40560-020-00466-z

22. Marcos SZ, Antelo ML, Galbete A, Etayo M, Ongay E, García-Erce JA. Infection and thrombosis associated with COVID-19: possible role of the ABO blood group. *Medicina Clínica (English Edition)*. 2020;155(8):340–343. doi:10.1016/j.medcle.2020.06.013

23. Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet Respir Med*. 2020;8(4):e21. doi:10.1016/S2213-2600(20)30116-8

24. Sardu C, Gargiulo G, Esposito G, Paolisso G, Marfella R. Impact of diabetes mellitus on clinical outcomes in patients affected by Covid-19. *Cardiovasc Diabetol*. 2020;19(1):1–4. doi:10.1186/s12933-020-01047-y

25. Sardu C, D'Onofrio N, Balestrieri ML, et al. Hyperglycaemia on admission to hospital and COVID-19. *Diabetologia*. 2020;63(11):2486–2487. doi:10.1007/s00125-020-05216-2

26. Liu K, Chen Y, Lin R, Han K. Clinical features of COVID-19 in elderly patients: a comparison with young and middle-aged patients. *J Infect*. 2020;80(6):e14–e18. doi:10.1016/j.jinf.2020.03.005

27. Ackermann M, Verleden SE, Kuehnel M, et al. Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in covid-19. *N Engl J Med*. 2020;383(2):120–128. doi:10.1056/NEJMoa2015432

28. Gale AJ. Continuing education course# 2: current understanding of hemostasis. *Toxicol Pathol*. 2011;39(1):273–280. doi:10.1177/0192623310389474

29. Golebiewska EM, Poole AW. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev*. 2015;29(3):153–162. doi:10.1016/j.blre.2014.10.003

30. Bautista-Vargas M, Bonilla-Abadía F, Cañas CA. Potential role for tissue factor in the pathogenesis of hypercoagulability associated with in COVID-19. *J Thromb Thrombolysis*. 2020;50:479–483. doi:10.1007/s11239-020-02172-x

31. Yuki K, Fujiogi M, Koutsogiannaki S. COVID-19 pathophysiology: a review. *Clin Immunol*. 2020;215:108427. doi:10.1016/j.clim.2020.108427

32. Wright FL, Vogler TO, Moore EE, et al. Fibrinolysis shutdown correlation with thromboembolic events in severe COVID-19 infection. *J Am Coll Surg*. 2020;231(2):193–203. doi:10.1016/j.jamcollsurg.2020.05.007

33. Goshua G, Pine AB, Meizlish ML, et al. Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. *Lancet Haematol*. 2020;7(8):e575–e82. doi:10.1016/S2352-3026(20)30216-7

34. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol*. 2013;13(1):34–45. doi:10.1038/nri3345

35. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. *Nat Rev Immunol*. 2020;20(6):363–374. doi:10.1038/s41577-020-0311-8

36. Puelles VG, Lütgehetmann M, Lindenmeyer MT, et al. Multiorgan and renal tropism of SARS-CoV-2. *New Engl J Med*. 2020;383(6):590–592. doi:10.1056/NEJMc2011400

37. Liu Y, Yang Y, Zhang C, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci China Life Sci*. 2020;63(3):364–374. doi:10.1007/s11427-020-1643-8

38. Wang J, Jiang M, Chen X, Montaner LJ. Cytokine storm and leukocyte changes in mild versus severe SARS-CoV-2 infection: review of 3939 COVID-19 patients in China and emerging pathogenesis and therapy concepts. *J Leukoc Biol*. 2020;108(1):17–41. doi:10.1002/JLB.3COVR0520-272R

39. Du F, Liu B, Zhang S. COVID-19: the role of excessive cytokine release and potential ACE2 down-regulation in promoting hypercoagulable state associated with severe illness. *J Thromb Thrombolysis*. 2020;51:1–17.

40. Magro C, Mulvey JJ, Berlin D, et al. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. *Transl Res*. 2020;220:1–13. doi:10.1016/j.trsl.2020.04.007

41. Fletcher-Sandersjöö A, Bellander B-M. Is COVID-19 associated thrombosis caused by overactivation of the complement cascade? A literature review. *Thromb Res*. 2020;194:36–41. doi:10.1016/j.thromres.2020.06.027

42. Forrester SJ, Booz GW, Sigmund CD, et al. Angiotensin II signal transduction: an update on mechanisms of physiology and pathophysiology. *Physiol Rev*. 2018;98(3):1627–1738.

43. Celi A, Cianchetti S, Dell'Omo G, Pedrinelli R. Angiotensin II, tissue factor and the thrombotic paradox of hypertension. *Expert Rev Cardiovasc Ther*. 2010;8(12):1723–1729. doi:10.1586/erc.10.161
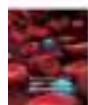
44. Guo T, Fan Y, Chen M, et al. Cardiovascular implications of fatal outcomes of patients with coronavirus disease 2019 (COVID-19). *JAMA Cardiol*. 2020;5(7):811–818. doi:10.1001/jamacardio.2020.1017

45. Becker RC. COVID-19 update: covid-19-associated coagulopathy. *J Thromb Thrombolysis*. 2020;50(1):54-67. doi:10.1007/s11239-020-02134-3.

46. Al-Ani F, Chehade S, Lazo-Langner A. Thrombosis risk associated with COVID-19 infection a scoping review. *Thromb Res*. 2020;192:152–160. doi:10.1016/j.thromres.2020.05.039

47. Zhang L, Feng X, Zhang D, et al. Deep vein thrombosis in hospitalized patients with COVID-19 in Wuhan, China: prevalence, risk factors, and outcome. *Circulation*. 2020;142(2):114–128. doi:10.1161/circulationaha.120.046702

48. Yang J, Zheng Y, Gou X, et al. Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. *Int J Infect Dis*. 2020;94:91–95. doi:10.1016/j.ijid.2020.03.017

49. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. *Lancet Haematol*. 2020;7(6): e438–e440. doi:10.1016/S2352-3026(20)30145-9

50. Thachil J, Tang N, Gando S, et al. ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thrombosis Haemostasis*. 2020;18(5):1023–1026. doi:10.1111/jth.14810

51. Nutescu EA, Burnett A, Fanikos J, Spinler S, Wittkowsky A. Pharmacology of anticoagulants used in the treatment of venous thromboembolism. *J Thromb Thrombolysis*. 2016;41(1):15–31. doi:10.1007/s11239-015-1314-3

52. Kim SY, Jin W, Sood A, et al. Characterization of heparin and severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) spike glycoprotein binding interactions. *Antiviral Res*. 2020;181:104873. doi:10.1016/j.antiviral.2020.104873

53. Thachil J. The versatile heparin in COVID-19. *J Thrombosis Haemostasis*. 2020;18(5):1020–1022. doi:10.1111/jth.14821

54. White D, MacDonald S, Bull T, et al. Heparin resistance in COVID-19 patients in the intensive care unit. *J Thromb Thrombolysis*. 2020;50 (2):287–291. doi:10.1007/s11239-020-02145-0

55. Ortega-Paz L, Capodanno D, Montalescot G, Angiolillo Dominick J. Coronavirus disease 2019–associated thrombosis and coagulopathy: review of the pathophysiological characteristics and implications for antithrombotic management. *J Am Heart Assoc*. 2021;10(3):e019650. doi:10.1161/JAHA.120.019650

56. Chen S, Zhang D, Zheng T, Yu Y, Jiang J. DVT incidence and risk factors in critically ill patients with COVID-19. *J Thromb Thrombolysis*. 2021;51(1):33–39. doi:10.1007/s11239-020-02181-w

57. Shah A, Donovan K, McHugh A, et al. Thrombotic and haemorrhagic complications in critically ill patients with COVID-19: a multicentre observational study. *Critical Care*. 2020;24(1):1–10. doi:10.1186/s13054-020-03260-3

58. Gibson CJ, Alqunaibit D, Smith KE, et al. Probative value of the d-dimer assay for diagnosis of deep venous thrombosis in the coronavirus disease 2019 syndrome. *Crit Care Med*. 2020;48(12): e1322–e1326. doi:10.1097/ccm.0000000000004614

59. Monfardini L, Morassi M, Botti P, et al. Pulmonary thromboembolism in hospitalised COVID-19 patients at moderate to high risk by Wells score: a report from Lombardy, Italy. *Br J Radiol*. 2020;93 (1113):20200407. doi:10.1259/bjr.20200407

60. Avruscio G, Camporese G, Campello E, et al. COVID-19 and venous thromboembolism in intensive care or medical ward. *Clin Transl Sci*. 2020;13(6):1108–1114. doi:10.1111/cts.12907

61. Al-Samkari H, Karp Leaf RS, Dzik WH, et al. COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection. *Blood*. 2020;136(4):489–500. doi:10.1182/blood.2020006520

62. Demelo-Rodríguez P, Cervilla-Muñoz E, Ordieres-Ortega L, et al. Incidence of asymptomatic deep vein thrombosis in patients with COVID-19 pneumonia and elevated D-dimer levels. *Thromb Res*. 2020;192:23–26. doi:10.1016/j.thromres.2020.05.018

63. Piazza G, Campia U, Hurwitz S, et al. Registry of arterial and venous thromboembolic complications in patients with COVID-19. *J Am Coll Cardiol*. 2020;76(18):2060–2072. doi:10.1016/j.jacc.2020.08.070

64. Brosnahan SB, Jonkman AH, Kugler MC, Munger JS, Kaufman DA. COVID-19 and respiratory system disorders: current knowledge, future clinical and translational research questions. *Arterioscler Thromb Vasc Biol*. 2020;40(11):2586–2597. doi:10.1161/ATVBAHA.120.314515

Waldenström's Macroglobulinemia: An Exploration into the Pathology and Diagnosis of a Complex B-Cell Malignancy

Elham Askari, Sara Rodriguez & Ramon Garcia-Sanz

Published online: 30 Aug 2021.

Submit your article to this journal 🗗

View related articles 🗗

View Crossmark data 🗗

REVIEW

Waldenström's Macroglobulinemia: An Exploration into the Pathology and Diagnosis of a Complex B-Cell Malignancy

Elham Askari[1]
Sara Rodriguez[2]
Ramon Garcia-Sanz [ID][3]

[1]Hematology Department, Fundación Jiménez Díaz, Centro de Investigación Biomédica en Red-Cáncer (CIBERONC) CB16/12/00369, Madrid, Spain; [2]Clinica Universidad de Navarra, Centro de Investigación Medica Aplicada (CIMA), Instituto de Investigación Sanitaria de Navarra (IDISNA), Accelerator project, Centro de Investigación Biomédica en Red-Cáncer (CIBERONC) CB16/12/00369, Pamplona, Spain; [3]Haematology Department, University Hospital of Salamanca, Research Biomedical Institute of Salamanca (IBSAL), Accelerator project, Centro de Investigación Biomédica en Red-Cáncer (CIBERONC) CB16/12/00369 and Center for Cancer Research-IBMCC (USAL-CSIC), Salamanca, Spain

Correspondence: Ramon Garcia-Sanz
Department of Hematology, University Hospital of Salamanca, Paseo de San Vicente, 58-182, Salamanca, 37007, Spain
Tel +34 923291100
Fax +34 923294624
Email rgarcias@usal.es

Abstract: After 77 years since the initial description, Waldenström macroglobulinemia (WM) remains as a bone marrow neoplastic disorder with lymphoplasmacytic differentiation oversecreting a monoclonal immunoglobulin M (IgM). However, many biological and genetic aspects of this entity have been unraveled and it is now easy to correctly diagnose patients with this illness. The diagnosis requires the presence of a monoclonal IgM component and bone marrow lymphoid infiltration must be demonstrated. In addition, other small B-cell lymphoid neoplasms with plasma cell differentiation must be discarded. Although the clinical picture is highly heterogeneous, the diagnosis is much easier today compared to the past, since now we can demonstrate the presence of somatic mutations, especially the L265P mutation in the MYD88 gene, highly characteristic of WM (>90% of the patients), followed by the WHIM-like mutations in the CXCR4 gene (~35%). The identification of these mutations is very important, because they can modulate the response to new treatments with Bruton's tyrosine kinase (BTK) inhibitors. Thus, the conventional prognostic factors that predict the outcome of these patients (anemia, thrombopenia, high M component, high B2M, and advanced age), must be complemented with the genetic evaluation of the patient, that can help us in the prediction of the risk of transformation from asymptomatic to symptomatic forms (Del6q) and/or from indolent forms of the disease to aggressive lymphomas (CD79b mutations).

Keywords: Waldenström's macroglobulinemia, IgM-MGUS, pathology, biology, diagnosis, prognosis

Introduction

Waldenström's macroglobulinemia (WM) is a B-cell lymphoproliferative disorder which is defined by bone marrow (BM) infiltration by small lymphocytes, lymphoplasmacytoid cells and plasma cells together with the presence of a detectable monoclonal immunoglobulin M (IgM).[1,2] According to the 2008 World Health Organization (WHO) classification system of lymphoid neoplasms, the pathological disorder underlying WM is a lymphoplasmacytic lymphoma (LPL).[3] By the end of 2011, a remarkable fact that has changed the view of WM was presented at this year annual meeting of the American Society of Hematology, as it was the presence of the MYD88[L265P] mutation in most cases of this disease,[4] which has been highlighted in the last 2016 WHO classification of lymphoid malignancies.[5] Other recent advances in the genomic profiling of patients with Waldenström macroglobulinemia (WM) have enhanced our understanding of its pathogenesis.[6,7] Thus, the highly recurring somatic

mutation in the MYD88^{L265P} is now recognized in >95% of cases, while CXCR4 mutations are present in 30–40% of WM patients.[6–8] Such studies have also shown that the genomic status can impact not only the diagnosis but also the clinical presentation, treatment outcome and overall survival of patients.[6,7]

Classification and Epidemiology

WM patients may have a long asymptomatic course, called Smoldering WM, also known as asymptomatic WM (AWM). Although some authors have defined AWM as a WM with a serum monoclonal IgM ≥30 g/l and/or ≥10% BM lympho-plasmacytic infiltration without end-organ damage,[9] the International Waldenström's Macroglobulinemia Group (IWMG)[1,2] and the WHO[1,2] consider that it would be inap-propriate to suggest disease definitions based on arbitrary values for laboratory parameters, such as IgM concentration and percentage of BM lymphocytes. Thus, patients with an IgM monoclonal protein and unequivocal evidence of BM infiltration by lymphoplasmacytic lymphoma should be con-sidered to have WM irrespective of the IgM concentration. Then, when we are in front of an asymptomatic patient with an IgM monoclonal component in the absence of unequivocal BM or other tissue infiltration, an IgM Monoclonal Gammopathy of Uncertain Significance (IgM-MGUS) must be diagnosed. We still can have a small group of patients with an IgM monoclonal component, with no demonstrable tissue infiltration but pathological consequences of the IgM protein, such as peripheral neuropathy, amyloidosis, or skin lesions among others; in these cases, we will be in front of a so-called IgM related disorder. Finally, we also can see some isolated patients with IgM monoclonal component and some tissue infiltration by LPL but in the absence of BM infiltration; such patients should be considered as 'pure' LPL, together with those rare cases in whom the M-component is an IgG and/or IgA. These definitions can be found in Table 1.

WM represents approximately 1–2% of all hematologic malignancies with 150–200 new cases per year in Spain.[10] WM mostly occurs in adult Caucasians with a median age in the seventh decade of life. The annual incidence of WM is 3–4 cases per million persons per year and increase with the age or familial predisposition that is observed in approximately 20% of patients.[11,12] Virus C Hepatitis is also associated with WM.[13] IgM monoclonal gammopathy of undetermined sig-nificance (IgM-MGUS) and AWM are defined as clinical precursor states to symptomatic WM.[14] Thus, patients with IgM-MGUS have a 10.8 higher relative risk of developing WM.[15]

Clinical Features

The clinical presentation of WM is variable (Tables 2 and 3). Symptoms can be related to tumor infiltration, either in the BM or as extramedullary infiltration, or they can be related to specific immunological or chemical properties of the monoclonal component.

Symptoms and Signs Derived from Lymphoma Infiltration
B Symptoms

In symptomatic WM patients, the presence of B symptoms including fever, night sweats, and weight loss is not uncommon.

Table 1 Classification of Immunoproliferative Disorders with Monoclonal IgM

	Symtoms[a] Attributtable to Tissue Infiltration	Symtoms[b] Attributable to IgM	Bone Marrow Infiltration[c]	Ig Monoclonal Protein[b]
Symptomatic WM	(+)	(+)	(+)	IgM
Asymptomatic WM	(−)	(−)	(+)	IgM
IgM-related disorders[d]	(−)	(+)[c]	(−)[b]	IgM
IgM MGUS	(−)	(−)	(−)[b]	IgM
Lymphoplasmacytic lymphoma[e]	(+/−)	(+/−)	(+/−)	IgM > IgG > IgA or absent

Notes: [a]Symptoms attributable to tumor infiltration will include any of the following manifestations: B symptoms, cytopenia(s), or symptomatic organ infiltration (ie central nervous system: Bing-Neel syndrome). [b]There is no definition of an IgM concentration threshold to differentiate between MGUS and WM. [c]Patients with demonstrated bone marrow infiltration by lymphoplasmacytic lymphoma have a WM, while patients without evidence of infiltration have an MGUS. Detection of clonal B-cells by flow cytometry or MYD88 L265P mutation by polymerase chain reaction will help in this definition, but in the absence of morphological evidence of bone marrow infiltration these patients should still be classified as having an MGUS. [d]There are patients who have symptoms attributable to the IgM monoclonal protein but no overt evidence of tissue infiltration by LPL. These patients may show peripheral neuropathy, Schnitzler Syndrome, symptomatic cryoglobulinemia, amyloidosis, or cold agglutinin disease. [e]Anatomo-clinical entity present in patients in whom, having a lesion with histological diagnosis of LPL, there are no criteria of WM (they lack on IgM M-component and/or bone marrow infiltration). As other indolent lymphomas they can have or not of symptoms related to the infiltration or M-component.

Table 2 Inside the Bone Marrow: Histology, Immunophenotype and Molecular Features

Bone Marrow	Relevant Findings
Morphological evaluation	Pleomorphic B lineage cells at different stages of maturation: B lymphocyte cells with lymphoplasmacytic differentiation with small population of clonal plasma cells. The presence of prominent mast cells is frequent.
Flow cytometry	1) B-cell monoclonal population: sIgM +,CD19+,CD20+,CD22 +,CD79 +, CD25+, CD27+, FMC7+, BCL-2+, Up to 10–20% of WM cases may be CD5+, CD10+, or CD23+. 2) Plasma cell population: CD138+ CD38++, CD19+, CD45+, CD56-, CD117-
Molecular aspects	MYD88 L265P mutation: > 90%. The presence of MYD88 L265P mutation supports the diagnosis of WM CXCR4 mutation: 30–40%
Cytogenetics	6q21 deletion, is the most common cytogenetic abnormality reported in 30–60% of WM patients (can be seen by fluorescence in situ hybridization, FISH)
Histology	Diffuse, interstitial and /or nodular infiltration of small B lymphocytes, lymphoplasmacytoid and plasma cells, with the predominately intertrabecular pattern associated with the restriction of light chains.

Table 3 Outside the Bone Marrow: IgM Related Symptoms

Clinical Features	IgM Related Disease
New onset headaches, blurred vision, mucosal bleeding, hearing loss, tinnitus, neurologic disorders, retinopathy and retinal hemorrhage	Hyperviscosity
Distal, symmetric, progressive, sensorio-motor peripheral neuropathies with predominantly demyelinating features in the nerve conduction studies. (50% of cases anti-MAG +) IgM antibodies against other neural targets: gangliosides GM1, GD1a, GD1b, GT1b, GM2 and GM3 and the paragloboside, sulphate-3-glucuronyl para-globoside (SGPG).	Peripheral neuropathies related to IgM
Raynaud like symptoms, acrocyanosis, ulcerations on extremities, purpura, cold urticaria	Cryoglobulinemia I
Arthralgias, sensorimotor neuropathies, purpura, renal failure.	Cryoglobulinemia II
Extra vascular Hemolytic anemia (cold exposure), Raynaud phenomenon, acrocyanosis.	Cold agglutinins
Recurrent thrombotic events	Antiphospholipid syndrome
Peripheral sensory neuropathies with axonal pattern associated with autonomic nerve dysfunction. Other organ involvements: kidneys, heart, lung, liver and Gastrointestinal tracts.	Amyloidosis AL
Chronic urticarial eruptions, recurrent fever, arthralgia ± lymphadenopathy	Schnitzler's Syndrome, (Auto-inflammatory disease)
Renal Failure ± Proteinuria (Moncolnal- IgM +/_ Light chain deposition)	Proliferative glomerulonephritis, Amyloidosis AL Cryoglobulinaemia II

Bone Marrow Failure

Diagnosis of WM requires the presence of lymphoplasmacytic lymphoma in the BM (Table 2) with or without other histological infiltration. A BM aspiration and biopsy, together with immunophenotyping and genetics will establish the diagnosis of WM and will help to differentiate it from IgM MGUS, IgM

multiple myeloma (MM) and other IgM-secreting lymphoproliferative disorders, such as marginal zone lymphoma (MZL) and chronic lymphocytic leukemia (CLL).[16–18] A typical effect of BM infiltration is anemia that is the most common symptom of WM and the usual cause to start therapy. Anemia is due to BM infiltration, but iron metabolism dysfunction and

hemolysis can play a role. Iron deficiency is very common in WM because hepcidin, a negative regulator of iron absorption, is elevated in the serum of WM patients, probably related with the MYD88 alterations.[19,20] Other cytopenias, as leukopenia and thrombocytopenia can also be present in around 15% of patients.

Extra-Medullary Disease (EMD)

At diagnosis, 10–15% of WM patients have EMD: lymphadenopathy, hepatosplenomegaly, pleural or abdominal effusions, among others (Table 3). At relapse, nearly 60% of patients can present adenopathy. The IWMG recommends an initial assessment of EMD by imaging in all new diagnosed patients, and at the time of relapse.[21] In a retrospective single center study in Dana-Farber Cancer Institute, among 985 patients with WM that were evaluated at diagnosis, only 4.4% of patients have extranodal/extramedullary disease; 21% of them presented EMD at diagnosis and 79% developed EMD during the outcome. Most frequent EMD sites involved were lungs (30%), soft tissue (21%), cerebrospinal fluid (23%), kidneys (8%), and bones (9%).[22]

Bing-Neel Syndrome (BNS) is a rare but interesting presentation of WM, observed in about 1% of patients. BNS should be suspected in patients with WM who develop central neurological symptoms. Such symptoms include motor deficits, balance disorder, gait abnormalities, cranial nerve deficits, seizures, headaches, and atypical peripheral neuropathy. The gold standard for BNS diagnosis is the demonstration of WM cells on cerebro spinal fluid (CSF) examination or brain biopsy. The evaluation of these patients should include brain and whole spine magnetic resonance imaging with gadolinium enhancement, and CSF sampling. CSF should be evaluated by morphology, flow cytometry and molecular studies.[23] Flow cytometry of CSF is the most sensitive technique to detect tumor cells in the central nervous system,[24] but PCR assays to detect *IGH* gene rearrangements and mutated MYD88[L265P] on CSF can also help to support the diagnosis of BNS. In patients with focal brain lesions but no CSF involvement, a stereotaxic biopsy should be programmed.[23]

Symptoms Related to IgM

IgM paraprotein can cause specific complications due to its physical-chemical properties, autoantibody activity, tissue deposition and non-specific interactions with other proteins.

Hyperviscosity Syndrome (HVS)

Hyperviscosity syndrome (HVS) related to high IgM levels is a hallmark of symptomatic WM. It has been described in 10–15% of cases with variable manifestations that include headache, blurred vision, confusion, and mucosal bleeding.[25] We should identify patients at high risk of symptomatic hyperviscosity that might support the decision to treat asymptomatic patients before irreversible damage occurs. Although the size of the monoclonal component is not exactly related to symptoms, they are rarely observed with a serum IgM level below 3 g/dL, while they are frequent in patients with a serum IgM ≥6 g/dL (median time to symptomatic hyperviscosity of ~3 months).[26] The funduscopic examination is very reliable to detect clinically significant hyperviscosity by seeing changes in the retinal vessels. Plasmapheresis should be carried out as an emergency procedure in high-risk HVS patients. The panel of the 8th International workshop on WM recommended that patients with serum IgM levels >3g/dL should undergo funduscopic evaluation by an experienced ophthalmologist to identify vessel tortuosity/retinal hemorrhages.[21] These findings would suggest the need for immediate therapy.

Cryoglobulins

Cryoglobulinemia refers to the presence of serum proteins (immunoglobulins) with heterogeneous etiopathogenetic and immunochemical properties that precipitate at the temperatures below 37°C [98.6 F] and redissolve at 37°C. Cryoglobulins can deposit in medium and large-sized blood vessels, leading to a systemic inflammatory syndrome characterized by fatigue, arthralgia, purpura, neuropathy, glomerulonephritis, endothelial injury and end-organ damage. Brouet criteria classify cryoglobulinemia into three (I, II & III) subgroups based on their immunoglobulin (Ig) composition.[27] Type I cryoglobulinemia, which develops in the setting of protein-secreting monoclonal gammopathies, is the one usually associated to WM. It is characterized by Raynaud phenomenon, acrocyanosis ulcers, purpura and cold urticaria. In contrast, in type II or mixed cryoglobulinemia, the cryoglobulins are composed of a mix of monoclonal IgM with rheumatoid factor (RF) activity and polyclonal IgG, usually associates Hepatitis C virus infection, and leads to purpura, renal failure, arthralgias and sensorimotor neuropathies.[28] Type III is characterized by polyclonal IgM with RF activity and polyclonal IgG, and it is not associated to WM.

Accordingly, in WM patients suspected of having cryoglobulins, serum samples should be obtained in a warm bath to avoid cryoprecipitation. Plasmapheresis will be a choice of therapy in WM patients with severe cryoglobulinemia. A blood warmer will be necessary to prevent cryoglobulin precipitation during plasmapheresis.[27]

Cold Agglutinin Syndrome (CAD)

Another cause of Anemia is hemolysis in WM patients. A hemolytic panel including reticulocyte counts, lactate dehydrogenase, haptoglobin, direct Coombs test, and cold agglutinins should be performed, in all patients with anemia and WM. We should think about of CAD, if the WM patient presents hemoglobinuria after cold exposure.[29] CAD is produced by IgM with immunological activity: IgM acts as an autoantibody against red blood cell antigens, producing Hemolytic anemia, and sometimes Raynaud phenomenon, acrocyanosis, and livedo reticularis. In patients with severe cold agglutininemia, plasmapheresis should be started promptly to remove cold agglutinins.

Polyneuropathy

The prevalence of peripheral neuropathy (PNP) in WM at diagnosis is around 30%, but 50% of them can be affected at some time during the course of their disease.[30] Although PNP may be related to lymphoplasmacytic infiltration nerve fibers, it most commonly is due to IgM deposition, presence of autoantibodies, cryoglobulinemia or amyloidosis.

When a WM patient is suspected to be affected by PNP, the first point to evaluate is whether or not the monoclonal gammopathy is the cause of PNP. The second point will be to distinguish if the PNP can be associated to specific plasma cell disorders, such as primary amyloidosis, that could have a specific approach and treatment.[31]

Usually, IgM monoclonal gammopathy associated PNP presents as a distal, acquired, demyelinating, and symmetric neuropathy with M protein (DADS-M).[31] Nerve conduction tests should be performed in all WM patients, and different patterns of nerve damage can appear: demyelinating, axonal or mixture pattern. All these patients should be tested for myelin-associated globulin antibodies (anti-MAG antibodies). A clinically significant result [>70.000 Bühlmann Titer Units (BTU)] strongly suggests a PNP caused by the M-component. Up to 50% of patients with IgM-associated demyelinating PNP have anti-MAG antibodies in the setting of IgM MGUS or WM.[31] If anti-MAG are negative, patients should be tested for antibodies

against other neural targets, including the gangliosides GM1, GD1a, GD1b, GT1b, GM2 and GM3 and the para-globoside, sulphate-3-glucuronyl para-globoside (SGPG). GM1 antibodies may be causally associated with a multifocal motor neuropathy, as well as IgM GD1b antibodies. IgM disialosyl antibodies could be associated with CANOMAD: Chronic Ataxic Neuropathy with Ophthalmoplegia, M-protein, cold Agglutinins and Disialosyl ganglioside (IgM Anti-GD1b/ GT1b/GQ1b) antibodies.[32] In CANOMAD, nerve conduction tests show a mixed pattern of axonal loss and demyelinating features. 30–40% of IgM-related demyelinating neuropathies have no identifiable antibodies.[30] In these cases, clinical judgment is the only possibility to connect IgM and PNP. It is important to identify them, because treatment could be needed to avoid a prolonged demyelination, that can induce irreversible axonal damage. In addition, other causes of PNP should be always ruled out: diabetes, cobalamin deficiency, thyroid dysfunction, etc.[33]

Amyloidosis

Systemic immunoglobulin light-chain (AL) amyloidosis is a rare complication of WM caused by the aggregation of misfolded proteins that deposit as fibrils in several organs, including kidneys, heart, peripheral nerves, liver, and gastrointestinal tract. Monoclonal IgM-related light chain AL accounts for 6% of all cases of AL.[34] Several studies have shown that the pattern of organ involvement is different from non-IgM Amyloidosis, with higher frequencies of lung, soft tissue, and peripheral nervous system involvement, and lower frequencies of heart.[34,35] In these cases, PNP is usually due to amyloid deposition in the nerves. The neuropathy in AL amyloidosis often has an axonal pattern whereas IgM monoclonal gammopathy associated PNP without amyloidosis often has a demyelinating nature. Autonomic function also tends to be impaired in neuropathy associated with AL amyloidosis, which is not frequent in non-amyloidotic WM neuropathy. The prognosis of patients with IgM-related AL amyloidosis might be more favorable than those with non-IgM AL amyloidosis.[36]

Diagnosis

The diagnosis of WM is clinicopathological, requiring the histologic evidence of BM infiltration by LPL as well as the presence of an IgM monoclonal gammopathy detected, at least, by immunofixation.[1,21,37] A summary of the tests that are essential or highly recommended to be performed

Table 4 Essential Evaluation of Patients with Waldenström Macroglobulinemia (WM)

Evaluation	If clinically indicated
- Clinical history and physical examination - Familial history for WM and other B-cell lymphoproliferative disorders - Funduscopic examination: fotographic documentation - Review of systems	- Cryoglobulins - Cold agglutinin titre - Serum viscosity - Screening for von Willebrand disease 24-h urine protein quantification
Laboratory studies - Complete blood count - Complete metabolic panel - Serum immunoglobulin levels (IgA, IgG, IgM) - Serum and urine electrophoresis with immunofixation - Serum free light assessment - Serum beta-2-microglobulin level	Bone marrow aspiration and biopsy - Immunohistochemistry - Flow cytometry - Testing for MYD88 L265P gene mutation - FISH analysis of del(6q21) and del(17p) Total body CT-scan with IV contrast - In patients being considered for therapy - PET-CT if transformation is suspected

Notes: Modified from reference 37: Castillo JJ, Garcia-Sanz R, Hatjiharissi E, Kyle RA, Leleu X, McMaster M, Merlini G, Minnema MC, Morra E, Owen RG, Poulain S, Stone MJ, Tam C, Varettoni M, Dimopoulos MA, Treon SP, Kastritis E. Recommendations for the diagnosis and initial evaluation of patients with WaldenströmMacroglobulinaemia: A Task Force from the 8th International Workshop on WaldenströmMacroglobulinaemia. Br J Haematol. 2016 Oct;175(1):77–86. doi: 10.1111/bjh.14196. Epub 2016 Jul 5. PMID: 27378193; PMCID: PMC5154335.

during the diagnostic work-up of patients with WM is shown in Table 4.

Laboratory Findings

Hemogram

50–70% of patients have normocytic and central anemia, although a peripheral component can also be found (blood loss, cold agglutinins, hemodilution).[21] White blood cells are usually normal, but lymphocytosis ($>4\times10^9$/L) is present in one-fourth of patients, usually monoclonal, and neutropenia is possible. Thrombopenia only appears in <20% of cases. Blood smear shows stacks or aggregations of red blood cells (rouleaux phenomenon) due to paraproteinemia, and sed rate (SR) is increased.

Biochemistry

The biochemistry of patients with WM is usually normal. Renal damage can occur in patients with WM, but they guide more towards the diagnosis of IgM myeloma. The biochemical profile can also provide data of hemolysis and/or other organ lesions, related or not to WM.[21]

Viscosity is usually increased in WM because in serum and plasma it is mainly determined by proteins. Among them, IgM has one of the highest molecular weight (950 kDa), with a high length/width ratio and the possibility of pentamerize.[25] This provides a very high intrinsic viscosity to IgM, being responsible of plasma viscosity in a higher way than any other immunoglobulin. Thus, while 5000 mg/L of IgM easily

promote HVS, an IgA component would require a serum level higher than 7000 mg/dL.

Serum and Urinary Proteinogram

Total proteins are increased by IgM paraprotein. Electrophoresis reveals the existence of a monoclonal IgM band that must be identified by immunofixation. Unlike MM, the levels of polyclonal Ig (IgG and IgA) are usually normal (immunoparesis is rare in WM),[1] but this could change with novel tests, such as free Heavy/Light chain assessment.[38] Finally, 30–50% of WM patients present with Bence Jones proteinuria, generally low (<2 g/24 hours).

The determination of serum-free light chains (sFLC) is a useful study in MW, since it avoids interpretation problems derived from the polymerization of the IgM chains. sFLC assessment has a prognostic value, because patients with >60 mg/L of the involved light chain have less hemoglobin (Hb) and more Beta$_2$microglobulin (B2M),[39] and patients with >80 mg/L of involved light chain had more progressive disease and shorter treatment-free survival.[38,40] The Heavy/Light chain (HLC) test could help to identify cases with special risk of progression.[38]

Detection of Tumor Traits in Peripheral Blood

Flow cytometry has demonstrated that WM cells can be detected in PB, but the efficacy is lower than that we can observe in BM.[41] The MYD88^{L265P}can also be detected in PB, but we need to use advanced techniques to increase the detection capacity, for instance by using CD19+

selected cells, cell free DNA (liquid biopsy)[42] or droplet digital PCR.[43] Probably, the most efficient approach could be a combination of cfDNA and ddPCR, although we cannot forget that the presence of the mutation is not sufficient for WM diagnosis, while the absence of MYD88[L265P] does not completely excludes it.

Histology

The 2008 World Health Organization (WHO) classification defines LPL as a B-cell lymphoid neoplasm composed of small B lymphocytes, lymphoplasmacytoid cells and plasma cells, usually involving BM and sometimes lymph nodes and spleen, which does not fulfill the criteria for any of the other small B-cell lymphoid neoplasms.[2] Thus, LPL remains an exclusionary diagnosis because there are no unique and uniform clinically applicable features that characterize the small lymphoid cells or the plasma cells.[44] LPL is characterized by BM infiltration of small B lymphocytes with a predominately intertrabecular pattern. In addition, an interstitial, nodular or diffuse pattern can be observed. A paratrabecular pattern of infiltration is unusual, which help to distinguish LPL from other subsets of lymphoma, such as MZL that exhibits this paratrabecular pattern much more frequently.[21] For the diagnosis of WM, variable numbers of plasmacytoid lymphocytes and plasma cells, often with positive periodic acid–Schiff intranuclear pseudoinclusions (Dutcher bodies), must also be present.[44]

BM biopsy (Table 2) shows the presence of light chain restricted lymphocytes, lymphoplasmacytoid and plasma cells, in which monoclonality is easy to be demonstrated by molecular or flow cytometric techniques in simple BM aspirates.[45] A typical feature of LPL is the presence of prominent mast cells that are often recognized in the spicules of aspirate smears with Giemsa stain or in the histologic sections with tryptase immunohistochemical stain. Finally, LPL may also be associated with immunoglobulin deposition, amyloid, or crystal storing histiocytosis.[2]

Outside the BM, the presence of LPL is not frequently seen. Lymphoid tissue infiltration is often difficult to be assessed, especially when differential diagnosis includes nodal MZL, splenic MZL, or other small B-cell neoplasms with plasmacytic differentiation. Three patterns of lymph node involvement by LPL have been described, the most classic being lymph nodes with intact sinuses, a relatively monotonous lymphoplasmacytic

proliferation, small residual follicles without large germinal centers or prominent follicular colonization.[44] In the spleen, LPL typically involves both the splenic white and red pulp with diffuse pattern. Periarteriolar aggregates of plasmacytoid cells, small lymphocytes, immunoblasts are consistent in LPL. Increased mast cells, and hemosiderin can also be seen. When BM is not involved and monoclonal IgM is not present (IgG/IgA/absent M-component), MYD88[L265P] mutation is less frequently seen.[46,47]

Large cells are uncommon in LPL-IgM unless histological transformation occurs. If transformation is suspected, a lymph node biopsy is recommended,[48] especially after a PET-CT scan to select one of the most actives adenopathies from the metabolic point of view.[49]

Immunophenotypic Features

Immunophenotypic evaluation is of great value in the differential diagnosis of B-cell lymphoproliferative disorders and must be performed on BM samples (Table 2). The immunophenotypic profile of lymphoplasmacytic cells in WM expresses pan B-cell antigens CD19, CD20, CD22, and CD79, as well as CD25+, CD27+ and light chain-restricted surface IgM.[50,51] It is also typically characterized by the lack of expression of CD5, CD10, CD11c, CD23 and CD103, which is in contrast with most other mature lymphoid malignancies, except MZL. However, several studies have observed that in 10–20% of WM cases tumor cells can express CD5, CD23 or CD10.[52–54]

Multiparametric flow cytometric immunophenotyping also demonstrates the presence of monoclonal plasma cells (PC) with the same restricted light-chain expression as the lymphoplasmacytic population.[50] The antigenic profile of these plasma cells shows a phenotypic profile similar to normal PC and clearly different from that of myeloma patients: CD138^{+++}, CD19^{+}, CD45^{+}, CD38^{+}, CD56^{−}, with high proportion of PC with the same light-chain restriction as the IgM monoclonal component seen in the serum.[50] This means that the genetic program of the final B-cell differentiation is altered in WM cells, which can explain why Robert et al observed the abnormal co-expression of CD138 and PAX5 in 23% of plasma cells in samples from a series of LPL patients. This finding contrasts with MZL, where PAX5 cells (B lymphocytes) completely lack of CD138, while CD138+ cells (plasma cells) have switch off PAX5 expression.[50]

Cytogenetic Features

Conventional karyotypic analysis is not mandatory for the routine diagnostic assessment of WM patients as it is very difficult to obtain tumor metaphases in vitro. 6q deletion (usually from 6q21 to 6q23), that can be seen by fluorescence in situ hybridization (FISH), is the most common cytogenetic abnormality reported in 30–60% of WM patients.[55–58] Such deletions are associated with more aggressive IgM gammopathies and a high probability of symptomatic transformation.[58] Chromosome 6q deletions involve genes that modulate Nuclear Factor-κB (NF-KB), BCL2, Bruton Tyrosine Kinase (BTK), apoptosis and differentiation, which could help to explain why this deletion is associated with poor clinical features and a higher risk of symptomatic evolution. Other genes whose deletion could justify such relationship are genes with important regulatory functions, such as IBTK, HIVEP2, and FOXO3.[8]

FISH studies may be also be useful to detect some abnormalities that could help in the differential diagnosis, such as the detection of the t(11;14), very frequent in IgM myeloma[60,61] and virtually absent in WM.[46] In addition, it can detect several other abnormalities that are in common to other B-cell lymphoproliferative disorders: del(13q14) in 3–16% of cases, del(17p23) in 7–15%, del(11q22) in 8%, +8 in 11%, and +4 in 8%.[57,59–61]

Molecular Genetics

MYD88 Mutations

Whole genome sequencing (WGS) in WM patients has identified several somatic mutations in WM.[8] However, a mutation in the myeloid differentiation primary response 88 (*MYD88*) gene, more specifically, the MYD88[L265P] mutation, is now considered the hallmark of WM (and LPL), since it is present in more than 90% of the patients.[6] MYD88 is a protein adapter that activates the IL-1 receptor signaling pathway via interleukin-1 promoting BTK constitutive activation. BTK is a kinase with a critical role in B-cell receptor (BCR) signaling that regulates immune response, cell proliferation and cell death and seems to be directly related to B-cell lymphoproliferative disorders as WM. Although rarely, MYD88[L265P] is also present in other B cells disorders, so it is not completely specific of WM, by now. Some WM patients in whom MYD88[L265P] mutation is negative can have a MYD88 variant but placed in a different point respect the L265 position.[62] Thus, some series reach a -100%[41] of incidence of WM patients and 87% of IgM-

MGUS patients.[46] These frequencies, together with the differential response to first generation BTK inhibitors hast prompted to some authors to consider that wild type MYD88 (MYD88[WT]) WM could be a different disease.[63] In addition, MYD88[WT] WM patients have a different genomic landscape that shows other NF-κB activating mutations, impart epigenomic dysregulation, or impair DNA damage repair (DDR). These patients show a shorter survival, especially if they have DDR mutations and a higher incidence diffuse large B-cell lymphoma transformation when compared with WM patients who carry the conventional MYD88 mutation.[64]

The MYD88[L265P] mutation is consider pathogenic in WM, since it leads to an amino-acid change in the MYD88 protein and is present in most cells and cases. It is an activating change and triggers interleukin-1 receptor–associated kinase (IRAK), Bruton's tyrosine kinase (BTK), and hematopoietic cell kinase (HCK) growth and survival signaling, that in turn activate NF-KB-p65 dependent nuclear translocation and malignant cell survival.[64] The presence of MYD88 mutation in IgM-MGUS reveals its role as a potential early oncogenic factor, but most of these IgM-MGUS patients never evolve into WM or other lymphoproliferative disorders, so this mutation cannot be considered as a unique pathogenic factor in WM.

The method and DNA source for the MYD88 mutation detection can affect the result.[65] There is no standardized method for MYD88[L265P] mutation detection and several methods can be used with various approaches and detection limits. This justify why the detection rate can range between 40% and 87% in IgM MGUS,[46,66] and from 71% to 100% in WM.[6,46,65] Currently, most authors recommend allele-specific polymerase chain reaction (AS-PCR)[67] which usually provides sensitivities beyond 1% and are sufficient for most diagnostic purposes,[68] although droplet digital PCR are becoming more and more popular.[43]

CXCR4 Mutations

In 30–40% of patients with WM, tumor cells have somatic activating mutations in the C-terminal domain of C-X-Chemokine receptor type 4 (*CXCR4*) gene, similar to nonsense (NS) and frameshift (FS) germline mutations found in the "warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome.[8,69] CXCR4 is a classical G protein coupled receptor (GPCRs) located on chromosome 2 and acts as a conventional chemokine receptor, the normal ligand of CXCR4 is CXCL2 who coupled to CXCR4 initiate intracellular signaling cascades

that control chemotaxis, migration, proliferation and stemness.[70] *CXCR4* mutations in WM were the first ever reported in human cancer with a wide range of possibilities, which makes difficult to develop of a PCR-based diagnostic test. The most frequent (50%) mutated region is the amino acid S338X at position 1013 with nucleotide changes C > G, and C > A, both resulting in a stop codon. The second one is a S338 frameshift mutation in 21% of the cases, but there are more than 40 different mutations described.[71] Most of such mutations introduce a premature stop codon or a frameshift that cuts the end of the CXCR4 protein avoiding its metabolization, thus prolonging its effects.

This over function of CXCR4 explains why patients with such mutations have a higher BM disease burden, higher serum immunoglobulin M levels and more cases of symptomatic hyperviscosity.[7] Asymptomatic patients also present mutations in CXCR4, but this fact seems to increase the risk of progression into symptomatic disease. In WM patients treated with a BTK-inhibitor the presence of CXCR4 mutations reduce the therapeutic effectiveness, and in vitro CXCR4 mutant cells treated with BTK-inhibitor are rescued by CXCL12 from apoptosis.[71] Accordingly, CXCR4 mutation emerges as a relevant molecular abnormality to be taken into account in WM not only for diagnosis, but also as a potential actionable target.

Other Somatic Mutations

Near 50% of WM harbor recurrent mutations in other genes.[72–74] Somatic mutations in *ARID1A* are present in 17% of patients with WM, including nonsense and frameshift variants. Although this frequency has not been reproduced by others,[74] patients with *ARID1A* mutations are presumed to have more advanced disease which concurs with the fact that it is located at chromosome 6q, as its homolog *ARID1B*. In addition, ARID1A could modulate TP53 and is thought to act as an epigenetic tumor suppressor in ovarian cancer.[67] *TP53* mutations are rare in WM (<5%), but they are associated with poor survival.[67]

Mutations in *CD79A* and *CD79B* can be found in 8–12% of patients with WM.[67,73,74] Both are components of the BCR pathway and can form heterodimers with each other, so activating mutations of these components could contribute to the chronic BCR signaling observed in WM cells.[67] In one study, mutations in *CD79A* and *CD79B* were nearly exclusive of *CXCR4* mutations, suggesting that *CD79A/B* mutations may also have an

independent role in facilitating mutated MYD88-directed progression in WM.[75] In addition, *CD79B* mutations have been associated with disease transformation in a some WM patients.[76] The number of detectable genetic abnormalities in IgM monoclonal gammopathies is increased as the aggressiveness of the disease is increased. In a recent study evaluating the 12 most frequently mutated genes in WM, the percentage of patients with alterations was increasing as the monoclonal gammopathy progressed: 21% in IgM MGUS, 35% in AWM and 50% in symptomatic WM.[74]

Risk Assessment & Prognosis

According to the type of IgM monoclonal gammopathy, we have different classifications of risk assessment that are specific for the different stages.

Risk Assessment in IgM-MGUS

When we are in front of a patient with a MGUS, the first thing that we have to consider is that, among the different heavy chain isotypes of the heavy chain that can exist, IgM is the one with the highest risk of malignant transformation.[15] These patients can evolve to non-Hodgkin lymphoma, WM, AL amyloidosis or CLL with a risk 11-fold higher than a matched control population. Probability of progression is higher for patients with an abnormal serum-free light-chain ratio (outside normal limits) and a high serum monoclonal protein level (≥1.5 g/dL), especially if both are associated. Thus, the 20 year probability of malignant progression is 55% when both risk factors are present, 41% if there is one, and 19% there is none of them.[15]

Risk Assessment in Asymptomatic WM

When a patient fits with the AWM criteria (Table 1), the risk assessment to score the probability of transformation into a symptomatic disease can be done through several systems, but the most recent one is based on four risk factors: Immunoglobulin M ≥4500 mg/L, BM lymphoplasmacytic infiltration ≥70%, $\beta_2$microglobulin ≥4.0 mg/dL, and albumin ≤3.5 g/dL.[77] With these risk factors, the current progression risk-classification identifies three groups of asymptomatic WM with a different median time to develop symptomatic disease: patients whose risk scores were below the first quartile (low risk, have a median time from diagnosis to transformation of 9.3 years), patients whose risk scores were in the interquartile range (intermediate risk, 4.8 years) and those whose risk

scores were above the third quartile (high risk, 18 years).[77] This new classification, whose access is facilitated through a ready-to-use webpage tool (http://www.awmrisk.com) could be of help to identify patients with high-risk AWM who may need closer follow-up or benefit from early intervention.

Risk Assessment in Symptomatic WM

Finally, if our patient fits the criteria of symptomatic WM, we should use the International Prognostic Scoring System (IPSS)[78] that includes five variables associated with poor prognosis:

Anemia: Hb ≤115 g/L,

Thrombopenia: ≤100×109/L,

Monoclonal component: >70 g/L,

Age: >65 years

β2-microglobulin: >3 mg/L.

Using these risk factors we can construct three risk-groups:

1. Low risk: when there is no one risk-factor or only one, excluding the age. The median survival is 14y
2. Intermediate risk: if the patient is older than 65 or there are 2 risk-factors. The median survival is 8y
3. High risk: if there are more than two risk factors. The median survival is 4y

Since this IPSS is based on patients treated with old-fashioned therapies, more recently Kastritis et al developed a revised score (rIPSSWM) that found four parameters associated with poor prognosis: age (66–75 and ≥76 years), $\beta_2$microglobulin ≥4 mg/L, serum albumin <3.5 gr/dL, and LDH ≥250 IU/L (ULN < 225). Accordingly, five different prognostic groups were constructed with a 3-year WM-related death rate of 0, 10, 14, 38, and 48% (p < 0.001) and 10-year survival rate of 84%, 59%, 37%, 19%, and 9%. This system includes two extremes: very-low risk and very-high risk groups. It is conceivable to recommend the modification of current strategies a better management approach in them.[5] However, we should take into account that IPSSWM and rIPSSWM, are merely based on clinical and biochemical parameters, lacking on molecular and genetic characteristics that could be crucial for the future risk assessment in WM.[18]

Summary

Clinical-biological work-up in WM must include a deep pathological evaluation that help in the correct diagnostic assignment of the patient in order to correctly prescribe the patient treatment. Diagnosis should be precise and identify what specific IgM monoclonal gammopathy is present in our patient, including as much as immunophenotypic and genetic information is possible to be obtained, including bone biopsy and BM cell data. In addition, a prognostic risk group assignment must be provided. Both, genomic and prognostic information will be used for a correct therapeutic indication. According to the 10th IWWM updated consensus, first line of therapy for newly diagnosed symptomatic patients WM could include alkylating drugs (Cyclophosphamide and Bendamustine) plus Rituximab, proteasome inhibitors (Bortezomib, Carfilzomib) plus Rituximab, or BTK inhibitors (Ibrutinib) with or without Rituximab. Second and further therapeutic lines will depend on the former response duration and toxicities, making possible the reuse of previous regimens or changing into others. The treatment initiation should be strictly guided by a correct diagnosis (especially differentiating between symptomatic and asymptomatic forms) knowing that that responses to novel BTK inhibitors could be influenced by the molecular characteristics of the disease.[79] This reinforces the necessity of an exquisite evaluating approach for these patients as we have pointed out in this review.

Acknowledgments

Disclosure

The authors declare that they have no potential conflicts of interest.

References

1. Owen RG, Treon SP, Al-Katib A, et al. Clinicopathological definition of waldenstrom's macroglobulinemia: consensus panel recommendations from the second international workshop on waldenstrom's macroglobulinemia. *Semin Oncol.* 2003;30(2):110–115. doi:10.1053/sonc.2003.50082
2. Swerdlow S, Campo E, Pileri S, et al. The 2016 revision of the world health organization classification of lymphoid neoplasms. *Blood.* 2016;127(20):2375–2390. doi:10.1182/blood-2016-01-643569
3. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood.* 2011;117 (19):5019–5032. doi:10.1182/blood-2011-01-293050

4. Hunter Z, Xu L, Zhou Y, et al. Whole-genome sequencing results from 30 patients with waldenstrom's macroglobulinemia. *Blood*. 2011;118(21):434. doi:10.1182/blood.V118.21.434.434

5. Lynch RC, Gratzinger D, Advani RH. Clinical impact of the 2016 update to the WHO lymphoma classification. *Curr Treat Options Oncol*. 2017;18(7):45. doi:10.1007/s11864-017-0483-z

6. Treon SP, Xu L, Yang G, et al. MYD88 L265P somatic mutation in Waldenström's macroglobulinemia. *N Engl J Med*. 2012;367 (9):826–833. doi:10.1056/NEJMoa1200710

7. Treon SP, Cao Y, Xu L, Yang G, Liu X, Hunter ZR. Somatic mutations in MYD88 and CXCR4 are determinants of clinical presentation and overall survival in Waldenstrom macroglobulinemia. *Blood*. 2014;123(18):2791–2796. doi:10.1182/blood-2014-01-550905

8. Hunter ZR, Xu L, Yang G, et al. The genomic landscape of Waldenstrom macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. *Blood*. 2014;123 (11):1637–1646. doi:10.1182/blood-2013-09-525808

9. Pophali PA, Bartley A, Kapoor P, et al. Prevalence and survival of smouldering Waldenström macroglobulinaemia in the United States. *Br J Haematol*. 2019;184(6):1014–1017. doi:10.1111/bjh.15201

10. García-Sanz R, Montoto S, Torrequebrada A, et al. Waldenström macroglobulinaemia: presenting features and outcome in a series with 217 cases. *Br J Haematol*. 2001;115(3):575–582. doi:10.1046/j.1365-2141.2001.03144.x

11. Kristinsson SY, Koshiol J, Goldin LR, et al. Genetics- and immune-related factors in the pathogenesis of lymphoplasmacytic lymphoma/Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma*. 2009;9(1):23–26. doi:10.3816/CLM.2009.n.004

12. Treon SP, Tripsas C, Hanzis C, et al. Familial disease predisposition impacts treatment outcome in patients with Waldenström macroglobulinemia. *Clin Lymphoma Myeloma Leuk*. 2012;12(6):433–437. doi:10.1016/j.clml.2012.08.006

13. Giordano TP, Henderson L, Landgren O, et al. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. *JAMA*. 2007;297(18):2010–2017. doi:10.1001/jama.297.18.2010

14. Mailankody S, Landgren O. Monoclonal gammopathy of undetermined significance and Waldenström's macroglobulinemia. *Best Pract Res Clin Haematol*. 2016;29(2):187–193. doi:10.1016/j.beha.2016.08.015

15. Kyle RA, Larson DR, Therneau TM, et al. Long-term follow-up of monoclonal gammopathy of undetermined significance. *N Engl J Med*. 2018;378(3):241–249. doi:10.1056/NEJMoa1709974

16. Swerdlow SH, Kuzu I, Dogan A, et al. The many faces of small B cell lymphomas with plasmacytic differentiation and the contribution of MYD88 testing. *Virchows Arch*. 2016;468(3):259–275. doi:10.1007/s00428-015-1858-9

17. Sabattini E, Bacci F, Sagramoso C, Pileri SA. WHO classification of tumours of haematopoietic and lymphoid tissues in 2008: an overview. *Pathologica*. 2010;102(3):83–87.

18. Dimopoulos MA, Kastritis E. How I treat Waldenström macroglobulinemia. *Blood*. 2019;134(23):2022–2035. doi:10.1182/blood.2019000725

19. Ciccarelli BT, Patterson CJ, Hunter ZR, et al. Hepcidin is produced by lymphoplasmacytic cells and is associated with anemia in Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma Leuk*. 2011;11(1):160–163. doi:10.3816/CLML.2011.n.038

20. Samba-Mondonga M, Calvé A, Mallette FA, Santos MM. MyD88 regulates the expression of SMAD4 and the iron regulatory hormone hepcidin. *Front Cell Dev Biol*. 2018;6:105. doi:10.3389/fcell.2018.00105

21. Castillo JJ, Garcia-Sanz R, Hatjiharissi E. Recommendations for the diagnosis and initial evaluation of patients with waldenström macroglobulinaemia: a task force from the 8th international workshop on waldenström macroglobulinaemia. *Br J Haematol*. 2016;175:77.

22. Banwait R, Aljawai Y, Cappuccio J, et al. Extramedullary Waldenström macroglobulinemia. *Am J Hematol*. 2015;90 (2):100–104. doi:10.1002/ajh.23880

23. Castillo JJ, Treon SP. How we manage Bing-Neel syndrome. *Br J Haematol*. 2019;187(3):277–285. doi:10.1111/bjh.16167

24. Muñiz C, Martín-Martín L, López A, et al. Contribution of cerebrospinal fluid sCD19 levels to the detection of CNS lymphoma and its impact on disease outcome. *Blood*. 2014;123(12):1864–1869. doi:10.1182/blood-2013-11-537993

25. Gertz MA. Acute hyperviscosity: syndromes and management. *Blood*. 2018;132(13):1379–1385. doi:10.1182/blood-2018-06-846816

26. Gustine JN, Meid K, Dubeau T, et al. Serum IgM level as predictor of symptomatic hyperviscosity in patients with Waldenström macroglobulinaemia. *Br J Haematol*. 2017;177(5):717–725. doi:10.1111/bjh.14743

27. Muchtar E, Magen H, Gertz MA. How I treat cryoglobulinemia. *Blood*. 2017;129(3):289–298. doi:10.1182/blood-2016-09-719773

28. Saadoun D, Sellam J, Ghillani-Dalbin P, Crecel R, Piette JC, Cacoub P. Increased risks of lymphoma and death among patients with non-hepatitis C virus-related mixed cryoglobulinemia. *Arch Intern Med*. 2006;166(19):2101–2108. doi:10.1001/archinte.166.19.2101

29. Berentsen S. New insights in the pathogenesis and therapy of cold agglutinin-mediated autoimmune hemolytic anemia. *Front Immunol*. 2020;11:590. doi:10.3389/fimmu.2020.00590

30. D'Sa S, Kersten MJ, Castillo JJ, et al. Investigation and management of IgM and Waldenström-associated peripheral neuropathies: recommendations from the IWWM-8 consensus panel. *Br J Haematol*. 2017;176(5):728–742. doi:10.1111/bjh.14492

31. Chaudhry HM, Mauermann ML, Rajkumar SV. Monoclonal gammopathy-associated peripheral neuropathy: diagnosis and management. *Mayo Clin Proc*. 2017;92(5):838–850. doi:10.1016/j.mayocp.2017.02.003

32. Le Cann M, Bouhour F, Viala K, et al. CANOMAD: a neurological monoclonal gammopathy of clinical significance that benefits from B-cell-targeted therapies. *Blood*. 2020;136(21):2428–2436. doi:10.1182/blood.2020007092

33. Castillo JJ, Garcia-Sanz R, Hatjiharissi E, et al. Recommendations for the diagnosis and initial evaluation of patients with waldenström macroglobulinaemia: a task force from the 8th international workshop on waldenström macroglobulinaemia. *Br J Haematol*. 2016;175 (1):77–86. doi:10.1111/bjh.14196

34. Sidana S, Larson DP, Greipp PT, et al. IgM AL amyloidosis: delineating disease biology and outcomes with clinical, genomic and bone marrow morphological features. *Leukemia*. 2020;34 (5):1373–1382. doi:10.1038/s41375-019-0667-6

35. Milani P, Merlini G. Monoclonal IgM-related AL amyloidosis. *Best Pract Res Clin Haematol*. 2016;29(2):241–248. doi:10.1016/j.beha.2016.08.013

36. Sachchithanantham S, Roussel M, Palladini G, et al. European collaborative study defining clinical profile outcomes and novel prognostic criteria in monoclonal immunoglobulin m-related light chain amyloidosis. *J Clin Oncol*. 2016;34(17):2037–2045. doi:10.1200/JCO.2015.63.3123

37. Kastritis E, Leblond V, Dimopoulos MA, et al. Waldenström's macroglobulinaemia: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2019;30(5):860–862. doi:10.1093/annonc/mdy466

38. Andrade-Campos M, Murillo-Flórez I, García-Sanz R, Giraldo P. Immunoparesis in IgM gammopathies as a useful biomarker to predict disease progression. *Clin Chem Lab Med*. 2017;55 (10):1598–1604. doi:10.1515/cclm-2016-0748

39. Leleu X, Moreau AS, Weller E, et al. Serum immunoglobulin free light chain correlates with tumor burden markers in Waldenstrom macroglobulinemia. *Leuk Lymphoma*. 2008;49(6):1104–1107. doi:10.1080/10428190802074619

805

40. Itzykson R, Le Garff-tavernier M, Katsahian S, Diemert MC, Musset L, Leblond V. Serum-free light chain elevation is associated with a shorter time to treatment in Waldenstrom's macroglobulinemia. *Haematologica*. 2008;93(5):793–794. doi:10.3324/haematol.12107

41. Xu L, Hunter ZR, Yang G, et al. MYD88 L265P in Waldenström macroglobulinemia, immunoglobulin M monoclonal gammopathy, and other B-cell lymphoproliferative disorders using conventional and quantitative allele-specific polymerase chain reaction. *Blood*. 2013;121(11):2051–2058. doi:10.1182/blood-2012-09-454355

42. Bagratuni T, Ntanasis-Stathopoulos I, Gavriatopoulou M, et al. Detection of MYD88 and CXCR4 mutations in cell-free DNA of patients with IgM monoclonal gammopathies. *Leukemia*. 2018;32 (12):2617–2625. doi:10.1038/s41375-018-0197-7

43. Drandi D, Genuardi E, Dogliotti I, et al. Highly sensitive mutation detection by droplet digital polymerase chain reaction in Waldenström macroglobulinemia. *Haematologica*. 2018;103 (6):1029–1037. doi:10.3324/haematol.2017.186528

44. Lin P, Molina TJ, Cook JR, Swerdlow SH. Lymphoplasmacytic lymphoma and other non-marginal zone lymphomas with plasmacytic differentiation. *Am J Clin Pathol*. 2011;136(2):195–210. doi:10.1309/AJCP8FOIVTB6LBER

45. Paiva B, Montes MC, García-Sanz R, et al. Multiparameter flow cytometry for the identification of the Waldenström's clone in IgM-MGUS and Waldenström's Macroglobulinemia: new criteria for differential diagnosis and risk stratification. *Leukemia*. 2014;28 (1):166–173. doi:10.1038/leu.2013.124

46. Jiménez C, Sebastián E, Chillón MC, et al. MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia. *Leukemia*. 2013;27 (8):1722–1728. doi:10.1038/leu.2013.62

47. Hamadeh F, MacNamara SP, Aguilera NS, Swerdlow SH, Cook JR. MYD88 L265P mutation analysis helps define nodal lymphoplasmacytic lymphoma. *Mod Pathol*. 2015;28(4):564–574. doi:10.1038/modpathol.2014.120

48. Durot E, Kanagaratnam L, Zanwar S, et al. A prognostic index predicting survival in transformed Waldenström macroglobulinemia. *Haematologica*. 2020. doi:10.3324/haematol.2020.262899

49. Durot E, Tomowiak C, Michallet AS, et al. Transformed Waldenström macroglobulinaemia: clinical presentation and outcome. A multi-institutional retrospective study of 77 cases from the French innovative leukemia organization (FILO). *Br J Haematol*. 2017;179(3):439–448. doi:10.1111/bjh.14881

50. García-Sanz R, Jiménez C, Puig N, et al. Origin of Waldenstrom's macroglobulinaemia. *Best Pract Res Clin Haematol*. 2016;29 (2):136–147. doi:10.1016/j.beha.2016.08.024

51. Paiva B, Corchete LA, Vidriales MB, et al. The cellular origin and malignant transformation of Waldenström macroglobulinemia. *Blood*. 2015;125(15):2370–2380. doi:10.1182/blood-2014-09-602565

52. Morice WG, Chen D, Kurtin PJ, Hanson CA, McPhail ED. Novel immunophenotypic features of marrow lymphoplasmacytic lymphoma and correlation with Waldenström's macroglobulinemia. *Mod Pathol*. 2009;22(6):807–816. doi:10.1038/modpathol.2009.34

53. Konoplev S, Medeiros LJ, Bueso-Ramos CE, Jorgensen JL, Lin P. Immunophenotypic profile of lymphoplasmacytic lymphoma/Waldenström macroglobulinemia. *Am J Clin Pathol*. 2005;124 (3):414–420. doi:10.1309/3G1XDX0DVHBNVKB4

54. San Miguel JF, Vidriales MB, Ocio E, et al. Immunophenotypic analysis of Waldenstrom's macroglobulinemia. *Semin Oncol*. 2003;30(2):187–195. doi:10.1053/sonc.2003.50074

55. Ocio EM, Schop RF, Gonzalez B, et al. 6q deletion in Waldenström macroglobulinemia is associated with features of adverse prognosis. *Br J Haematol*. 2007;136(1):80–86. doi:10.1111/j.1365-2141.2006.06389.x

56. Braggio E, Fonseca R. Genomic abnormalities of Waldenström macroglobulinemia and related low-grade B-cell lymphomas. *Clin Lymphoma Myeloma Leuk*. 2013;13(2):198–201. doi:10.1016/j.clml.2013.02.015

57. Schop RF, Fonseca R. Genetics and cytogenetics of Waldenstrom's macroglobulinemia. *Semin Oncol*. 2003;30(2):142–145. doi:10.1053/sonc.2003.50075

58. García-Sanz R, Dogliotti I, Zaccaria GM, et al. 6q deletion in Waldenström macroglobulinaemia negatively affects time to transformation and survival. *Br J Haematol*. 2020;192:843.

59. Ocio EM, Hernandez JM, Mateo G, et al. Immunophenotypic and cytogenetic comparison of Waldenstrom's macroglobulinemia with splenic marginal zone lymphoma. *Clin Lymphoma*. 2005;5 (4):241–245. doi:10.3816/CLM.2005.n.007

60. Chang H, Samiee S, Li D, et al. Analysis of IgH translocations, chromosome 13q14 and 17p13.1(p53) deletions by fluorescence in situ hybridization in Waldenstrom's macroglobulinemia: a single center study of 22 cases. *Leukemia*. 2004;18(6):1160–1162. doi:10.1038/sj.leu.2403369

61. Nguyen-Khac F, Lambert J, Chapiro E, et al. Chromosomal aberrations and their prognostic value in a series of 174 untreated patients with Waldenström's macroglobulinemia. *Haematologica*. 2013;98 (4):649–654. doi:10.3324/haematol.2012.070458

62. Treon SP, Xu L, Hunter Z. MYD88 Mutations and Response to Ibrutinib in Waldenström's Macroglobulinemia. *N Engl J Med*. 2015;373(6):584–586. doi:10.1056/NEJMc1506192

63. Gracia-sanz R. WM, MYD88, and CXCR4: following the thread. *J Am Society Hematol*. 2016;128:746.

64. Hunter ZR, Xu L, Tsakmaklis N, et al. Insights into the genomic landscape of MYD88 wild-type Waldenström macroglobulinemia. *Blood Adv*. 2018;2(21):2937–2946. doi:10.1182/bloodadvances.2018022962

65. Varettoni M, Arcaini L, Zibellini S, et al. Prevalence and clinical significance of the MYD88 (L265P) somatic mutation in Waldenstrom's macroglobulinemia and related lymphoid neoplasms. *Blood*. 2013;121(13):2522–2528. doi:10.1182/blood-2012-09-457101

66. Poulain S, Roumier C, Decambron A, et al. MYD88 L265P mutation in Waldenstrom macroglobulinemia. *Blood*. 2013;121 (22):4504–4511. doi:10.1182/blood-2012-06-436329

67. Treon SP, Xu L, Guerrera ML, et al. Genomic landscape of waldenström macroglobulinemia and its impact on treatment strategies. *J Clin Oncol*. 2020;38(11):1198–1208. doi:10.1200/JCO.19.02314

68. Jiménez C, Chillón M, Balanzategui A, et al. Detection of MYD88 L265P mutation by real-time allele-specific oligonucleotide polymerase chain reaction. *Appl Immunohistochem Mol Morphol*. 2014;22 (10):768–773. doi:10.1097/PAI.0000000000000020

69. Schmidt J, Federmann B, Schindler N, et al. MYD88 L265P and CXCR4 mutations in lymphoplasmacytic lymphoma identify cases with high disease activity. *Br J Haematol*. 2015;169(6):795–803. doi:10.1111/bjh.13361

70. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res*. 2010;16(11):2927–2931. doi:10.1158/1078-0432.CCR-09-2329

71. Kaiser LM, Hunter ZR, Treon SP, Buske C. CXCR4 in Waldenström's Macroglobulinema: chances and challenges. *Leukemia*. 2021;35(2):333–345. doi:10.1038/s41375-020-01102-3

72. Hunter ZR, Yang G, Xu L, Liu X, Castillo JJ, Treon SP. Genomics, signaling, and treatment of waldenström macroglobulinemia. *J Clin Oncol*. 2017;35(9):994–1001. doi:10.1200/JCO.2016.71.0814

73. Varettoni M, Boveri E, Zibellini S, et al. Clinical and molecular characteristics of lymphoplasmacytic lymphoma not associated with an IgM monoclonal protein: a multicentric study of the Rete Ematologica Lombarda (REL) network. *Am J Hematol*. 2019;94 (11):1193–1199. doi:10.1002/ajh.25600

74. Jiménez C, Prieto-Conde MI, García-álvarez M, et al. Unraveling the heterogeneity of IgM monoclonal gammopathies: a gene mutational and gene expression study. *Ann Hematol*. 2018;97(3):475–484.

75. Poulain S, Roumier C, Venet-Caillault A, et al. Genomic landscape of CXCR4 mutations in waldenström macroglobulinemia. *Clin Cancer Res*. 2016;22(6):1480–1488. doi:10.1158/1078-0432.CCR-15-0646

806

DovePress

Journal of Blood Medicine 2021:12

76. Jiménez C, Alonso-álvarez S, Alcoceba M, et al. From Waldenström's macroglobulinemia to aggressive diffuse large B-cell lymphoma: a whole-exome analysis of abnormalities leading to transformation. *Blood Cancer J.* 2017;7(8):e591. doi:10.1038/bcj.2017.72

77. Bustoros M, Sklavenitis-Pistofidis R, Kapoor P, et al. Progression risk stratification of asymptomatic waldenström macroglobulinemia. *J Clin Oncol.* 2019;37(16):1403–1411. doi:10.1200/JCO.19.00394

78. Morel P, Duhamel A, Gobbi P, et al. International prognostic scoring system for Waldenstrom macroglobulinemia. *Blood.* 2009;113 (18):4163–4170. doi:10.1182/blood-2008-08-174961

79. Castillo JJ, Advani RH, Branagan AR, et al. Consensus treatment recommendations from the tenth international workshop for waldenström macroglobulinaemia. *Lancet Haematol.* 2020;7(11): e827–e837. doi:10.1016/S2352-3026(20)30224-6

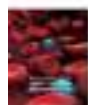# The Level of vWF Antigen and Coagulation Markers in Hospitalized Patients with Covid-19

Hadeel Al Otair, Khalid AlSaleh, Fatmah S AlQahtany, Khalid Al Ayed, Hessah Al Ammar, Noura Al Mefgai & Faisal Al Zeer

ORIGINAL RESEARCH

The Level of vWF Antigen and Coagulation Markers in Hospitalized Patients with Covid-19

Hadeel Al Otair [1]
Khalid AlSaleh[2]
Fatmah S AlQahtany[3]
Khalid Al Ayed[1]
Hessah Al Ammar[4]
Noura Al Mefgai[4]
Faisal Al Zeer[5]

[1]Department of Medicine, King Saud University Medical city (KSUMC), King Saud University, Riyadh, Saudi Arabia; [2]Department of Medicine, Hematology/oncology division, College of Medicine, King Saud University, Riyadh, Saudi Arabia; [3]Department of Pathology, Hematopathology Unit, College of Medicine, King Saud University, King Saud University Medical City, Riyadh, Saudi Arabia; [4]Department of Medicine, King Saud University, Riyadh, Saudi Arabia; [5]College of Medicine, King Saud University, King Saud University, Riyadh, Saudi Arabia

Correspondence: Hadeel Al Otair
Department of Medicine, King Saud University Medical City, King Saud University, P.O. Box 2925(38), Riyadh, 11461, Saudi Arabia
Tel +966(1) 8066519
Fax +966(1) 4692365
Email halotair@ksu.edu.sa

Background: The coagulopathy of COVID-19 still awaits more clarification, and one approach that has not been investigated is to compare the hemostatic changes between COVID-19 and non-COVID-19 infected patients.

Objective: This study aims to study COVID-19 coagulopathy by measuring markers of endothelial injury and coagulation, including anticoagulants (TFPI, protein C, protein S, and AT) in COVID-19 patients and compare them with non-COVID-19 patients early in the course of the disease.

Methodology: This is an observational, prospective cross-sectional study comparing the levels of protein C, protein S, antithrombin (AT) III, clotting factor (F) VIII, von Willebrand factor (vWF) and coagulation screening tests (PT and a PTT), fibrinogen, D-dimer in COVID-19 patients admitted during the same time with non-COVID-19 infections. The demographic and clinical data of the patients were collected from electronic medical records during admission. Blood tests were extracted within 24 hours of admission for both groups.

Results: Fifty-four (66.7% males) consecutive COVID-19 patients and 24 (59% males) non-COVID-19 controls were enrolled in the study from October 2020 till December 2020. COVID-19 patients were significantly older than non-COVID-19 (57.7 ± 14.2 vs 50 ± 19.8 years, $p=0.005$). Fibrinogen level was significantly higher in COVID-19 patients compared to controls (5.9 ± 1.48 vs 3.9 ± 1.57, $p<0.001$). There was no statistically significant difference in the level of FVIII, protein C, S, ATIII, and D-dimer between the two groups. The level of vWF Ag was statistically higher in COVID-19 patients (276.7 ± 91.1 vs 184.7 ± 89.4, $p=0.0001$). There was significant thrombocytopenia and lymphopenia among COVID-19 patients. Inflammatory markers, CRP, ferritin, and LDH, were increased in COVID-19 patients compared to non-COVID-19, but the difference was not statistically significant. High fibrinogen and vWF AG levels were the two independent variables found in COVID-19 patients.

Conclusion: The level of vWF Ag is increased early in the course of COVID-19 infection. This can be used as a biomarker for endothelial injury, which is peculiar to COVID-19 infection.

Keywords: COVID-19, vWF, fibrinogen, coagulopathy, endothelium

Introduction

Coronavirus disease (COVID-19) started in Wuhan, China, as multiple cases of pneumonia of unclear cause.[1–3] On 11th March 2020, WHO declared COVID-19 as a pandemic after affecting more than 118,319 patients globally.[4] Currently, more than 127 million cases are confirmed worldwide with 2,799,030 deaths.[5] In the Kingdom of Saudi Arabia, the total number of cases has reached 388,860 with 6656 deaths, according to the last report (29th March 2021) from the Saudi Ministry of Health and the Saudi Centre of Disease Prevention and Control (CDC).[6]

809

It is well known now that severe acute respiratory syndrome corona virus2 (SARS-CoV-2) utilizes angiotensin-converting enzyme 2 (ACE2) receptor to enter human host cells.[31] Specifically, SARS-CoV-2 surface spike protein binds to human ACE2 on the surface of the cell through its receptor-binding domain (RBD) which is activated by trans-membrane protease serine 2 (TMPRSS2). This in turn induces virus-plasma membrane fusion and subsequent cell entry. Therefore, it plays a fundamental role in SARS-CoV-2 cellular infectivity as well as reduced viral recognition by neutralizing antibodies. Its expression by endothelial cells of the respiratory and digestive tracts explains many of the clinical presentations of COVID-19 infection.[32–34]

Patients with DM have an upregulation of ACE2 expression (total and glycosylated forms) on the surface of the cells secondary to the renin-angiotensin system activation.[35] This contributes to the higher prevalence and worse prognosis of COVID-19 infection in patients with type 2 DM in conjunction with microvascular damage and overt inflammation mediated by high plasma levels of IL-6 and other pro-inflammatory cytokine.[36]

Similarly, the higher binding of COVID-19 and ACE2 could explain the higher rate of hypertension among patients infected with covid-19 and their complicated course. ACE2 is a known modulator of the renin-angiotensin system (RAS) and responsible for many of the pathways underlying hypertension.[37]

Recently, many papers have reported an increased prevalence of venous and arterial thrombosis in COVID-19 patients.[7–10] This is especially true in patients with non-O blood groups who have higher risk for arterial and venous thrombosis. Possibly related to alteration in hemostatic markers, vWF and FVIII, and over-inflammation.[38]

Similarly, postmortem studies have demonstrated the presence of widespread multiple microthrombi.[11] Pulmonary embolism and deep vein thrombosis have also been reported in-69%of critically ill patients.[9]

During the last year, COVID-19 coagulopathy has been the subject of numerous publications, and it is now well established that the laboratory findings in COVID-19 coagulopathy are quite different from the usual findings of disseminated intravascular coagulation (DIC) seen commonly in septicemia.[12,13]

In hospitalized COVID-19 patients, the most observed abnormalities are elevations of plasma fibrinogen and D-dimer, along with a parallel rise in markers of inflammation (eg, CRP and cytokines), and minimum prolongation of prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and mild thrombocytopenia (platelet count ~100 x10^9/L).[14,15] This does not fit in the findings noted in classical DIC.[16]

These unique features of COVID-19 have been researched extensively in the last few months, and no consensus on its pathophysiology has been reached. A recent article in Nature has described it rightly as the COVID-19 mystery.[17,18]

With this background in mind, we conducted a cross-sectional study to explore the mechanism of clot formation in COVID-19, specifically the level of clotting factor (F) VIII, von Willebrand factor (vWF), and natural anticoagulants in COVID-19 infection on admission to the hospital and compared it to the non-COVID-19 patients. We believe this approach of comparing markers of endothelial injury and coagulation between patients with COVID-19 pneumonia and bacterial pneumonia would bring out differences in the coagulopathy between these two groups of patients and thereby shed some light on the peculiar mechanism of the COVID-19 coagulopathy and better understanding of its pathophysiology, which may pave the way for novel therapeutic and/or preventive measures to prevent this potentially fatal complication•

Materials and Methodology

This study is a cross-sectional prospective observational study comparing COVID-19 patients confirmed by positive real-time polymerase chain reaction rt-PCR test, Roche Light Cycler® 480, of nasopharyngeal swabs, and non-COVID-19 patients admitted at King Khalid University Hospital, Riyadh, Saudi Arabia, between October 2020 and December 2020.

Informed consent was obtained from all patients or their next of kin for reviewing their electronic medical records and collection of blood samples for the laboratory coagulation tests. The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of the College of Medicine-King Saud University, clinical trial number E-20-5099.

Patient Selection

The study included patients aged 18–80 years. We excluded incompetent or mentally disabled patients, oncology patients, pregnant or lactating women, patients known to have nephrotic syndrome and liver cirrhosis, and patients recently diagnosed with venous thromboembolism (<3 months) and those on anticoagulants.

For the control patients (non-COVID-19) two negative screening rt-PCR test and diagnosis of community-acquired pneumonia (ATS definition) was required for enrolment.[19]

Demographic and clinical data were collected from the patients' electronic charts and recorded in a data entry form. These included age, sex, basal metabolic rate (BMI), smoking, comorbidities, medication, and clinical presentation for COVID-19.

The metrics for all the baseline laboratory investigations were collected from the system (HbA1C, D-dimer, CBC with differential count, serum ferritin, LDH, Cr, BUN, AST, ALT, Albumin, Bilirubin LDH and CRP).

Using a citrated tube, blood samples for natural coagulation factors inhibitors (Protein C, S, Antithrombin (AT) III) were extracted by the attending nurse, within 24 hours of admission, for both COVID-19 and non-COVID-19 patients. Samples were then transferred to Hematopathology Laboratory at King Khalid University Hospital.

Coagulation Tests

The performed assays included PT, aPTT, fibrinogen, D-dimer, quantification of coagulation FVIII, and physiological inhibitor proteins (protein C, free protein S, and AT). The PT, aPTT, and plasma fibrinogen assays were determined on the NeoPTimal using STA®, PTT A ⑤, Liquid FIB respectively and D-Dimer assay on the Liatest® D-Di PLUS. The Protein S, Protein C, antithrombin assays were determined on the Staclot, Stachrom, Stachrom ATIII STA®, respectively. The ristocetin cofactor activity of vWF-Rco was determined by vWF: RCo and vWF: Ag using Liatest ® vWF: Ag STA®.

Statistical Analysis

For descriptive and inferential statistical data analyses, Statistical Package for Social Sciences software, version 25.0 (IBM SPSS Inc., Chicago, IL), was used. Both descriptive and inferential statistics involving the Chi-square test and T-independent Test were used to present the results. For each test, a p-value of less than 0.05 was considered statistically significant. Multiple logistic regression analysis and ROC curves were used to identify the independent variable.

Results

Fifty-four (66.7% males) consecutive COVID-19 patients and 24 (59% males) non-COVID-19 controls were enrolled in the study, from October 2020 till December 2020. The control group patients were diagnosed with community-acquired pneumonia, and 6 of them had acute decompensated heart failure. Seven patients (7.4%) of COVID-19 patients died, 2 patients developed PE and one patient DVT during hospitalization. The severity of Infection with Covid 19 was moderate in 15 patients, severe in 20 patients who required high flow oxygen or CPAP. Out of nineteen patients (35%) admitted to ICU, seven were put on mechanical ventilation (37%), and eight patients received anti-IL-6 (tocilizumab) therapy. Blood group O positive was the predominant ABO phenotype in both Covid-19 and non-COVID-19 patients, 56% and 48%, respectively.

COVID-19 patients were significantly older than non-COVID-19 patients (57.7 + 14.2 years vs 50 ±19.8 years, p=0.005) and were more obese (BMI = 31.3 ±7.5 vs 25.7 ±6.9 kg/m², P=0.003). Their mean Glycosylated Hb (HbA1C) was 7.69±2.1% (Table 1). Thirty-one patients (57%) of Covid-19 patients had type2DM and were on anti-hyperglycemic drugs, while 24 patients (44.4%) were hypertensive There were more smokers in the control group compared to the COVID-19 group (Table 2). There was no difference in comorbid conditions between the COVID-19 and non-COVID-19 groups apart from chronic lung disease, which was more common in the COVID-19 group (18.5% vs 0%, p=0.024) (Table 3). Forty-nine patients (90.7%) of COVID-19 patients versus 18 patients (75%) of controls received LMWH, enoxaparin for VTE prophylaxis, but the difference was not statistically significant (p=0.065). Additionally, the use of anti-platelets was similar among the 2 groups (22.2% vs 33.3%, p=0.3)(Table 3)

Laboratory Results

Plasma fibrinogen was significantly higher in COVID-19 patients compared to controls (5.9 ±1.5 vs 3.9 ±1.57, p=0.000). There was no statistically significant difference in the level of proteins C, S, ATIII between the two groups. Similarly, the level of FVIII, although it was elevated in both groups high, it did not differ significantly between the 2 two groups (196.8 ±83.3% vs 227.4±82.9%, p=0.138). However, the level of factor vWF AG was statistically higher in COVID-19 patients (276.7 ±91.01 vs 184.7 ±89.4, p=0.0001) (Table 1). There was a trend towards increased vWF activity in Covid-19 patients, but this did not reach statistical significance, probably due to the small sample size (191,5.31±68,8.18% vs 177.1 ±64.5%, p=0.08). The level of clotting FVIII was

Table 1 The Laboratory Result Values of COVID-19 Patients and Control Subjects

Parameters	Reference Values	COVID-19 Patients		NON COVID-19 Controls		Calculated T-Value	Sig. (P-value)
		Mean	S.D.	Mean	S.D.		
WBC	4.000–11.000 *10^9/L	8.17	6.24	9.23	4.75	−0.742	0.46
Lymph	1.0–5.0*10^9/L	1.25	1.17	2.12	1.49	−2.65	0.010*
Haemoglobin	130–180 gm/L	12.75	1.76	11.22	1.82	3.47	0.001*
Platelets	140–450*10^9/L	215.62	100.61	333.16	113.03	−4.58	0.0001*
PT	sec 12.1–15.7	14.05	1.41	14.64	2.05	−1.21	0.236
aPTT	sec 25.7–39.5	42.01	8.00	38.15	9.92	1.76	0.081
D dimer	ug/mL 0.2–0.45	1.85	4.19	1.51	1.15	0.357	0.72
Fibrinogen	g/l 2--4	5.90	1.48	3.93	1.57	4.95	0.0001*
Protien c	% 70–130	92.52	18.85	94.45	21.95	−0.395	0.69
Protien S	% 55–143	66.39	19.91	65.91	26.49	0.08	0.93
APCR	sec >120	152.47	43.79	162.99	29.47	−1.07	0.29
AT III	% 80–120	88.98	14.49	84.20	16.37	1.28	0.203
FVIII	% 60–150	196.77	83.29	227.37	82.93	−1.49	0.14
VWF Activity	% 50–160	191,5.3	68,8.2	177.08	64.54	−1.74	0.08
VWF Ag	% 50–160	276.70	91.02	184.70	89.36	4.14	0.0001*
HgA1C	4.0–6.0%	7.69	2.07	8.61	3.32	−0.918	0.38
Creatinine	53–115 mcmole/L	98.0	51.22	97.79	104.94	0.011	0.991
ALT	16–61 unit/L	51.96	34.06	58.50	99.83	0.5	0.76
AST	15–37 unit/L	58.02	66.64	45.95	87.63	0.624	0.54
Albumin	34.00–50.00 gm/L	30.09	4.37	30.84	6.64	−0.509	0.61
Bilirubin	3.00–17.00 umole/L	8.90	5.51	17.12	22.58	−1.76	0.09
Ferritin	30–400 mcg/L	585.67	817.22	106.075	371,67	−0.501	0.61
LDH	84–246 unit/L	392.88	139.24	251,125	375.67	−1.73	0.09
CRP	0.00–3.00 mg/L	85.07	73.170	51.34	44.77	1.26	0.21
Troponin	0.00–19.00 ng/L	20.16	53.27	15.64	28.74	0.357	0.72
BNP	< 100 pg/mL	756.48	1122.29	2616.53	6222.86	−1.07	0.30

Notes: Data are presented as mean ± standard deviation or number (%). WBC – White blood cells. *Indicates values with statistical significance. Significant differences in lab results between patients and control subjects include lymphocyte count, Hemoglobin, Platelets, fibrinogen and VWF Ag.
Abbreviations: PT, prothrombin time; aPTT, activated partial thromboplastin time; PC, protein c; PS, protein S; ATIII, antithrombin III; APCR, activated protein C resistance; F VIII, FACTOR VIII; VWFAg, Von Willebrand Factor Antigen; VWFACT, Von Willebrand Factor Activity; HgA1C, hemoglobin A1C; ALT, alanine trans aminase; AST, aspartate trans aminase; LDH, lactate dehydrogenase; CRP, C reactive protein; BNB, brain natriuretic peptide.

increased in COVID-19 as well as in non-COVID-19 patients, with no significant difference between the two groups (p=0.138).

There was significant thrombocytopenia and lymphopenia in the COVID-19 group, but there were no differences found in coagulation tests PT, aPTT, and D-dimers levels (Table 1). Inflammatory markers CRP, Ferritin in, and LDH were highly elevated in both COVID-19 and non-COVID-19 patients, but there was no statistical difference between both COVID-19 and non-COVID-19 patients (Table 1).

In the multivariate logistic regression analysis of the laboratory values, high fibrinogen and vWF: AG levels were the 2 independent variables found in COVID-19 patients (Table 4). Plasma fibrinogen (OR = 2.552; 95% CI= 1.2835.077; P <0.05) and vWF Ag (OR = 1.011; 95%)

COVID-19 patients were used to generate ROC curves (Figure 1). The area under the ROC curve of 0.0.652 for age (P >0.05), of 0.738 for BMI (P <0.05), of 0.678 for smoking (P <0.05), of 0.309 for sex (P <0.05), of 0.202 for lymphopenia (P 5), of 0.797 for Hg (P <0.05), of 0.404 for PT (P <0.05), of 0.862 for fibrinogen (P < 0.05) and of 0.795 for VWF Ag (P <0.05) (Figure 1).

Discussion

This study investigated some of the markers of endothelial dysfunction, coagulation factors, and level of natural anticoagulants early in the course of COVID-19 infection in comparison to non-COVID-19 patients admitted with community-acquired pneumonia CAP during the same time period. We found that the level of vWF Ag, which is a marker of endothelial injury, was significantly higher

Table 2 Demographic Profile of Study Participants

Parameters	Cases		Controls			
	Mean ± S.D		Mean ± S.D			
Age(year)	57.69 ± 14.23		50 ± 19.79			P=0.005
BMI(Kg/m²)	31.34 ± 7.55		25.69 ± 6.91			P=0.003
Age Group	**Frequency**	**Percentage**	**Frequency**	**Percentage**	**Chi-Square**	**P-value**
≤ 46 (year)	7	13%	10	41%		
4756(year)	21	38.9%	3	12.5%		
57–65(year)	12	22.2%	2	8.3%	12.58	0.005
66 + (year)	14	25.9%	9	37.5%		
Total	54	100%	24	100%		
Smoking	**Frequency**	**Percentage**	**Frequency**	**Percentage**	**Chi-Square**	**P-value**
Ex-smoker	4	7.4%	3	12.5%		
Non smoker	27	50%	19	79.2%		
Smoker	2	3.7%	2	8.3%	12.9	0.005
Unknown	21	38.9%	0	0%		
Total	54	100%	24	100%		
Gender	**Frequency**	**Percentage**	**Frequency**	**Percentage**	**Chi-Square**	**P-value**
Male	36	66.7%	10	59.0%		
Female	18	33.7%	14	41.0%	4.26	0.035
Total	54	100%	24	100%		
Nationality	**Frequency**	**Percentage**	**Frequency**	**Percentage**	**Chi-Square**	**P-value**
Saudi	39	73.6%	23	95.8%		
Others	14	26.4%	1	4.2%	5.12	0.022

Abbreviation: BMI, body mass index.

in COVID-19 patients than in bacterial infections. Its release following SARS-CoV-2 infection of endothelial cells leads to platelet activation and increased levels of FVIII. We also found an Increased level of D-dimer and fibrinogen early in COVID-19 infection. Our findings highlight the important role of endotheliitis in COVID-19 coagulopathy.

The high level of vWF Ag and activity may indicate that endothelial stimulation has taken place very early in the course of COVID-19, resulting in the release of vWF from the endothelium. This process is mediated by ACE2 receptors for SAR-Cov-2 on the surface of endothelial cells[20] and contributes to the upregulation of fibrinogen and other procoagulants. This goes in parallel with the increase in the inflammatory markers IL6, ferritin, LDH. CRP and suggests that VWF can be a predictive marker of severe infection.[21,39] The direct infection of the endothelial cells also leads to platelet activation and increased levels of VWF and FVIII, all of which contribute to

thrombin generation and fibrin clot formation.[22] The resultant endothelial cell activation can, to a great extent, explain the pulmonary microvascular thrombosis found in post-mortem examination of deceased patients with COVID-19,[23,24] The level of FVIII in this study cohort of COVID-19 patients was increased early in the disease and the platelet count was mildly reduced.

Interestingly, the levels of natural anticoagulants (Pr C, S, ATIII) in COVID-19 patients were low normal but were not different from that found in patients with non-COVID -19 patients with CAP. This could indicate that depletion of natural anticoagulants occurs in both bacterial and viral infection at a later stage.

In addition, this study found that biomarkers of coagulation (such as D-dimer, fibrinogen, platelet count) were affected early in the COVID-19 infection. Previous studies reported that D-dimer could be used to differentiate between COVID-19 patients with severe versus mild disease.[22,25]

Table 3 Comparison of Co-Morbid Conditions Between (COVID-19 Patients and Control Individuals)

Parameters	Patients		Control		Chi-Square	P-value
	Frequency	**Percentage**	**Frequency**	**Percentage**		
Diabetes Mellitus	31	57.4%	13	54.2%	0.071	0.7
DM Type 1	0	0%	1	4.2%		
DM Type 2	31	57.4%	12	55.1%	2.56	0.277
Hypertension	24	44.4%	12	50%	0.206	0.650
Heart failure	6	11.1%	6	25%	2.46	0.11
Diastolic	4	7.4%	4	16.7%		
Systolic	2	3.7%	1	4.2%	4.03	0.25
Atrial Fibrillation	1	1.9%	0	0%	0.74	0.39
Ischemic heart disease	4	7.4%	4	12.5%	0.501	0.48
Stroke	2	3.7%	1	4.2%	0.01	0.92
Chronic Kidney Disease	5	9.3%	4	16.7%	0.85	0.35
On dialysis	38	66.7%	0	0%	29.7	0
Chronic Lung Disease	10	18.5%	0	0%	5.1	0.024*
Name of VTE prophylaxis	**Frequency**	**Percentage**	**Frequency**	**Percentage**	**Chi-Square**	**P-value**
Enoxaparin	49	90.7%	18	75%	3.39	0.065
Unfractionated Heparin	5	9.3%	6	25%		
Antiplatelet	12	22.2%	8	33.3%	1.08	0.3

Notes: *Indicates values with statistical significance. No difference in co-morbid conditions was found between patients and control subjects apart from Chronic Lung disease. No significant difference was found regarding the type of VTE prophylaxis.
Abbreviation: VTE, venous thromboembolism.

Table 4 Multivariate Logistic Regression Analysis of the Laboratory Result Values Obtained from COVID-19 Patients and Control

Parameter	Univariate Analysis				Multivariate Analysis			
	OR	**95% C.I. for OR**		**Sig. (P-value)**	**OR**	**95% C.I. for OR**		**Sig. (P-value)**
		Lower	**Upper**			**Lower**	**Upper**	
Age	1.029	0.998	1.061	0.063	0.976	0.914	1.042	0.470
BMI	1.138	1.039	1.247	0.005*	1.071	0.940	1.220	0.305
Smoking	2.925	1.411	6.063	0.004*	1.649	0.318	8.555	0.552
Sex	0.357	0.133	0.960	0.041	0.439	0.039	4.925	0.504
Lymphocyte	0.614	0.395	0.953	0.030*	0.816	0.452	1.473	0.500
Hemoglobin	1.565	1.173	2.089	0.002*	1.462	0.854	2.505	0.166
Platelets	0.812	0.602	1.095	0.172	0.879	0.435	1.778	0.721
Fibrinogen	2.528	1.565	4.085	0.0001*	2.552	1.283	5.077	0.008*
VWF Ag	1.011	1.005	1.018	0.001*	1.011	0.999	1.024	0.080*

Notes: *Indicates values with statistical significance. The independent variables found in Covid-19 patients are Fibrinogen and VWF Ag.
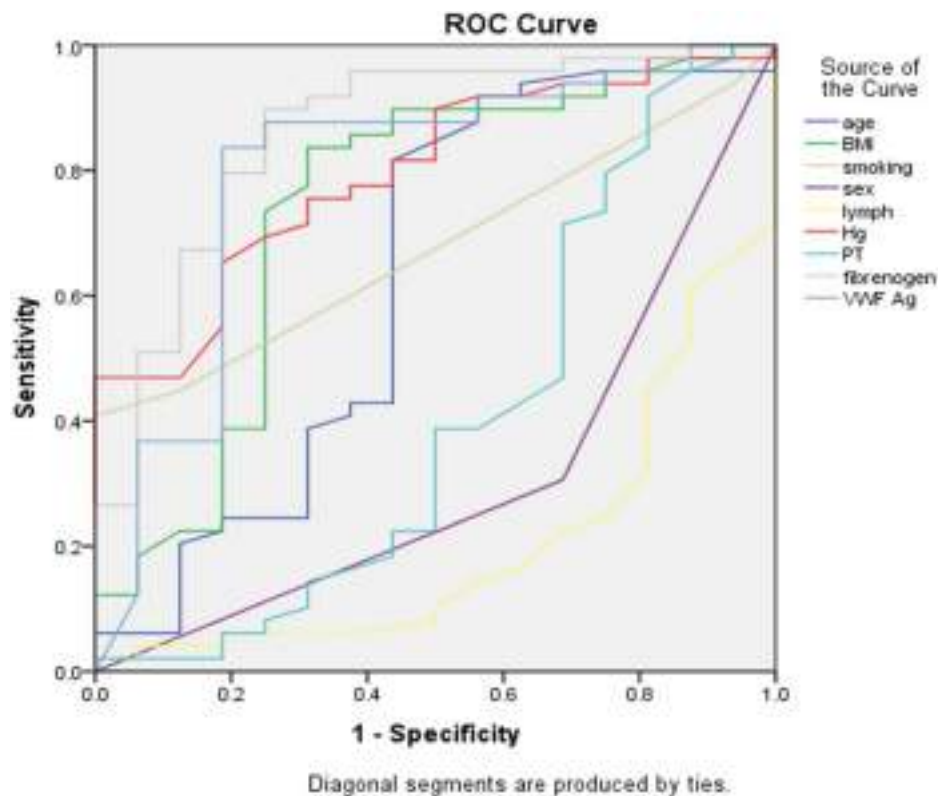**Abbreviations:** BMI, body mass index; VWFAg, Von Willebrand Factor Antigen.

Figure 1 ROC curves for multivariate logistic regression models of significant variables among COVID-19-patients.

A cut-off value of D-dimer of ≥ 2 µg/mL (fourfold increase) within 24 hours after hospital admission was reported by Zhang et al to predict in-hospital mortality with a sensitivity of 92.3% and a specificity of 83.3%.[26] Of note, the two study groups were not different in the anticoagulant agent used for VTE prophylaxis (Table 3); therefore, the changes noted in D-dimer, fibrinogen and coagulation factors were not related to the type of anticoagulant agent.

The increase in fibrinogen noticed early in COVID-19 infection helps differentiate bacterial sepsis or DIC from COVID-19 induced coagulopathy.[27] Besides, the associated thrombocytopenia and prolonged activated partial thromboplastin time tend to be mild.[18] This supports the theory that arterial thrombosis in COVID-19 is the result of direct endotheliitis caused by SARS-CoV-2 infection of endothelial cells through the two receptors of angiotensin-converting enzyme which results in disseminated microthrombosis, reactive endotheliitis and release of von Willebrand (vWF) multimers.[18,39] This seems to be peculiar for COVID-19 and not shared with other viruses that present with decreased plasma fibrinogen concentrations,

such as Ebola or Dengue, responsible for hemorrhagic fever and associated with the hypercoagulable state.[28]

In this study, the lymphocyte count of COVID-1919 pts was statistically lower than non-COVID-19 and occurred early in the disease. This is in agreement with previous studies that reported lymphopenia in 70.3% of hospitalized COVID-19 patients[29] and can be considered as a biomarker of adaptive immune response and was found to be associated with COVID-19 severity.[30]

Limitations of This Study

Including the small sample size and being a single-center study, other inflammatory markers, eg, ferritin. IL 6 and procalcitonin were not compared among the two groups. Future studies in a larger number of patients are needed to confirm our findings and probably try to identify other soluble and cellular markers of early endothelial derangement. This will help to further reveal the role of endotheliitis in the procoagulant mechanism of SARs-cov2 infection, eg, plasma VWF propeptide (VWF pp).

Conclusion

Endothelial injury activation markers are increased early in COVID-19 infection, which is peculiar to COVID-19. The levels of VWF-Ag and fibrinogen are higher in COVID-19 infection than in non-COVID-19 bacterial infections.

We probably need to target endothelial injury in early COVID-19 to halt the activation of the coagulation system and consumption of natural anticoagulants and triggering of inflammatory response.

VWF Ag can be used as a biomarker for endothelial injury in COVID-19 early in the course of infection and may play a role as a prognostic indicator as demonstrated in other recent studies.

Ethics Approval and Consent to Participate

The research proposal for this study was approved by the Institutional Review Board (IRB) of the King Saud University, Riyadh Saudi Arabia (IRB Approval Project No. E-205099) for human studies. Informed consents were obtained from the subjects or authorized family representatives with strict confidentiality of information gathered. The study was conducted in accordance with the Declaration of Helsinki.

Acknowledgments

Funding

Disclosure

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

References

1. Zhu N, Zhang D, Wang W, et al.; China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. *N Engle J Med*. 2020;382 (8):727–733. doi:10.1056/NEJMoa2001017

2. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the Chinese center for disease control and prevention. *JAMA*. 2020;323(13):1239. doi:10.1001/jama.2020.2648

3. World Health Organization. A novel coronavirus (2019-nCoV). Situation report - 1. 2020.

4. World Health Organization. B Coronavirus disease 2019 (COVID-19). Situation report – 51. 2020.

5. World Health Organization. Coronavirus disease (COVID-19) weekly epidemiological update and weekly operational update. March. 2021.

6. Weqaya A. *COVID-19-19 Daily Update of Cases*. Riyadh, Saudi Arabia: Weqaya-Saudi Center for Disease Control and Prevention; 2021.

7. Klok FA, Kruip MJHA, van der Meer NJM, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res*. 2020;191:145–147. doi:10.1016/j.thromres.2020.04.013

8. Cui S, Chen S, Li X, Liu S, Wang F. Prevalence of venous thromboembolism in patients with severe novel coronavirus pneumonia. *J Thromb Haemost*. 2020;18(6):1421–1424. PMID: 32271988. doi:10.1111/jth.14830

9. Llitjos JF, Leclerc M, Chochois C, et al. High incidence of venous thromboembolic events in anticoagulated severe COVID-19 patients. *J Thromb Haemost*. 2020;22(10):1111.

10. Lodigiani C, Iapichino G, Carenzo L, et al. Humanitas COVID-19 task force. Venous and arterial thromboembolic complications in COVID-19 patients admitted to an academic hospital in Milan, Italy. *Thromb Res*. 2020;191:9–14. PMID: 32353746. doi:10.1016/j.thromres.2020.04.024

11. Wichmann D, Sperhake JP, Lütgehetmann M, Steurer S, Edler C, Heinemann A. Autopsy findings and venous thromboembolism in patients with COVID-19. *Ann Intern Med*. 2020:M20–2003. PMID: 32374815.

12. Mucha SR, Dugar S, McCrae K, et al. Coagulopathy in COVID-19. *Cleve Clin J Med*. 2020. PMID: 3240943.

13. Becker RC. COVID-19 update: COVID-19-associated coagulopathy. *J Thromb Thrombolysis*. 2020;50(1):54–67. PMID: 32415579. doi:10.1007/s11239-020-02134-3

14. Görlinger K, Dirkmann D, Gandhi A, Simioni P. COVID-19-associated coagulopathy and inflammatory response: what do we know already and what are the knowledge gaps? *Anesth Analg*. 2020;131(5):1324–1333. PMID: 33079850; PMCID: PMC7389937. doi:10.1213/ANE.0000000000005147

15. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost*. 2020;18(4):844–847. doi:10.1111/jth.14768

16. Levi M, Jonge E, van der Poll T. de Sepsis and disseminated intravascular coagulation. *J Thromb Thrombolysis*. 2003;16(1/2):43–47. doi:10.1023/B:THRO.0000014592.27892.11

17. Callaway E, Ledford H, Mallapaty S. Six months of coronavirus: the mysteries scientists are still racing to solve. *Nature*. 2020;583 (9):178–179. doi:10.1038/d41586-020-01989-z

18. Shalhub S. The mystery of COVID-19-associated arterial thrombosis. *J Vasc Surg*. 2021;73(2):390–391. doi:10.1016/j.jvs.2020.08.022

19. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J. Diagnosis and treatment of adults with community-acquired pneumonia. an official clinical practice guideline of the American Thoracic Society and Infectious Diseases Society of America. *Am J Respir Crit Care Med*. 2019;200(7).

20. Escher R, Breakey N, Lämmle B. Severe COVID-19 infection associated with endothelial activation. *Thromb Res*. 2020;190:62. doi:10.1016/j.thromres.2020.04.014

21. Levi M, van der Poll T. Inflammation and coagulation. *Crit Care Med*. 2010;38(2 Suppl):S26–34. doi:10.1097/CCM.0b013e3181c98d21
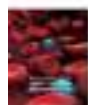
22. Abou-Ismail MY, Diamond A, Kapoor S, Arafah Y, Nayak L. The hypercoagulable state in COVID-19: incidence, pathophysiology, and management. *Thromb Res*. 2020;194:101–115. doi:10.1016/j.thromres.2020.06.029

23. Ward SE, Curley GF, Lavin M; Irish COVID-19 Vasculopathy Study (ICVS) Investigators. Von Willebrand factor propeptide in severe coronavirus disease 2019 (COVID-19): evidence of acute and sustained endothelial cell activation. *Br J Haematol*. 2020;16. PMID: 33326604. doi:10.1111/bjh.17273

24. Elsoukkary S, Mostyka S, Dillard A. Autopsy findings in 32 patients with COVID-19: a single-institution experience. *Pathobiology*. 2021;88(1):56–68. doi:10.1159/000511325

25. Gao Y, Li T, Han M, et al. Diagnostic utility of clinical laboratory data determinations for patients with the severe COVID-19. *J Med Virol*. 2020;92(7):791–796. doi:10.1002/jmv.25770

26. Zhang L, Yan X, Fan Q, et al. D-dimer levels on admission to predict in-hospital mortality in patients with COVID-19. *J Thromb Haemost*. 2020;18(6):1324–1329. doi:10.1111/jth.14859

27. Stief TW, Ijagha O, Weiste B, Herzum I, Renz H, Max M. Analysis of hemostasis alterations in sepsis. *Blood Coagul Fibrinolysis*. 2007;18(2):179–186. doi:10.1097/MBC.0b013e328040bf9a

28. Scarlatescu E, Lance MD. Lance Crimean-Congo haemorrhagic fever: test early with ROTEM? *Lancet Infect Dis*. 2019;19 (8):796–797. doi:10.1016/S1473-3099(19)30298-1

29. Wang D, Hu B, Hu C, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan China. *JAMA*. 2020;323(11):1061–1069. doi:10.1001/jama.2020.1585

30. Henry BM. COVID-19, ECMO, and lymphopenia: a word of caution. *Lancet Respir Med*. 2020;8(4):e24. doi:10.1016/S2213-2600(20)30119-3

31. Shang J, Wan Y, Luo C, et al. Cell entry mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A*. 2020;117(21):11727–11734. doi:10.1073/pnas.2003138117

32. Brojakowska A, Narula J, Shimony R, Bander J. Clinical implications of SARS-CoV-2 interaction with renin angiotensin system: JACC review topic of the week. *J Am Coll Cardiol*. 2020;75 (24):3085–3095. doi:10.1016/j.jacc.2020.04.028

33. Matarese A, Gambardella J, Sardu C, Santulli G. miR-98 regulates TMPRSS2 expression in human endothelial cells: key implications for COVID-19. *Biomedicines*. 2020;8(11):462. doi:10.3390/biomedicines8110462

34. Glowacka I, Bertram S, Muller MA, et al. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J Virol*. 2011;85(9):4122–4134. doi:10.1128/JVI.02232-10

35. D'Onofrio N, Scisciola L, Sardu C, et al. Glycated ACE2 receptor in diabetes: open door for SARS-COV-2 entry in cardiomyocyte. *Cardiovasc Diabetol*. 2021;20(1):99. PMID: 33962629; PMCID: PMC8104461. doi:10.1186/s12933-021-01286-7

36. Sardu C, Gargiulo G, Esposito G, et al. Impact of diabetes mellitus on clinical outcomes in patients affected by Covid-19. *Cardiovasc Diabetol*. 2020;19(1):76. doi:10.1186/s12933-020-01047-y

37. Sardu C, Maggi P, Messina V, et al. Could anti-hypertensive drug therapy affect the clinical prognosis of hypertensive patients with COVID-19 infection? Data from centers of southern Italy. *J Am Heart Assoc*. 2020;9(17):e016948. Epub 2020 Jul 7. PMID: 32633594; PMCID: PMC7660768. doi:10.1161/JAHA.120.016948

38. Sardu C, Marfella R, Maggi P, et al. Implications of AB0 blood group in hypertensive patients with covid-19. *BMC Cardiovasc Disord*. 2020;20(1):373. doi:10.1186/s12872-020-01658-z

39. López Reboiro ML, Suárez Fuentetaja R, Gutiérrez López R, et al. Role of lupus anticoagulant and von Willebrand factor in chronic reactive endotheliitis in COVID-19. *J Infect*. 2021;82(6):e27–e28. Epub 2021 Mar 15. PMID: 33737115; PMCID: PMC7959681. doi:10.1016/j.jinf.2021.03.006

Changes in Hematological, Clinical and Laboratory Parameters for Children with COVID-19: Single-Center Experience

Mahasen Saleh, Amani Alkofide, Anfal Alshammari, Khawar Siddiqui & Tarek Owaidah

Published online: 04 Sep 2021.

Submit your article to this journal ⬏

Article views: 200

View related articles ⬏

View Crossmark data ⬏

Citing articles: 10 View citing articles ⬏

ORIGINAL RESEARCH

Changes in Hematological, Clinical and Laboratory Parameters for Children with COVID-19: Single-Center Experience

Mahasen Saleh[1]
Amani Alkofide[1]
Anfal Alshammari[1]
Khawar Siddiqui[1]
Tarek Owaidah[2,3]

[1]Department of Pediatric Hematology Oncology, King Faisal Specialist Hospital and Research Center, Riyadh, 11533, Kingdom of Saudi Arabia; [2]Department of Pathology and Laboratory Medicine, King Faisal Specialist Hospital and Research Center, Riyadh, 11211, Kingdom of Saudi Arabia; [3]Department of Pathology, Alfaisal University, Riyadh, 11533, Kingdom of Saudi Arabia

Background: COVID-19 wreaked havoc on the healthcare system, with more than 36 million cases reported globally. Although the pediatric population makes up a lesser proportion of total COVID-19 patients than adults, the clinical status, age and comorbidities warrant identifying possible prognostic factors associated with disease severity in this group. The current study aimed to explore the incidence of thrombosis, overall outcome, and different hematological and coagulation markers in children with COVID-19.

Methods: This is a single-center prospective study of 43 patients (age < 14 years) with confirmed COVID-19 diagnosis recruited from April to August 2020. Data for clinical presentation were collected and analyzed. The samples were tested for different hematological and coagulation markers.

Results: Twenty-nine (67.4%) were symptomatic at presentation, with fever being the most common symptom (n = 23, 53.5%), followed by respiratory (n = 5, 11.6%) and gastrointestinal symptoms (n = 3, 7%). Co-morbid conditions were recorded in 26 (60.5%) patients, with malignancy being the commonest (n = 9, 20.9%). In this cohort of patients with age <14 years, hypertension, respiratory symptoms and ABO group-A were significantly associated with pediatric intensive care unit (PICU) admission during the course of treatment. Patients with elevated FVIII and fibrinogen levels at presentation were more likely to have an extended length of hospital stay (LOS) (*P*–value =0.036 and 0.032 respectively). No thrombotic event was observed in our cohort. D-dimer values were higher (above 0.5 µg/mL) in 24 (55.8%) patients at admission. We found an association between high D-dimer and PICU admission and LOS.

Conclusion: Although we did not observe thrombosis in our cohort, serial measurements of D-dimer and elevated FVIII bear a prognostic value in predicting the need for critical care in children with COVID-19. Further studies with larger sample size can aid in the establishment of prognostic factors for the pediatric COVID-19 population.

Keywords: COVID-19, children, hematological and coagulation markers

Introduction

A novel β coronavirus, 2019-nCoV or SARS-CoV-2 infection leading to Coronavirus disease-19 (COVID-19), emerged in Wuhan, China, in December 2019.[1] Despite exceptional patient care and healthcare technology, the alarming mortality rate demands research to identify prognostic factors associated with COVID-19. The clinical severity of the disease is variable, from mild respiratory symptoms to severe cases requiring intensive care. The virus has caused more than 2 million deaths globally.[2]

Correspondence: Tarek Owaidah
Email towaidah@kfshrc.edu.sa

819

One of the various manifestations of COVID-19 is hypercoagulability.[3] There are no clear criteria to predict the cases that will progress to severe clinical symptoms that may require intensive care. Venous and arterial thrombosis has been reported in COVID-19, with the involvement of microcirculatory systems.[4] Different mechanisms induced by thrombosis coupled with intra-alveolar fibrin deposition can lead to lethal respiratory failure.[5–7] Several hematological markers may aid in optimizing treatment at earlier stages and may predict prognosis in real-time.[8,9] Changes in coagulation test, including elevated D-dimers, had been reported in (3.7–68%) and fibrinogen in (5.7% in mild and 19.1% severe cases) and have been shown to be reliable predictors of poor outcome in hospitalized COVID-19 adult patients.[8,10]

In the general population, venous thrombosis occurs at an annual incidence of about 1/1000 in adults[11] and 0.07–0.14/10,000 in children. In hospitalized children, VTE occurs in 5.3/10,000.[12] In children <13 years with COVID-19, the rate of VTE is reported to be 1.3%.[13] In a larger cohort of 180 pediatric patients, although a 2% mortality with Covid-19 was reported, yet no incidence of VTE was found.[14] Data regarding the prognostic factors in the pediatric age group are scarce. D-dimers, a probable biomarker of disease severity and mortality in COVID-19, have not been tested regularly in pediatric patients. While several hematological societies have published recommendations for the anticoagulation of hospitalized symptomatic adult patients with COVID-19,[15] such guidelines, however, cannot be established for pediatric patients due to a dearth of data in that population.

The current study aimed to evaluate the changes in hematological and coagulation markers, such as CBC, different WBCs, D-dimer, Fibrinogen (FIB), Antithrombin (AT), Protein C (PC), Protein S (PS) von Willebrand factor antigen (vWF Ag), and factor VIII as prognostic factors in pediatric patients with COVID-19.

Methods
Patient Population
From April to August 2020, a total of 43 children (age <14 years) were hospitalized with COVID-19 related symptoms. The study complied with the Declaration of Helsinki, and informed consent was obtained from the patients' guardians after appropriate approval by the Institutional Review Board of the hospital. In this prospective study, patients' demographics, details on symptoms, COVID-19 stage, sequential hematological profile, treatment, and outcome were recorded throughout their stay in the hospital. Inclusion criteria were all PCR confirmed COVID-19 patients <14 years with the need for in-hospital treatment.

Excluded were patients transferred from other hospitals and patients already admitted or intubated at admission; outpatient patients and those with negative COVID-19 testing were excluded from data analysis.

Blood Sample Collection
Blood Samples comprising 10 cc of EDTA, 10 cc of Citrated blood (at 3.2%), and 5 cc sodium heparin were collected at admission, 3rd day, 7th day, and 14th day for those who stayed for up to 14 days. CBC and complete 5 parts differential were tested from the EDTA samples using an automated SYSMEX XN-10 instrument (Sysmex Corporation, Kobe, Japan). Serum creatinine and C reactive protein were measured using an automated chemistry analyzer COBAS 601 (Roche Diagnostics, Basel, Switzerland). ABO blood grouping was carried out via Diamed Gel card (Changsha Yingtai Instrument Co., Ltd).

The samples for the coagulation tests were centrifuged within two hours and aliquoted for testing different coagulation markers, including prothrombin time (PT), international normalization ratio (INR), activated thromboplastin time (APTT), D-dimer, FIB, AT, PC, PS, vWF Ag, and FVIII using STAR Max® (Diagnostica Stago, Marseille, France).

COVID-19 Clinical Stages
To explore the correlation between disease severity and coagulation markers, we used a hospital-based clinical staging system for COVID-19 to assess disease severity. The system divides patients into stages A to E, as shown in Table 1.

Statistical Analysis
After performing quality checks on the dataset, descriptive statistics were calculated. Measures of central tendency and dispersion for continuous variables are provided as the mean and standard deviation for normally distributed data, while median with range for data that did not conform to normality assumptions and categorical variables as number and percentage. To test the significance of association between categorical variables, we used the Chi-square test or Fisher's exact. Independent-samples Mann–

Table I Hospital-Based Clinical Staging System for COVID-19 to Assess Disease Severity

Stage	Clinical Presentation	Characteristic Features
Stage A	Asymptomatic	Patients with no signs or symptoms of infection
Stage B	Mild Infection	Upper respiratory tract infection symptoms and other mild symptoms (including fever and gastrointestinal symptoms) without evidence of pneumonia
Stage C	Moderate Infection	Patients with hypoxia with oxygen saturation less than 93% at rest or presence of pneumonia not requiring ICU admission
Stage D	Severe Infection	Pneumonia requiring ICU admission or any of the following: 1. Respiratory rate of 30 breaths/min 2. Arterial oxygen partial pressure to fractional inspiratory oxygen ratio (PaO2/FiO2) less than 300 3. More than 50% lung involvement on imaging within 24–48 hours 4. Critical respiratory failure requiring mechanical ventilation, septic shock, or multi-organ dysfunction

Whitney *U*-Test and Kruskal–Wallis Test were used to test the significance of the difference between two and more than two categories of continuous variables. Correlation between various continuous variables was calculated using Spearman correlation coefficient. A p-value of less than 0.05 was considered to be statistically significant. All data analysis was performed using SPSS version v23 (IBM Corp, Armonk NY, USA).

Results
Demography
Our cohort consisted of 43 children with confirmed COVID-19 diagnoses. The male-to-female ratio was 1:1.3 with a median age of 5.8 years (range "r"= 4 months – 13.3 years). Twenty-nine (67.4%) were symptomatic at presentation, with fever being the most common symptom (n=23, 53.5%), followed by respiratory (n=5, 11.6%) and gastrointestinal symptoms (n=3, 7%). Co-morbid conditions were recorded in 26 (60.5%) patients, with malignancy being the commonest (n=9, 20.9%). The COVID-19 staging was done for all the patients using standard COVID-19 staging, as shown in Table 1.[16–19] COVID-19 stage B was the most common stage at the time of presentation (n=27, 62.8%), followed by stage A (n=14, 32.6%).

Length of Stay at the Hospital and Pediatric Intensive Care Unit (PICU)
The median duration of hospital stay was nine days (*r* =1–48). PICU admission rate was 11.6% (n=5), with an average time to transfer to PICU from the pediatric care wards

of 2.6 ±3.2 days (*r*= 0–7 days). Patients' demographics and clinical characteristics associated with PICU admission are presented in Table 2, showing that younger patients, hypertension, respiratory symptoms, and ABO group-A were significantly associated with PICU admission during treatment. Of the 41 patients with known ABO groups, no significant association between the COVID-19 stage and ABO groups was observed at presentation (*P*–value =0.638). Overall, the average length of stay (ALOS) was significantly higher in patients with COVID-19 stage B (9.8 days, r= 2–23) as compared to Stage A (6.8 days, r= 1–12, *P*–value=0.046). Contrarily, ALOS was not significantly different across different ABO groups (*P*–value =0.374). There is no association between stage B and ALOS (*P*–value =0.081). Patients with elevated FVIII, fibrinogen, and D-dimer at presentation were more likely to have an extended length of hospital stay (*P*–value =0.036, 0.032, and 0.034, respectively).

Risk Factors and Associations with Hospital Stay and PICU Admission
None of our patients experienced any thrombotic event during the admission period, even though D-dimer values were higher (above 0.5 µg/mL) in 24 (55.8%) patients at admission (Figure 1), with 4 out of those 24 (16.7%) requiring PICU admission. An associative trend was observed between high D-dimer values and COVID-19 stage at admission, with 75% (18 of 27) of stage B patients having high D-dimer (*P*–value=0.081). There was also a trend for patients with higher D-dimer at admission to have an ICU visit (4 of 24, 16.7%)

Table 2 Demographics and Clinical Parameters of COVID-19-Infected Patients

Parameters	All Cases (n=43)	No ICU Visit (n=38)	With ICU Visit (n=5)	P–value
Demographic and clinical				
Age (years)	5.8 (0.04–13.3)	5.9 (0.06–13.32)	1.4 (0.04–7.12)	0.04
Gender (female/male)	24/19	20/18 (52.6%)	4/1 (80.0%)	0.363
BMI	15.7±2.9	15.8±2.9	15.0±2.8	0.58
Comorbidities (positive/negative)	26/17 (60.5%)	22/16 (57.9%)	4/1 (80.0%)	0.633
Hypertension	2/41 (4.7%)	0/38 (0%)	2/3 (40.0%)	0.011
Diabetes	1/42 (2.3%)	1/37 (2.6%)	0/5 (0%)	1.000
Bleeding	0/43 (0%)	0/38 (0%)	0/5 (0%)	–
Previous VTE	0/43 (0%)	0/38 (0%)	0/5 (0%)	–
Malignancy	9/34 (20.9%)	8/30 (21.1%)	1/4 (20.0%)	1.000
Hyperlipidemia	1/42 (2.3%)	1/37 (2.6%)	0/5 (0%)	1.000
Stem Cell Transplantation	6/37 (14.0%)	4/34 (10.5%)	2/3 (40%)	0.135
Symptoms (positive/negative)	27/16 (62.8%)	23/15 (60.5%)	4/1 (80.0%)	0.635
Fever	23/20 (53.5%)	19/19 (50.0%)	4/1 (80.0%)	0.351
Respiratory	5/38 (11.6%)	2/36 (5.3%)	3/2 (60.0%)	0.008
GIT related	3/40 (7.0%)	3/35 (7.9%)	0/5 (0%)	1.000
COVID-19 Stage				0.072
Stage A	14 (32.6%)	14 (36.8%)	0 (0%)	
Stage B	27 (62.8%)	23 (60.5%)	4 (80%)	
Stage C	1 (2.3%)	0 (0%)	1 (20%)	
Stage D	1 (2.3%)	1 (2.6%)	0 (0%)	
Stage E	0 (0%)	0 (0%)	0 (0%)	
ABO groups (n=41)				0.049
A	13 (31.7%)	9 (25.0%)	4 (80.0%)	
AB	2 (4.9%)	2 (5.6%)	0 (0%)	
B	8 (19.5%)	7 (19.4%)	1 (20.0%)	
O	18 (43.9%)	18 (50.0%)	0 (0%)	

Notes: The variable BMI was normally distributed while age followed a non-normal distribution. Data are presented as mean±standard deviation for normally distributed and median (minimum-maximum) for non-normally distributed variable.
Abbreviations: GIT, gastrointestinal tract; DIC, disseminated intravascular coagulopathy; INR, international normalized ratio; PT, prothrombin time; HCT, hematopoietic cell transplant.

compared to their counterparts (1 of 19, 5.3%, *P*–value =0.363). More than half of our patients continued to have high D-dimer throughout the course of their admission (Supplementary Table 1). No significant change in neutrophil, lymphocyte count, C-Reactive protein levels, or hemoglobin was observed during the hospital stay, as shown in Supplementary Table 2.

VTE Prophylaxis
VTE with Enoxaparin 1 mg/kg daily was given to only six (14%) patients; two were PICU admission. Clinical profile and symptoms observed at admission, day 3, 7, and 14 are presented in Supplementary Table 1, while sequential laboratory profile is detailed in Supplementary Table 2. Only one patient with malignancy was positive for Lupus anticoagulant; was put on VTE prophylaxis (1 mg/kg) for

11 days and discharged afterward without any remarkable event.

PICU Support Prerequisites
The median PICU length of stay was 3 days (r= 2–11), where one patient required intubation and ventilation; however, the patient recovered well. All patients were discharged from the hospital in a generally good condition. There was no association between age at diagnosis, BMI, D-dimer, and hypertension at presentation. The median length of stay at the hospital was not significantly different for patients with D-dimer higher than 0.5 µg/mL (median 9 days, r= 1–48 days) compared to their counterparts (median 8 days, r= 2–20 days, *P*–value=0.094). There was a weak positive correlation between D-dimer at presentation and hospital stay (Spearman's rho 0.325, *P*–value=0.034).
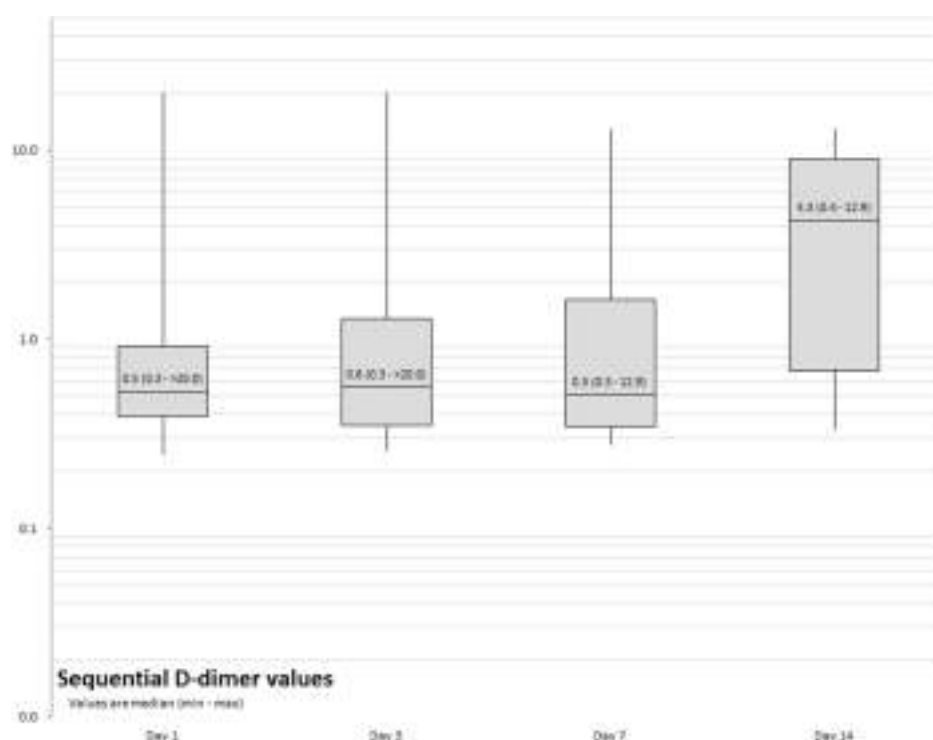
Figure 1 Sequential D-dimer values.

Five patients out of 43 patients needed PICU support. Four (80%) had high D-dimer compared to only one with normal D-dimer (*P*–value=0.363). The average length of stay at the PICU for these four patients was 6.5 ±4.7 days (*r*= 2–11), while it was two days for the one with normal D-dimer (≤0.5 μg/mL). A weak association was found between COVID-19 stage at presentation and the level of D-dimer (*P*–value=0.081).

Laboratory and Coagulation Factors

WBC showed an increasing trend from the day of admission (7.5 ±4.3) to Day 14 (8.1±4.7) at admission, whereas HgB was declining (111.7±19.1 vs 76±36.1). No such trend was observed in platelet, neutrophils and lymphocytes. The mean and median of other laboratory parameters are presented in Supplementary Table 2.

No statistically significant correlation was found for low PC and PS or high vWF Ag at presentation with respect to D-dimer or outcome (Table 3).

Median D-dimer values at admission were found to be higher in patients with malignant disorders (0.78 μg/mL, n=9) compared to those with non-malignant diseases (0.52 μg/mL, n=34); however, the difference was not found to

be statistically significant (*P*–value =0.369). No significant correlation or association was noted between D-dimer and FVIII at admission (P-values 0.393 and 0.262).

Discussion

COVID-19 has wreaked havoc on the healthcare system and caused over one million deaths globally, which indicates disease severity and a lack of prognostic factors to estimate the patient outcomes and plan treatment strategies. Thrombosis had been linked to disease severity in the adult population, for which management guidelines are released by multiple hematology organizations.[20,21] Children appear to account for a much lesser proportion of total COVID-19 infected patients. Various studies have reported a low incidence of thromboembolic complications in children with COVID-19. In a study containing 91 pediatric patients, one developed VTE.[22] None of our patients experienced any thrombotic event, even though only six (14%) received VTE prophylaxis. It is paramount to unfold prognostic biomarkers in children with COVID-19 as this population is unique due to its potential vulnerability, younger age, co-existing and co-morbid conditions with a lack of data to guide and determine therapeutic

Table 3 Length of Stay by Coagulation Biomarkers at Admission

At Presentation	Hospital Stay				P-value
	< 3 Days	3–7 Days	7–14 Days	14 and Above	
Protein C					0.802
Low	0 (0%)	1 (14.3%)	1 (4.5%)	0 (0%)	
Normal	3 (100%)	6 (85.7%)	20 (90.9%)	5 (100%)	
Protein S					1.000
Low	-	-	-	-	
Normal	3 (100%)	7 (100%)	21 (95.5%)	5 (100%))	
vW Ag					0.388
Low	-	-	-	-	
Normal	3 (100%)	4 (57.1%)	15 (68.2%)	2 (40%)	
High	0 (0%)	3 (42.9%)	7 (31.8%)	3 (60%)	
FVIII					0.036
Low	-	-	-	-	
Normal	3 (100%)	6 (85.7%)	18 (81.8%)	1 (20%)	
High	0 (0%)	1 (14.3%)	4 (18.2%)	4 (80.0%)	
Fibrinogen					0.151
Low (n=5)	0 (0%)	0 (0%)	5 (23.8%)	0 (0%)	
Normal (n=25)	3 (100%)	7 (100%)	12 (57.1%)	3 (50%)	
High (n=7)	0 (0%)	0 (0%)	4 (19.0%)	3 (50%)	

measures and prognostic factors. Dong et al, in an epidemiological study, reported 34.1% laboratory-confirmed and 65.9% suspected pediatric patients of COVID-19.[23]

Elevated D-dimer of >1 μg/mL is associated with poor prognosis and mortality in adult COVID-19 patients.[24] Our data demonstrate that higher D-dimer can predict disease severity leading to longer hospitalization and PICU stay, which is in contrast to Del Borrello et al prospective study on 35 pediatric COVID-19 patients, where he found D-dimer values apparently do not distinguish mildly affected COVID-19 patients from more severely affected cases.[25]

D-dimer values were higher in 55.8% of patients at admission. A weak positive correlation was found between D-dimer at presentation and hospital stay at an individual level in our cohort. No significant association between high D-dimer at admission with COVID-19 stages or risk factors was found in our study. Tang et al[20] had reported an overall mortality of 11.5%, significantly higher D-dimer and fibrin degradation product (FDP) levels, longer prothrombin and activated partial thromboplastin times in non-survivals compared to survivors on admission.[7] This discrepancy in results indicates that the prognostic factors in COVID-19 are different between adult and pediatric populations, warranting further investigation.

Zou et al[26] have also reported a significant increase in fibrinogen levels in 5.7% of COVID-19 patients with mild disease and 19.1% patients with severe disease. In critically ill COIVD-19 patients, a prothrombotic diathesis has been reported with significantly higher fibrinogen levels.[27] Our study findings are similar to the reported data whereby the fibrinogen levels increase with time in admitted patients.

The attributable risk for severe disease from COVID-19 in children is challenging to discern. Feldstein et al reported that 92% of patients aged <1–20 years had positive biomarkers like elevated erythrocyte sedimentation rate or C-reactive protein level, lymphocytopenia, neutrophilia, elevated ferritin level, hypoalbuminemia, elevated alanine aminotransferase level, anemia, thrombocytopenia, and an elevated D-dimer level indicating inflammation.[14] Our reported results are in concordance with the study of Feldstein et al.

None of the pediatric subjects included in this study died, suggesting minimal disease mortality among this age group. The susceptibility of children for being infected

with SARS-CoV-2 is similar to those of adults; however, the disease is less severe among children, and thus mortality is also low.[28–30] Various immunological hypotheses have been put forward to support these findings. Firstly, the angiotensin-converting enzyme 2 (ACE2) receptor, a binding site for SARS-CoV-2, is lower in children than adults.[31] Secondly, children usually have few comorbidities.[32,33] However, the morbidity associated with COVID-19 is significantly higher in populations with a high prevalence of childhood obesity.[34] These findings suggest that the risk of being infected is similar for children and adults; however, children are less prone to severe disease outcomes.

Another similarity between adult and pediatric infected patients admitted to PICU is the high percentage of blood group A as previously reported by Yaylac et al; a similar trend was observed in our study.[35] Additionally, comorbidities affected the PICU admission and length of stay significantly. In our cohort, co-morbid conditions were recorded in 26 (60.5%) patients, with malignancy being the commonest (20.9%). One of the limitations of our study was the small sample size presenting some weak associations.

Conclusion

We observed a very low incidence of VTE in children with COVID-19, with a weak association between D-dimer and COVID-19 stage at presentation, but no significant correlation of natural anticoagulants with PC, PS, and AT. We suggest that serial measurements of D-dimer and FVIII bear a prognostic value in predicting the need for critical care in children with COVID-19 patients. Further studies with a larger sample size can aid in the establishment of prognostic factors in this patient group.

Data Sharing Statement

Furnished upon request to the corresponding author.

Ethics Approval and Consent to Participate

The study was approved by the Institutional Review Board of King Faisal Specialist Hospital and Research Center, Kingdom of Saudi Arabia, under approval # RAC KFSHRC (2201086). Informed consent was obtained from the research subjects' guardians prior to study commencement.

Consent for Publication

All authors consent to publication.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors report no conflicts of interest in this work.

References

1. Zhou P, Yang X-L, Wang X-G, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. 2020;579(7798):270–273. doi:10.1038/s41586-020-2012-7

2. Worldometers. COVID-19 coronavirus pandemic; 2021 [cited July 3, 2021]. Available from: https://www.worldometers.info/coronavirus/?utm_campaign=homeAdvegas1?. Accessed August 18, 2021.

3. Becker RC. COVID-19 update: Covid-19-associated coagulopathy. *J Thromb Thrombolysis*. 2020;50(1):54–67. doi:10.1007/s11239-020-02134-3

4. Grewal S, Harjo B, Aykut G, et al. Case report: sublingual microcirculatory alterations in a Covid-19 patient with subcutaneous emphysema, venous thrombosis, and pneumomediastinum. *Front Med*. 2020;7:624695. doi:10.3389/fmed.2020.624695

5. Helms J, Tacquard C, Severac F, et al. High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. *Intensive Care Med*. 2020;46(6):1089–1098.

6. Iba T, Levy JH, Levi M, Connors JM, Thachil J. Coagulopathy of coronavirus disease 2019. *Crit Care Med*. 2020;48(9):1358–1364. doi:10.1097/CCM.0000000000004458

7. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost*. 2020;18(4):844–847. doi:10.1111/jth.14768

8. Yao Y, Cao J, Wang Q, et al. D-dimer as a biomarker for disease severity and mortality in COVID-19 patients: a case control study. *J Intensive Care*. 2020;8(1):1–11. doi:10.1186/s40560-020-00466-z

9. Zhang L, Yan X, Fan Q, et al. D-dimer levels on admission to predict in-hospital mortality in patients with Covid-19. *J Thromb Haemost*. 2020;18(6):1324–1329. doi:10.1111/jth.14859

10. Eljilany I, Elzouki A-N. D-dimer, fibrinogen, and IL-6 in COVID-19 patients with suspected venous thromboembolism: a narrative review. *Vasc Health Risk Manag*. 2020;16:455. doi:10.2147/VHRM.S280962

11. Cushman M, editor. Epidemiology and risk factors for venous thrombosis. *Semin Hematol*. 2007;44(2):62–69. Elsevier.

12. Celkan T, Dikme G. Thrombosis in children: which test to whom, when and how much necessary? *Turk Arch Pediatr*. 2018;53(1):1. doi:10.5152/TurkPediatriArs.2018.2586

13. Goldenberg NA, Sochet A, Albisetti M, et al. Consensus-based clinical recommendations and research priorities for anticoagulant thromboprophylaxis in children hospitalized for COVID-19–related illness. *J Thromb Haemost*. 2020;18(11):3099–3105. doi:10.1111/jth.15073

14. Feldstein LR, Rose EB, Horwitz SM, et al. Multisystem inflammatory syndrome in US children and adolescents. *N Engl J Med*. 2020;383(4):334–346. doi:10.1056/NEJMoa2021680

15. Loi M, Branchford B, Kim J, Self C, Nuss R. COVID-19 anticoagulation recommendations in children. *Pediatr Blood Cancer*. 2020;67(9):e28485. doi:10.1002/pbc.28485

16. Thachil J, Cushman M, Srivastava A. A proposal for staging COVID-19 coagulopathy. *Res Pract Thromb Haemost*. 2020;4 (5):731–736. doi:10.1002/rth2.12372

17. Fan L, Liu S. CT and COVID-19: Chinese experience and recommendations concerning detection, staging and follow-up. *Eur Radiol*. 2020;30(9):5214–5216.

18. Marchandot B, Trimaille A, Curtiaud A, et al. Staging severity of COVID-19 according to hemostatic abnormalities (CAHA score). *Thromb Haemost*. 2020;120(12):1716–1719.

19. Cordon-Cardo C, Pujadas E, Wajnberg A, et al. COVID-19: staging of a new disease. *Cancer Cell*. 2020;38(5):594–597. doi:10.1016/j.ccell.2020.10.006

20. Thachil J, Tang N, Gando S, et al. ISTH interim guidance on recognition and management of coagulopathy in COVID-19. *J Thromb Haemost*. 2020;18(5):1023–1026. doi:10.1111/jth.14810

21. Iba T, Levy JH, Levi M, Thachil J. Coagulopathy in COVID-19. *J Thromb Haemost*. 2020;18(9):2103–2109. doi:10.1111/jth.14975

22. Caro-Dominguez P, Shelmerdine SC, Toso S, et al. Thoracic imaging of coronavirus disease 2019 (COVID-19) in children: a series of 91 cases. *Pediatr Radiol*. 2020;50(10):1354–1368. doi:10.1007/s00247-020-04747-5

23. Dong Y, Mo X, Hu Y, et al. Epidemiology of COVID-19 among children in China. *Pediatrics*. 2020;145(6):e20200702. doi:10.1542/peds.2020-0702

24. Ogimi C, Englund JA, Bradford MC, Qin X, Boeckh M, Waghmare A. Characteristics and outcomes of coronavirus infection in children: the role of viral factors and an immunocompromised state. *J Pediatric Infect Dis Soc*. 2019;8(1):21–28. doi:10.1093/jpids/pix093

25. Del Borrello G, Giraudo I, Bondone C, et al. SARS-CoV-2 associated coagulopathy and thromboembolism prophylaxis in children: a Single Centre Observational Study. *J Thromb Haemost*. 2021;19 (2):522–530.

26. Zou Y, Guo H, Zhang Y, et al. Analysis of coagulation parameters in patients with COVID-19 in Shanghai, China. *Biosci Trends*. 2020;14 (4):285–289. doi:10.5582/bst.2020.03086

27. Thachil J, Agarwal S. Understanding the COVID-19 coagulopathy spectrum. *Anaesthesia*. 2020;75(11):1432–1436. doi:10.1111/anae.15141

28. Zimmermann P, Curtis N. COVID-19 in children, pregnancy and neonates: a review of epidemiologic and clinical features. *Pediatr Infect Dis J*. 2020;39(6):469. doi:10.1097/INF.0000000000002700

29. Leung CW, Kwan YW, Ko PW, et al. Severe acute respiratory syndrome among children. *Pediatrics*. 2004;113(6):e535–e543. doi:10.1542/peds.113.6.e535

30. Al-Tawfiq JA, Kattan RF, Memish ZA. Middle east respiratory syndrome coronavirus disease is rare in children: an update from Saudi Arabia. *World J Clin Pediatr*. 2016;5(4):391. doi:10.5409/wjcp.v5.i4.391

31. Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol*. 2020;5(4):562–569. doi:10.1038/s41564-020-0688-y

32. Patel AB, Verma A. COVID-19 and angiotensin-converting enzyme inhibitors and angiotensin receptor blockers: what is the evidence? *JAMA*. 2020;323(18):1769–1770.

33. Patel AB, Verma A. Nasal ACE2 levels and COVID-19 in children. *JAMA*. 2020;323(23):2386–2387. doi:10.1001/jama.2020.8946

34. Rivas-Ruiz R, Roy-García IA, Ureña-Wong KR, et al. Factors associated with death in children with COVID-19 in Mexico. *Gac Med Mex*. 2020;156(6):516–522.

35. Yaylacı S, Dheir H, Işsever K, et al. The effect of abo and rh blood group antigens on admission to intensive care unit and mortality in patients with COVID-19 infection. *Rev Assoc Med Bras*. 2020;66 (suppl 2):86–90. doi:10.1590/1806-9282.66.s2.86

Correlation of Transferrin Saturation and Serum Ferritin with Bone Mass Density in Adult Transfusion Dependent Beta-Thalassemia Patients

Tubagus Djumhana Atmakusuma & Jeffry Beta Tenggara

Open Access Full Text Article

ORIGINAL RESEARCH

Correlation of Transferrin Saturation and Serum Ferritin with Bone Mass Density in Adult Transfusion Dependent Beta-Thalassemia Patients

Tubagus Djumhana
Atmakusuma [ID][1,2]
Jeffry Beta Tenggara[2,3]

[1]Division of Hematology-Medical
Oncology, Department of Internal
Medicine, Dr. Cipto Mangunkusumo
General Hospital, Jakarta, Indonesia;
[2]Faculty of Medicine Universitas
Indonesia, Jakarta, Indonesia; [3]Division of
Hematology-Medical Oncology,
Department of Internal Medicine,
MRCCC Siloam Hospital Jakarta, Jakarta,
Indonesia

Background: The use of regular blood transfusions and iron chelation therapy to treat thalassemia has improved survival and increased the incidence of osteoporosis. Moreover, iron toxicity is one of the contributing factors that reduce bone mass density in adult transfusion-dependent beta-thalassemia patients. Therefore, this study aims to determine the proportion of low bone mass density in adult thalassemia patients and transferrin saturation, as well as serum ferritin, which correlates to the skeletal condition.

Methods: This is a cross-sectional study conducted in Thalassemia and Hematology Medical Oncology Clinics of Cipto Mangunkusumo Hospital in March 2016. The anthropometric data and hemoglobin levels were obtained before transfusion. Subsequently, the average ferritin levels, bone mineral density, and radiographic results were obtained.

Results: The percentage of adult thalassemia major and intermedia patients with low bone mass density was 68%. Also, there was a weak inverse correlation between bone mass density and transferrin saturation ($r = -0.329$, $p = 0.01$), while no correlation was shown between bone mass density and ferritin ($r = -0.088$, $p = 0.504$). The transferrin saturation cutoff point value used to distinguish the incidence of low and normal bone density in patients with transfusion-dependent beta-thalassemia was 89.5%. In addition, there was weak correlation between Singh index and bone mass density ($r = 0.273$, $p = 0.038$).

Conclusion: Among the transfusion-dependent beta-thalassemia patients, 68% had low bone mass density, which inversely correlated to transferrin saturation. Furthermore, the cutoff value of transferrin saturation to differentiate the incidence of low and normal bone density in thalassemia major compared to thalassemia intermedia was 89.5%. Singh Index correlates weakly with bone mass density and might be used to detect low bone mass density in remote healthcare facilities.

Keywords: bone mass density, ferritin, thalassemia, transfusion dependent beta, transferrin

Background

Thalassemia is a group of hereditary disorders associated with the defective synthesis of alpha or beta-globin, which are inherited as pathologic alleles of the globin genes on the chromosomes 11 (β) and 16 (α).[1] The damage of the membrane structure leads to accelerated apoptosis and premature destruction of the erythroid precursors in the bone marrow (ineffective erythropoiesis).[2] Meanwhile, ineffective erythropoiesis reduces bone mass density and increases the incidence of fractures.[3]

The provision of regular blood transfusions has been shown to increase the survival of patients with thalassemia; however, it leads to the occurrence of iron

Correspondence: Tubagus Djumhana
Atmakusuma
Division of Hematology-Medical
Oncology, Department of Internal
Medicine, Dr. Cipto Mangunkusumo
General Hospital, Jalan Diponegoro No.
71, Jakarta Pusat, DKI, Jakarta, Indonesia
Email hom_fkui@yahoo.com

overload, which progresses to iron toxicity. The iron toxicity is mediated through non-transferrin bound iron (NTBI), a free radical, which is among the factors that contribute to organ damage in thalassemia.[4,5] Since NTBI levels are measured only at several research centers abroad, therefore, to measure iron toxicity, surrogate markers such as saturation transferrin and serum ferritin are used.[6]

Iron toxicity functions as one of the contributing factors to bone density reduction in adult patients with thalassemia. Moreover, ineffective erythropoiesis causes expansion of the bone marrow and leads to mechanical disruption of the bone structure.[7] Currently, there have not been studies on the correlation between transferrin saturation and ferritin parameters with bone density or NTBI receptors in bone.

The use of transfusion and iron chelation therapy to treat thalassemia patients has improved survival; however, regular transfusions cause an increase in the incidence of osteoporosis.[8–11] Out of the population of patients with thalassemia that have received adequate transfusion therapy, the incidence of osteoporosis reported was approximately 66%.[12] A previous study by Hashemieh et al[13] reported a 23% increased incidence of osteopenia in thalassemia major and 8% in thalassemia intermedia.[13] Furthermore, a study also stated that incidence of osteoporosis is increased by 60% in thalassemia major and intermedia are 60% and 81.6%<, respectively.[13] Meanwhile, bone disorder as a complication of thalassemia causes negative psychological impact, lowers the quality of life, and increases treatment costs.

In Indonesia, the incidence of osteoporosis/low bone density is between 28% and 32%.[14] The pathogenesis of reduced bone density in thalassemia is associated with the expansion of bone marrow caused by ineffective erythropoiesis, sexual maturation disorders, hormonal disorders such as hypothyroidism, hypoparathyroidism, diabetes mellitus, and direct iron toxicity on the bone. Furthermore, the use of desferrioxamine iron chelation therapy also plays an important role in the incidence of low bone density.[15]

The measurement of bone mass dual X-ray absorptiometry (DXA) in the lumbar area, femoral neck, and forearm is a non-invasive examination that accurately assesses bone density,[16] however, these tests are not widely available in Indonesia. To overcome this limitation, a simple Singh Index measurement using a conventional X-ray modality is considered. Although this method is widely available, studies on the evaluation of bone density in patients with thalassemia had not been examined, especially in Asian population. Therefore, this study aims to determine the proportion of low bone mass density in adult thalassemia patients and the correlation between transferrin saturation and serum ferritin with the skeletal condition.

Methods

This is a cross-sectional study conducted in Thalassemia together with Hematology and Medical Oncology Clinics of Cipto Mangunkusumo Hospital in March 2016. The inclusion criteria were patients above 18 years, which were diagnosed with thalassemia based on High-Performance Liquid Chromatography (HPLC) or microcapillary, received a regular red blood transfusion at least once a month, and agreed to participate in the study. Subsequently, blood samples, inspection dual X-ray absorptiometry (DXA), and bone radiographs were acquired, while GE® Lunar iDXA™ system was used to measure bone densitometry. In this study, the exclusion criteria were patients that refused to participate, use drugs that affect bone density such as corticosteroids, and are with clinical signs of infection such as fever.

The data collected included name, age, complete address, phone number, weight, height, puberty status, hemoglobin level before transfusion, average ferritin levels during the last 6 months to 1 year, bone density measurement with DXA instruments, and bone radiographic examination.

The data obtained were analyzed using IBM™ SPSS Statistics® 20 and Statistical correlation tests were carried out using non-parametric Spearman test. The Z-score from bone densitometry was analyzed as a continuous variable that correlated with the serum ferritin, transferrin, and femoral Singh index. Dichotomous bone mass density data were only used to determine the percentage of patients with low bone density.

This study was conducted in line with the Declaration of Helsinki and was approved by the Ethical Committee of the Faculty of Medicine, Universitas Indonesia. Also, appropriate informed consent was from all subjects.

Results
Demographic Data

The data were from 60 patients, which consisted of 27 males (45%) and 33 females (55%). The median age was

25 years, which ranged from 18 to 68 years as shown in Table 1. Meanwhile, the median level of ferritin was 3881 ng/mL with the lowest value of 645 ng/mL and the highest value of 15,437 ng/mL. The median transferrin saturation was 86% with the lowest value of 20% and the highest value of 120%. These results showed that the median bone mass density values were −1.1 with the lowest value of −5.7 and the highest of 2.6. (Table 1).

The Proportion of Low Bone Mass Density

Bone mass density was evaluated using the Dual X-ray Absorptiometry (DXA). The results showed that 41 (68%) patients had low bone mass density (Z score <-2), while 19 (32%) had normal bone density (Z score ≥ 2).

The Correlation Between the Saturation of Transferrin or Ferritin in Bone Density

The transferrin saturation, ferritin, and bone mass density of 60 patients were examined with the Kolmogorov–Smirnov test for normality, which obtained a probability value of 0.000 (p<0.05); therefore, non-parametric tests were used. Spearman correlation test was used to determine the correlation between transferrin saturation and ferritin with the bone mass density.

There was a weak inverse correlation between transferrin saturation and bone mass density (r=−0.329, p=0.01), while there was no significant correlation between the levels of serum ferritin with bone mass density (r=−0.088, p=0.504) (Table 2).

The Cut-Off Point of Transferrin Saturation Associated with Low Bone Density

Curve analysis by the receiver operating curve (ROC) was generated to determine the portion of patients predicted to have a low bone mass density in adult thalassemia major and intermedia by the transferrin saturation values. Figure 1 showed a ROC curve value of transferrin saturation 0.727, p-value 0.005, and 95% CI 0.595–0.858, which indicated a good value. Moreover, the cut-off point to determine transferrin saturation to distinguish low and normal bone densities in adult patients with thalassemia major and intermedia was analyzed. Based on the result, a transferrin saturation value of 89.5% was obtained as the most excellent point (Figure 2).

Table 1 General Characteristics of Research Subjects

General Characteristics	N = 60
Gender, n (%)	
Male	27 (45%)
Female	33 (55%)
Classification of Thalassemia	
Thalassemia major (TM)	19 (32%)
Thalassemia intermedia (TI)	41 (68%)
Classification of thalassemia	
Thalassemia Beta Thalassemia	26 (43%)
Beta/HBE	34 (57%)
Iron status	
Ferritin (ng/mL), median (min-max)	3881 (645–15,437)
Transferrin saturation, median (min-max)	86 (20–120)
Anthropometry	
Weight (kg), median (min-max)	43 (25–85)
Height (cm), median (min-max)	154 (123–170)
Short stature, N (%)	28 (47%)
Facies Cooley, N (%)	32 (53%)
BMD Z score, median (min-max)	−1.1 (−5.7 to 2.6)
Laboratory values	
Pre-transfusion hemoglobin (mg/dL), mean (SD)	8.08 (1.09)
hsCRP (mg/L), mean (SD)	3.15 (0.34–4.4)
Magnesium (mEq/L), mean (SD)	2.02 (0.24)
Sodium (mEq/L), mean (SD)	137.38 (4.30)
Potassium (mEq/L), mean (SD)	4.06 (0.42)
Chloride (mEq/L), median (min-max)	99 (89–114)
Calcium (mg/dL), mean (SD)	8.91 (0.49)
Phosphate (mg/dL), median (min-max)	4 (2.2–6.20)
Transferrin saturation (%), median (min-max)	86 (20–112)
Serum ferritin (ng/mL), median (min-max)	3881 (645–15,437)
Femoral Singh Index, median (min-max)	5 (1–6)

Abbreviations: BMD, bone mass density; SD, standard Ddeviation; hsCRP, high-sensitive C-reactive protein.

Cut Point (Cut-Off) of Serum Ferritin Associated with Low Bone Density in Adult Thalassemia Patients

Based on previous analysis (Table 2), there was no significant correlation between ferritin and bone density, and can not be determined as cut-off point ferritin value to

Table 2 Correlation of Transferrin Saturation, Serum Ferritin and Singh Index with Bone Mass Density

Variables	r	p*
Transferrin saturation – BMD	−0.329	0.010
Serum Ferritin – BMD	−0.088	0.504
Femoral Singh Index – BMD	0.273	0.038

Note: *Spearman correlation test.
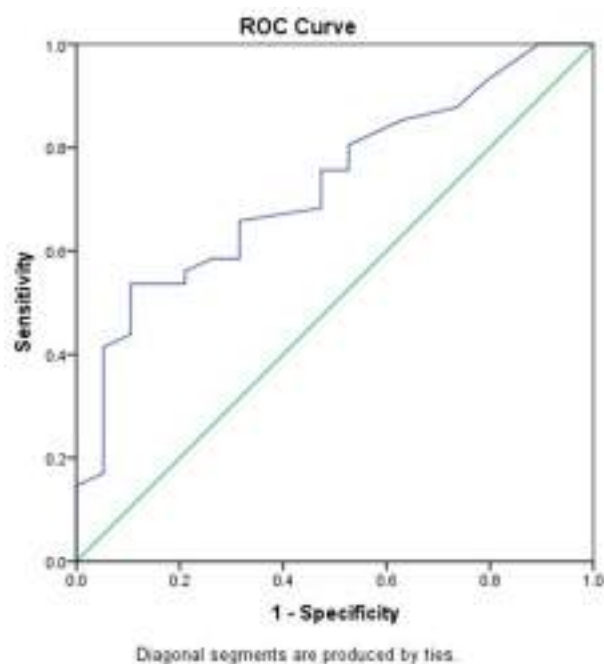**Abbreviation**: BMD, bone mass density.

Figure 1 Transferrin saturation values receiver operating characteristic (ROC) curve for prediction of future occurrence of low bone density in adult thalassemia patients.

distinguish low and normal bone density in patients with thalassemia major and intermedia adults.

The Correlation Between Bone Density Values with Singh Index in Thalassemia Adult Patients

The Singh index value and the bone mass density in 60 patients were examined based on the Kolmogorov–

Smirnov test to obtain a normality probability value of 0.000 (p < 0.05). Furthermore, the Spearman correlation test was conducted to evaluate the correlation between Singh index and bone mass density. The results showed that there was weak correlation (Table 2) between Singh Index and bone mass density (r = 0.273, p-value = 0.038).

Discussion

The wide range of serum ferritin and transferrin saturation values occurred due to the differences in patients' compliance with iron chelation therapy. The amount of blood transfusion should not be a confounding factor since the subjects receive regular blood transfusion every month. In this study, the ferritin value used was obtained by calculating the average ferritin values through several examinations in the previous year. Since the value can be influenced by many factors besides the iron content in the blood such as inflammation and hepatic impairment, averaging several measurements minimized that concern.

There were 68.3% subjects with low bone density. This value is higher than the incidence of low bone mass density in the general population (23%),[14] In addition, 47% of the patients had short stature. Increased incidence of low bone density and osteoporosis in patients with thalassemia was also reported by Hashemieh et al[10] in Iran (65%) and Merchant et al[17] in India (81%). Based on the similarity of our results with other studies, low bone mass density is considered a frequent complication in patients with thalassemia, which requires early diagnosis and prompt management.
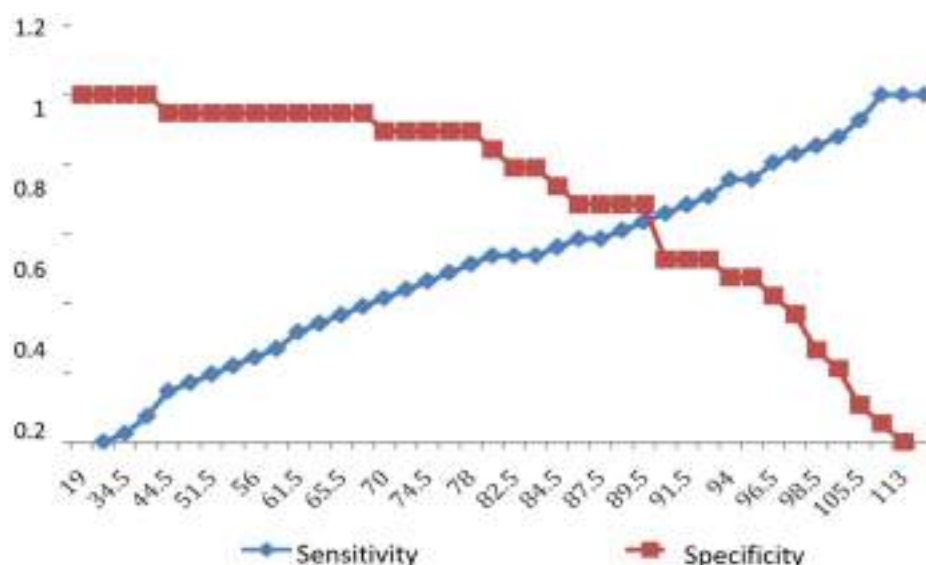

Figure 2 Cutoff point of transferrin saturation in distinguishing incidence of low bone density in adult patients with thalassemia.

This study analyzed the correlation between bone mass density with transferrin saturation, ferritin serum, and Singh index. The result showed an inverse correlation between bone mass density and transferrin saturation with a value of r=−0.329 and p=0.01. On the other hand, there was no significant correlation between bone mass density and ferritin (r=−0.088 p=0.504). This is the first study that obtained a significant correlation between the bone mass density and transferrin saturation in adult patients with thalassemia. Although the correlation was weak, it was statistically significant. Piga et al[18] showed a correlation between non-transferrin-bound iron (NTBI) with transferrin saturation (r=0.52 p <0.001), but there was no correlation with serum ferritin. These findings might suggest that transferrin saturation can be used as a surrogate of bone mass density if the examination is not available.

These results also showed that there was no significant correlation between ferritin and bone density, which was similar to a previous study by Jensen et al.[10] Ferritin is a protein that works as a storage of iron in the human body. Moreover, the levels are influenced by many factors besides iron overload, such as inflammatory reactions, liver damage, and malignancy.[19,20] To overcome this issue, hs-CRP values were measured to exclude inflammatory conditions, while the average values of ferritin were used by examining 3 to 5 measurements in the last one-year. Nonetheless, after excluding infection and averaging the serum ferritin levels, there was still no significant correlation between serum ferritin and bone mass density.

The Singh index is chosen since it can be obtained from plain femoral radiograph, which is available world-wide, even in remote healthcare facilities. Since bone mass density examination is not available throughout Indonesia, we want to determine if it can be used to detect low bone mass density. We found weak correlation between Singh Index and bone mass density (r = 0.273, p-value = 0.038). This study provides preliminary data for further research to strengthen the evidence supporting the use of Singh index for detection of low bone mass density in patients with thalassemia.

With the discovery of the correlation between transferrin saturation with bone mass density, we determined the cut-off point of transferrin saturation to distinguish the incidence of low and normal bone density in adult patients with thalassemia. The Receiver Operator Curve (ROC) obtained a value of Area Under the Curve (AUC) 0.727 (95% CI: 0.595–0.858), which showed that transferrin saturation has a good value to distinguish between low

and normal bone density. Based on the ROC curve obtained, transferrin saturation values best cut-off point was 89.5%.

The limitation of this study is the absence of hormonal assessment and its relation to bone mass density; therefore, it is recommended for future study. The clinical application of this study is to show that transferrin saturation can be used as a surrogate marker of low bone density in adult patients with thalassemia that has not pass through DXA examination.

Conclusion
The percentage of low bone mass density in patients with thalassemia major and intermedia adults in Cipto Mangunkusumo Hospital was 68%. Based on the results, there was a significant inverse correlation between bone mass density with transferrin saturation, while there was no correlation between bone mass density with ferritin saturation in adult thalassemia major and intermedia patients. There was weak correlation between the Singh index and femoral bone density. Transferrin saturation cutoff point value to distinguish between the incidence of low and normal bone density is 89.5%.

Data Sharing Statement
Additional data can be requested by contacting the corresponding author on the email address provided.

Disclosure
The authors report no conflicts of interest in this work.

References
1. Rachmilewtiz E, Giardina P. How I treat thalassemia. *Blood*. 2011;118:3479–3488. doi:10.1182/blood-2010-08-300335
2. Thein SL. Pathophysiology of β-thalassemia-A guide to molecular therapies. *Am Soc Hematol Educ Prog*. 2005;2005:31–37. doi:10.1182/asheducation-2005.1.31
3. Mahachoklertwattana P, Sirikulchayanonta V, Chuansumrit A, et al. Bone histomorphometry in children and adolescents with beta-thalassemia disease: iron-associated focal osteomalacia. *J Clin Endocrinol Metab*. 2003;88:3966–3972. doi:10.1210/jc.2002-021548
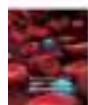
4. Kremastinos DT, Farmakis D. Iron overload cardiomyopathy in clinical practice. *Circ.* 2011;124:2253–2263. doi:10.1161/CIRCULATIONAHA.111.050773

5. Hershko C. Pathogenesis and management of iron toxicity in thalassemia. *Ann NY Acad Sci.* 2010;1202:1–9. doi:10.1111/j.1749-6632.2010.05544.x

6. Papanikolaou G, Pantopoulos K. Pantopoulos iron metabolism and toxicity. *App Toxicol Pharmacol.* 2005;202:199–211. doi:10.1016/j.taap.2004.06.021

7. Voskaridou E, Tepos E. New insights into the pathophysiology and management of osteoporosis in Patients with beta-thalassemia. *Br J Haematol.* 2004;127:127–139. doi:10.1111/j.1365-2141.2004.05143.x

8. Pootrakul P, Hungsprenges S, Fucharoen S, et al. Relation between erythropoiesis and bone metabolism in thalassemia. *N Eng J Med.* 1981;304:1470–1473. doi:10.1056/NEJM198106113042406

9. Michelson J, Cohen A. Incidence and treatment of fractures in thalassemia. *J Orthop Trauma.* 1988;2:29–32. doi:10.1097/00005131-198802000-00007

10. Jensen CE, Tuck BC, Agnew JE, et al. High prevalence of low bone mass in thalassemia major. *Brit J Haematol.* 1998;103:911–915. doi:10.1046/j.1365-2141.1998.01108.x

11. Vichinsky EP. The morbidity of bone disease in thalassemia. *Ann NY Acad Sci.* 1998;850:344–348. doi:10.1111/j.1749-6632.1998.tb10491.x

12. Abdollah SA, Bekheirnia MR, Kamgar M, et al. Metabolic and endocrinologic complications in beta-thalassemia major: a multicenter study in Tehran. *BMC Endocr Disord.* 2003;3:1–6.

13. Hashemieh M, Azarkeivan A, Radfar M, et al. Prevalence of osteoporosis among thalassemia patients with thalassemia clinic from Zafar adult, Iran. *J Iran Blood Cancer.* 2014;6:143–148.

14. Tirtarahardja G, Setyohadi B, Weynand LS, Zhou Q. Bone mineral density reference values for Indonesian men and women; 2006. Available from: https://www.researchgate.net/publication/297712953. Accessed March 10, 2016.

15. DiStefano M, Chiabotto P, Roggia C, Garofalo F, Laura R. Bone mass and metabolism in thalassemic children and adolescents treated with different iron-chelating drugs. *J Bone Miner Metab.* 2004;22:53–57. doi:10.1007/s00774-003-0449-z

16. Grampp S, Genant HK, Mathur A, et al. Comparisons of noninvasive bone mineral measurements in assessing age-related loss, fracture discrimination, and diagnostic classification. *J Bone Miner Res.* 1997;12:697–711. doi:10.1359/jbmr.1997.12.5.697

17. Merchant R, Udani A, Puri V, et al. Evaluation of osteopathy in thalassemia by bone mineral densitometry and biochemical indices. *Indian J Pediatr.* 2010;77:987–991. doi:10.1007/s12098-010-0158-2

18. Piga A, Longo F, Duca L, et al. High nontransferrin bound iron levels and heart disease in thalassemia major. *Am J Hematol.* 2009;84:29–33. doi:10.1002/ajh.21317

19. Ogilvie C, Fitzsimons K, Fitzsimons E. Serum ferritin values in primary care: high values are overlooked. *J Clin Pathol.* 2010;63:1124–1126. doi:10.1136/jcp.2010.083188

20. Li CK, Chik KW, Lam CWK, et al. Liver disease in transfusion-dependent thalassemia major. *Arch Dis Child.* 2002;86:344–347. doi:10.1136/adc.86.5.344

21. Tenggara JB, Atmakusuma TD, Yulisa ND, Abdullah M. Correlation of transferrin saturation and serum ferritin with bone mass density in adult transfusion dependent beta thalassemia patients. Thesis. 2016.

Primary HHV-8 (-) Effusion-Based Non-Germinal Center B Cell Diffuse Large B Cell Lymphoma Successfully Treated with Standard Anthracycline-Based Chemoimmunotherapy

Justin Kuhlman, Muhamad Alhaj Moustafa, Liuyan Jiang & Han W Tun

Published online: 09 Sep 2021.

Submit your article to this journal ☑

Article views: 115

View related articles ☑

View Crossmark data ☑

CASE REPORT

Primary HHV-8 (-) Effusion-Based Non-Germinal Center B Cell Diffuse Large B Cell Lymphoma Successfully Treated with Standard Anthracycline-Based Chemoimmunotherapy

Justin Kuhlman [1]
Muhamad Alhaj Moustafa [2]
Liuyan Jiang [3]
Han W Tun [2]

[1]Department of Internal Medicine, Mayo Clinic, Jacksonville, FL, USA; [2]Division of Hematology and Medical Oncology, Mayo Clinic, Jacksonville, FL, USA; [3]Department of Pathology and Laboratory Medicine, Mayo Clinic, Jacksonville, FL, USA

Abstract: Effusion-based lymphomas (EBL) are usually high-grade B cell non-Hodgkin's lymphomas which involve effusion fluid in a body cavity, typically presenting as a pleural effusion, without evidence of disease elsewhere. They are most frequently seen in HIV-infected individuals and are biologically driven by human herpesvirus-8 virus (HHV-8). HHV-8 (+) EBL is recognized as primary effusion lymphoma (PEL) under the World Health Organization classification. HHV-8 (-) EBL has been reported in association with Hepatitis C virus (HCV) infection, Epstein-Barr virus (EBV) infection, fluid overload, liver cirrhosis, renal dysfunction, cardiac arrhythmias, myocardial infarction, and heart failure. These cases can be labeled as primary EBL (PEBL). We describe a non-germinal center B cell diffuse large B cell lymphoma (NGCB-DLBCL) presenting as PEBL in an immunocompetent 81-year-old male who had an extensive cardiac history and tested negative for HIV, HHV-8, and EBV. He was treated with thoracentesis and standard anthracycline-based chemoimmunotherapy and has remained in complete remission for over 5 ½ years since his original diagnosis. Our case indicates that NGCB-DLBCL can present as PEBL and is potentially curable with the standard chemoimmunotherapeutic approach.

Keywords: HHV-8 negative EBL, non-germinal center diffuse large B cell lymphoma, primary effusion-based lymphoma, primary effusion lymphoma

Introduction

Diffuse large B-cell lymphoma (DLBCL) can frequently involve extra nodal tissues in a secondary process, including body cavities.[1] On rare occasions, however, such lymphomatous involvement of a body cavity can develop as a primary malignancy without evidence of lymphoma elsewhere. The 2016 revised WHO classification of lymphoid neoplasms describes primary effusion lymphoma (PEL) as a large B-cell lymphoma universally associated with human herpesvirus-8 (HHV-8) infection.[2] Conversely, primary HHV-8 negative effusion-based lymphoma (HHV-8 (-) EBL) is a distinct category of non-Hodgkin lymphoma (NHL) characterized by the absence of HHV-8 infection and has only been reported in various case reports, case series, and literature reviews.[3–23] HHV-8 (-) EBL typically manifests as a primary serous effusion in a body cavity without a distinct tumor mass and is often discovered in immunocompetent hosts who are HIV negative.[24] HHV-8 (-) EBL has been reported in

Correspondence: Han W Tun
Mayo Clinic Florida, Division of Hematology and Oncology, 4500 San Pablo Road S, Jacksonville, FL, 32224, USA
Tel +1904-953-2000
Email tun.han@mayo.edu

association with hepatitis C virus (HCV) infection, Epstein-Barr virus (EBV) infection, fluid overload, liver cirrhosis, renal dysfunction, cardiac arrhythmias, myocardial infarction, and heart failure.[5,7–9,16,25] Herein, we describe a rare case of NGCB-DLBCL presenting as primary HHV-8 (-) EBL in an immunocompetent 81-year-old male with a complex cardiac history who has been effectively cured following treatment with thoracentesis and anthracycline-based chemoimmunotherapy.

Case Presentation

An 81-year-old male with coronary artery disease, valvular heart disease status post mitral valve replacement and tricuspid annuloplasty ring implant, coronary artery bypass graft, persistent atrial fibrillation, hypertension, and congestive heart failure with preserved ejection fraction (EF 60%) presented to his cardiologist's office complaining of increased fatigue, cough, orthopnea, and exertional dyspnea for 3–4 weeks. He denied any significant weight loss, drenching sweats, increased lymph node size, fevers, or chills. Eight months prior to current presentation during hospitalization for a heart failure exacerbation, the patient was noted to have small bilateral pleural effusions (left greater than right) and underwent left-sided thoracentesis at that time, which revealed no evidence of infection or malignancy. Only one month following that time, the patient underwent mitral valve replacement surgery and during the procedure, the patient underwent bilateral drainage for re-accumulating pleural effusions. Only one month prior to current presentation, the patient was again noted to have

another right-sided pleural effusion accredited to heart failure, and the decision was made to up-titrate the patient's diuretic regimen instead of undergoing thoracentesis.

Upon current presentation, chest x-ray reconfirmed a large right-sided pleural effusion, and the patient subsequently underwent repeat diagnostic and therapeutic thoracentesis with serous fluid removed. Cytology was positive for large lymphoma cells (Figure 1A and B). By immunostaining, lymphoma cells were positive for CD20, PAX-5, BCL-2, BCL-6, and MUM-1, and were negative for CD138, CD10, and MYC. The Ki-67 proliferation index was high at >90% (Figure 1C–H). Fluorescence in situ hybridization (FISH) testing on lymphoma cells revealed that 55% of nuclei had three intact copies of MYC located at 8q24, indicative of trisomy 8 or an 8q duplication. Pathologic findings were consistent with NGCB-DLBCL. An extensive infectious workup, including Epstein-Barr virus (EBV) in situ hybridization testing, HHV-8 immunostaining, hepatitis C virus RNA quantitative serum testing, hepatitis B core antibody testing, HIV antibody screening, and QuantiFERON Tb-gold analysis were all negative. Computed tomography (CT) scan of the chest, abdomen, and pelvis showed no evidence of pleural-based abnormalities or lymphadenopathy. Full-body positron emission tomography (PET) scan further demonstrated no evidence for any fluorodeoxyglucose (FDG) avid process (Figure 2). Bone marrow aspirate and biopsy was negative for lymphoma. The diagnosis of primary effusion-based lymphoma (PEBL) was made.

Due to the patient's age and with concern towards his ability to tolerate standard anthracycline-based
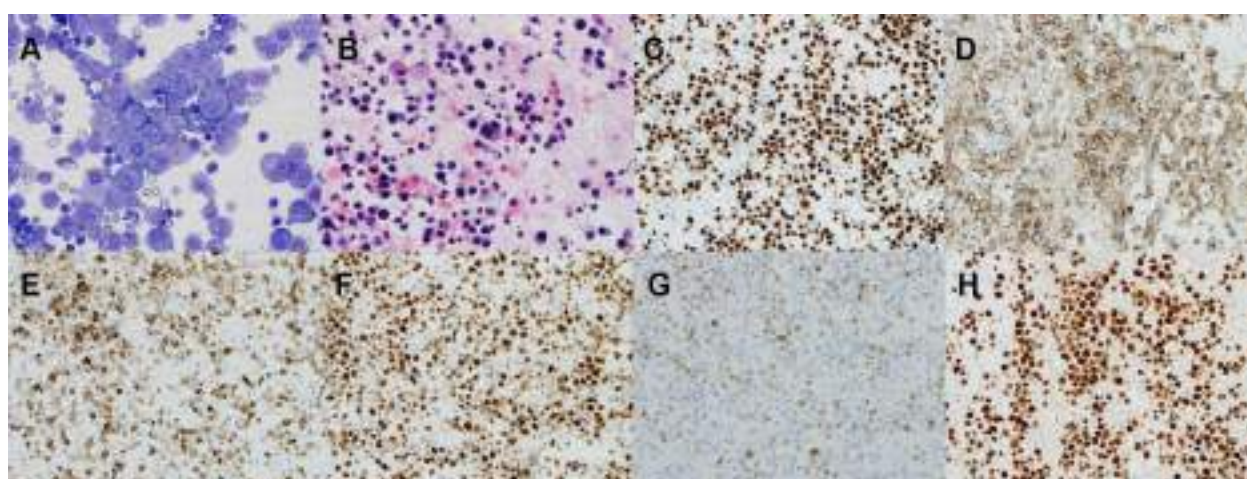

Figure 1 The pleural fluid contained numerous large atypical lymphocytes on the cytospin slide ((**A**) Giemsa and Wright stain, × 50) and cell block ((**B**) Hematoxylin and eosin stain, × 40). Immunohistochemistry studies showed the lymphocytes were positive for PAX 5, CD20, BCL2, BCL6, MUM1 (weak) with a high proliferative rate by Ki-67 90% ((**C**–**H**) × 20).

Figure 2 PET scan demonstrated no abnormal FDG processes throughout the body. Large right-sided pleural effusion was present, but without any hypermetabolic activity (arrows).

chemoimmunotherapy with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone), the patient was initially treated with one dose of rituximab (375mg/m2) as single agent therapy followed by rituximab and cyclophosphamide (750mg/m2) for two cycles, which were well tolerated. He was then transitioned to R-CHOP with a 30% reduction in doxorubicin dosing due to concerns for cardiac toxicity. Before the second cycle of R-CHOP, he was noted to have a persistent moderate right-sided pleural effusion, and he underwent thoracentesis with negative cytology for lymphoma cells. The patient subsequently completed an additional 5 cycles of

R-CHOP (for a total of 6 cycles). CT chest, abdomen, and pelvis following treatment completion revealed no suspicious lymphadenopathy with a small stable right-sided pleural effusion. Thereafter, the patient was monitored with surveillance imaging every 4–6 months and did not require any further thoracenteses due to resolution of his previous pleural effusion. Currently, he remains in complete remission 5½ years after the time of his original diagnosis.

Discussion

High-grade B cell lymphomas primarily involving effusion fluid can be divided into primary effusion lymphoma (PEL), which is positive for HHV-8 and usually found in association with HIV infection, and primary effusion-based lymphoma (PEBL), which is negative for HHV-8. Although they share similar clinical presentations, there are several key differentiating features between these two entities. PEBL notably occurs in patients above 70 years of age while the median diagnostic age of PEL has been reported to be as low as 44 years.[5,7,9,22] Unlike PEL, which often carries a poor prognosis with an estimated median survival of 6 months,[26] PEBL is associated with better survival outcomes. The largest review series involving 64 patients with PEBL recently reported an overall survival (OS) of 84.7% at two years.[7] PEBL has also been proven to show better response rates to chemotherapy in comparison to PEL despite similar treatment profiles, with first-line therapy typically consisting of CHOP-like therapy with or without rituximab. Alexanian et al also discovered a significant difference in treatment outcomes in response to systemic chemotherapy among PEBL and PEL patients when they reported a treatment response in 82.1% and 36.9% of patients, respectively.[5] This same review also demonstrated that PEBL carried a much higher rate of CD20 expression compared to PEL as 71% of PEBL patients demonstrated CD20 positivity versus only 15% of PEL patients. In the case of PEBL, it has been further reported that increased age and CD20 positivity appear to be favorable independent prognostic factors, both of which were present in our patient.[10] For this reason, and as we and others have suggested elsewhere, CD20 targeting therapy with rituximab in combination with thoracentesis of effusion fluid seems to be an acceptable alternative treatment option in CD20 positive patients who cannot withstand CHOP-like chemotherapy due to increased age or an inadequate performance functional status.[11,14,18] Our patient was able to successfully complete R-CHOP after

initial treatment with rituximab monotherapy and rituximab-cyclophosphamide.

Other reports of PEBL have also interestingly documented complete remission following thoracentesis alone as well as spontaneous regression without any treatment whatsoever, with remissions lasting up to multiple years in certain cases.[7,9,19,21,27] This response of PEBL to drainage alone is in clear discrepancy with PEL as Alexanian et al's review notably reported that 70% of PEBL patients experienced partial or complete remission following thoracentesis alone as compared to only 18% of PEL patients.[5] Although thoracentesis alone is a promising treatment option, we propose that thoracentesis alongside R-CHOP should be used in eligible patients since the data supporting thoracentesis alone is quite limited at this time. Moreover, most of the available data has demonstrated prolonged remissions primarily following reception of CHOP-like chemotherapy with or without rituximab. Most notably, in Kaji et al's review, 86% of patients who received systemic CHOP or CHOP-like therapy demonstrated an overall response of 95%, with 73% achieving complete remission.[7] These and other reports, including the current case, suggest that CHOP or CHOP-like chemotherapy with or without rituximab should be the first-line treatment for eligible patients with PEBL. It appears that therapeutic agents in R-CHOP can penetrate and achieve therapeutic concentrations in the effusion fluid, thereby killing lymphoma cells as well as modulating effusion fluid milieu. Nonetheless, the interesting phenomenon of spontaneous remission following thoracentesis alone, in combination with PEBL's favorable prognosis when compared to PEL, seems to suggest a unique mechanism of action in the lymphomagenesis of this entity that is clearly distinct from PEL. These findings suggest that PEBL is more biologically dependent on effusion fluid milieu when compared to PEL which is driven by HHV-8. Thoracentesis may be therapeutic in the case of PEBL due to the clearance of lymphoma cells as well as removal of the effusion fluid which seems to promote lymphomagenesis.

Although the exact etiology of PEBL remains undetermined, multiple conditions have been found in association with this distinct entity, including fluid overload states, HCV infection, EBV infection, cardiac arrhythmias, myocardial infarction, heart failure, renal dysfunction, liver cirrhosis, and hypothyroidism.[5,7–9,16,25] In two of the largest reviews analyzing PEBL patients, approximately half of cases had fluid overload preceding a lymphoma diagnosis.[5,7] This is also the case in our patient. It is important that clinicians include primary lymphomatous effusions in their differential diagnosis when patients present with recurrent pleural effusions as PEBL can be easily misdiagnosed as fluid overload. It is possible that conditions predisposing to fluid overload lead to effusions with milieu conducive to lymphomagenesis. Localized inflammation in a confined environment of effusion may play a role in the lymphomagenesis of PEBL, analogous to pyothorax-associated lymphoma (PAL) which develops in the setting of chronic uncontrolled pyothorax. Unlike PAL, PEBL typically lacks evidence of inflammation, such as serosal membrane thickening, and the effusion fluid is typically serous, not exudative nor purulent. Moreover, PAL usually occurs in those with a history of pulmonary tuberculosis and is strongly associated with EBV infection and positivity for EBV latent genes (LMP-1, EBNA-1, EBNA-2). Our patient demonstrated no evidence of serosal membrane thickening and his pleural fluid was serous, not purulent. He further lacked any history of tuberculosis and tested negative on QuantiFERON Tb-gold analysis and EBV in situ hybridization. Also included in our patient's differential diagnosis was extranodal Burkitt lymphoma, but the absence of MYC rearrangement on FISH analysis and the presence of large cell morphology on histological analysis was consistent with NGCB-DLBCL. Cytogenetically, trisomy 8 has been reported in certain cases of PEBL.[12,16,17,23,28,29] Our patient likely possesses trisomy 8 as FISH testing revealed three copies of MYC with negative staining for MYC on immunostaining.

Conclusion

In conclusion, NGCB-DLBCL can present as PEBL in the setting of cardiac conditions predisposing to fluid overload and is potentially curable with the standard anthracycline-based chemoimmunotherapeutic approach. Therapeutic agents in the R-CHOP regimen appear to have excellent penetration into effusion fluid with significant therapeutic impact. Effusion fluid removal by thoracentesis likely has therapeutic impact via eradication of lymphoma cells as well as removal of fluid milieu which promotes lymphomagenesis. We suggest that both thoracentesis and anthracycline-based chemoimmunotherapy should be employed when treating eligible patients with PEBL.

Ethics and Consent

Written informed consent was obtained from the patient for the publication of this manuscript and any accompanying images. Institutional approval was not required for publication.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Disclosure

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

References

1. Møller MB, Pedersen NT, Christensen BE. Diffuse large B-cell lymphoma: clinical implications of extranodal versus nodal presentation – a population-based study of 1575 cases. *Br J Haematol*. 2004;124(2):151–159.
2. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390. doi:10.1182/blood-2016-01-643569
3. Carbone A, Gloghini A. PEL and HHV8-unrelated effusion lymphomas: classification and diagnosis. *Cancer Cytopathol*. 2008;114 (4):225–227. doi:10.1002/cncr.23597
4. Adiguzel C, Bozkurt SU, Kaygusuz I, Uzay A, Tecimer T, Bayik M. Human herpes virus 8-unrelated primary effusion lymphoma-like lymphoma: report of a rare case and review of the literature. *Apmis*. 2009;117(3):222–229. doi:10.1111/j.1600-0463.2008.00005.x
5. Alexanian S, Said J, Lones M, Pullarkat ST. KSHV/HHV8-negative effusion-based lymphoma, a distinct entity associated with fluid overload states. *Am J Surg Pathol*. 2013;37(2):241. doi:10.1097/PAS.0b013e318267fabc
6. Chen BJ, Chen DYT, Kuo CC, Chuang SS. EBV-associated but HHV8-unrelated double-hit effusion-based lymphoma. *Diagn Cytopathol*. 2017;45(3):257–261. doi:10.1002/dc.23638
7. Kaji D, Ota Y, Sato Y, et al. Primary human herpesvirus 8–negative effusion-based lymphoma: a large B-cell lymphoma with favorable prognosis. *Blood Adv*. 2020;4(18):4442–4450. doi:10.1182/bloodadvances.2020002293
8. Kim Y, Huh J, Yoon S. Primary effusion lymphoma and human herpesvirus 8-negative effusion-based lymphoma: analysis of 17 cases in Korea. *Korean J Pathol*. 2013;47(Suppl 1):S100.
9. Kobayashi Y, Kamitsuji Y, Kuroda J, et al. Comparison of human herpes virus 8 related primary effusion lymphoma with human herpes virus 8 unrelated primary effusion lymphoma-like lymphoma on the basis of HIV: report of 2 cases and review of 212 cases in the literature. *Acta Haematol*. 2007;117(3):132–144. doi:10.1159/000097460
10. Kubota T, Sasaki Y, Shiozawa E, Takimoto M, Hishima T, Chong J-M. Age and CD20 expression are significant prognostic factors in human herpes virus-8-negative effusion-based lymphoma. *Am J Surg Pathol*. 2018;42(12):1607–1616. doi:10.1097/PAS.0000000000001168
11. Matsumoto Y, Nomura K, Ueda K, et al. Human herpesvirus 8-negative malignant effusion lymphoma: a distinct clinical entity and successful treatment with rituximab. *Leuk Lymphoma*. 2005;46 (3):415–419. doi:10.1080/10428190400018364
12. Saini N, Hochberg EP, Linden EA, Jha S, Grohs HK, Sohani AR. HHV8-negative primary effusion lymphoma of B-cell lineage: two cases and a comprehensive review of the literature. *Case Rep Oncol Med*. 2013;2013:292301.
13. Takahashi T, Hangaishi A, Yamamoto G, Ichikawa M, Imai Y, Kurokawa M. HIV-negative, HHV-8-unrelated primary effusion lymphoma-like lymphoma: report of two cases. *Am J Hematol*. 2010;85(1):85–87.

14. Yamaoka O, Matsui T, Nishiyama K, et al. Indolent primary effusion lymphoma-like lymphoma in the pericardium: a case report and review of the literature. *J Cardiol Cases*. 2019;19(5):148–152. doi:10.1016/j.jccase.2018.12.013
15. Ashihara E, Shimazaki C, Hirai H, et al. Human herpes virus 8-negative primary effusion lymphoma in a patient with a ventriculoperitoneal shunt tube. *Int J Hematol*. 2001;74 (3):327–332. doi:10.1007/BF02982069
16. Ichinohasama R, Miura I, Kobayashi N, et al. Herpes virus type 8-negative primary effusion lymphoma associated with PAX-5 gene rearrangement and hepatitis C virus: a case report and review of the literature. *Am J Surg Pathol*. 1998;22(12):1528–1537. doi:10.1097/00000478-199812000-00010
17. Kashiwagi T, Minagawa K, Kawano H, et al. HIV-negative, HHV-8-unrelated primary effusion lymphoma-like lymphoma with genotypic infidelity and c-MYC expression. *Ann Hematol*. 2014;93 (9):1609–1610. doi:10.1007/s00277-013-1999-3
18. Kuhlman JJ, Alhaj Moustafa M, Tun AJ, Menke DM, Tun HW, Jiang L. Primary age-related EBV-associated effusion-based lymphoma successfully treated with rituximab and thoracentesis. *Clin Case Rep*. 2021;9(5):e03971. doi:10.1002/ccr3.3971
19. Nakatsuka S-I, Kimura H, Nagano T, et al. Self-limited effusion large B-cell lymphoma: two cases of effusion lymphoma maintaining remission after drainage alone. *Acta Haematol*. 2013;130 (3):217–221. doi:10.1159/000350482
20. Rodriguez J, Romaguera JE, Katz RL, Said J, Cabanillas F. Primary effusion lymphoma in an HIV-negative patient with no serologic evidence of kaposi's sarcoma virus. *Leuk Lymphoma*. 2001;41(1–2):185–189. doi:10.3109/10428190109057969
21. Terasaki Y, Yamamoto H, Kiyokawa H, et al. Disappearance of malignant cells by effusion drainage alone in two patients with HHV-8-unrelated HIV-negative primary effusion lymphoma-like lymphoma. *Int J Hematol*. 2011;94(3):279–284. doi:10.1007/s12185-011-0906-8
22. Usmani A, Walts AE, Patel S, Alkan S, Kitahara S. HHV8-negative effusion based lymphoma: a series of 17 cases at a single institution. *J Am Soc Cytopathol*. 2015;4(1):37–43. doi:10.1016/j.jasc.2014.10.002
23. Wu W, Youm W, Rezk SA, Zhao X. Human herpesvirus 8–unrelated primary effusion lymphoma–like lymphoma: report of a rare case and review of 54 cases in the literature. *Am J Clin Pathol*. 2013;140 (2):258–273. doi:10.1309/AJCPHZ3CHO4HUWET
24. Carbone A, Gloghini A. *PEL and HHV8-unrelated Effusion Lymphomas: Classification and Diagnosis*. Wiley Online Library; 2008.
25. Kim M, Han S, Yu JR, et al. A case of primary effusion lymphoma in a patient with chronic kidney disease. *Korean J Med*. 2012;83 (3):385–389. doi:10.3904/kjm.2012.83.3.385
26. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Vol. 2. Lyon, France: International agency for research on cancer; 2008.
27. Kim M, An J, Yoon SO, et al. Human herpesvirus 8-negative effusion-based lymphoma with indolent clinical behavior in an elderly patient: a case report and literature review. *Oncol Lett*. 2020;20(6):1.
28. Mendeville M, Roemer MG, van den Hout MF, et al. Aggressive genomic features in clinically indolent primary HHV8-negative effusion-based lymphoma. *blood*. 2019;133(4):377–380. doi:10.1182/blood-2017-12-822171
29. Ohshima K, Ishiguro M, Yamasaki S, et al. Chromosomal and comparative genomic analyses of HHV-8-negative primary effusion lymphoma in five HIV-negative Japanese patients. *Leuk Lymphoma*. 2002;43(3):595–601. doi:10.1080/10428190290012100

Taylor & Francis

Effect of Gasoline Exposure on Hematological Parameters of Gas Station Workers in Mekelle City, Tigray Region, Northern Ethiopia

Gebre Teklu, Mikias Negash, Tsegay Asefaw, Feven Tesfay, Gebreslassie Gebremariam, Gebreyohannes Teklehaimanot, Mistire Wolde & Aster Tsegaye

Published online: 16 Sep 2021.

Submit your article to this journal

Article views: 833

View related articles

View Crossmark data

Citing articles: 5 View citing articles

ORIGINAL RESEARCH

Effect of Gasoline Exposure on Hematological Parameters of Gas Station Workers in Mekelle City, Tigray Region, Northern Ethiopia

Gebre Teklu[1]
Mikias Negash[2]
Tsegay Asefaw[1]
Feven Tesfay [ID][1]
Gebreslassie Gebremariam [ID][1]
Gebreyohannes Teklehaimanot [ID][1]
Mistire Wolde[2]
Aster Tsegaye [ID][2]

[1]Department of Medical Laboratory Science, College of Health Sciences, Mekelle University, Mekelle, Ethiopia; [2]Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

Background: The adverse health effects of chronic gasoline exposure may be related to impairment of the hematopoietic system with bone marrow suppression, an increased risk of blood cell morphology abnormality and developing cancer.

Objective: To assess the effect of gasoline exposure on hematological parameters among gas station workers in Mekelle City, Tigray Region, Northern Ethiopia.

Methods: This cross-sectional study was carried out on 43 subjects (exposed group) and 77 subjects (unexposed group) with matched age and sex. Socio-demographic characteristics and duration of exposure data were collected using a structured questionnaire and an observation checklist. Sysmex XP-300 was used for hematological analysis and stained peripheral blood smear was examined for any abnormality. Data were entered and analyzed using SPSS version 23.

Results: Of exposed individuals, 28/43 (65.1%) and 49/77 (63.6%) of controls were males. The average exposure time was 5.19 ± 4.38 years, with an average working hour of 11.74 ± 1.89 hours/day. The mean RBC count (10^{12}/L), HCT (%), HGB (g/dl) and platelets count (10^9/L) of the exposed group were significantly lower (4.88 ± 0.573, 43.29 ± 3.71, 15.04 ± 1.33 and 248.95 ± 58.19) compared with controls (5.35 ± 0.533, 44.95 ± 3.10, 15.59 ± 1.26 and 292.45 ± 62.17) at $p < 0.05$, respectively. The MCH (pg) (30.48 ± 2.06 vs 29.52 ± 1.66) and MCHC (g/dl) (34.83 ± 0.988 vs 34.32 ± 0.927) were significantly higher in the exposed group compared with controls ($p < 0.05$). HCT, RBC, HGB and platelet counts were significantly decreased with increased years of exposure ($p < 0.05$). The peripheral blood film examination revealed basophilic stippling and macrocytosis in 9.3% of the exposed group.

Conclusion: Long-term exposure to gasoline at gas stations affected RBC parameters and platelet count. A significant negative correlation was noted between duration of exposure and HGB, HCT and platelet count, warranting implementation of protective measures at gas stations.

Keywords: gas station, gasoline, hematological parameters, petroleum

Introduction

Changes in blood parameters are probably the early detectable variations under stress conditions like gasoline exposure and used for assessing the health condition of exposed individuals.[1,2]

Exposure to several chemicals is implicated in the derangement of hematological profile with characteristic pancytopenia, mainly aplastic anemia and an increased risk of developing cancer (acute myeloblastic leukemia). Morphological effect on red blood cells (RBCs) like microcytosis and inclusion also occurs.[3–5] Gas

Correspondence: Gebre Teklu
Email gebreteklu30@gmail.com

Aster Tsegaye
Email tsegayeaster@yahoo.com

839

station workers are exposed to gasoline by inhalation during refilling or through contaminated food at service stations.[6] Gasoline is a very volatile liquid, with several organic and inorganic constituents.[6] Some of its constituents are known to be highly toxic or carcinogenic to humans.[7,8] Many of the toxicological effects associated with the exposure to gasoline can be attributed to specific components of gasoline, such as benzene, toluene, ethylene and xylene, which are also known as volatile organic compounds (VOCs).[8]

Effects of gasoline exposure are time dependent. If unprotected individuals are exposed for long periods, it may lead to permanent suppression of bone marrow functioning, accompanied by reduction in the formation of new blood cells causing aplastic anemia.[1,9,10] Such disorders are believed to be caused by toxic Benzene metabolites. Benzene is metabolized in the liver to its primary metabolite phenol by cytochrome P4502E1 (CYP2E1) through the benzene oxide intermediate. It is subsequently metabolized by CYP2E1 to hydroquinone (HQ).[11,12] HQ is transported to the bone marrow and oxidized to benzoquinones, which eventually release reactive oxygen species (ROS) damaging hematopoietic cells.[14,15] Therefore, chronic exposure to benzene is believed to be associated with many bone marrow failures and hematological malignancies like acute myeloid leukemia (AML), aplastic anemia myelodysplastic syndrome, acute lymphoblastic leukemia and chronic myeloid leukemia.[13,16]

Several authors have reported that toxicity of gasoline comes mainly from benzene metabolites.[11–15] According to existing evidence, petroleum hydrocarbons have the potential to cause deoxyribo nucleic acid (DNA) damage in gasoline-exposed individuals and exposure to petrol vapor induces genotoxic effects, confirming that the gas station workers have a high risk of cancer due to their daily occupational exposure.[17,20,21] Once gasoline is inhaled, benzene, as the main ingredient of gasoline, enters the lungs then is passed to the blood stream from which it goes to the liver, where three main phenolic metabolites of benzene are released, transient phenol and accumulated hydroquinone and catechol, in relatively high concentrations.[18,19] Benzene is a lipophilic agent, so its metabolites go directly to fatty tissues such as bone marrow where actual toxic species are generated.[15,19,22]

It has been shown that genetic polymorphisms of xenobiotic metabolizing enzymes may modulate the susceptibility of individuals to toxic compounds like the glutathione S-transferase (GST) superfamily, which plays an important role in detoxification of various toxicants.[23] It is reported that these enzymes are involved in detoxification of several toxins, including some of the compounds present in gasoline.[24] Evidence is provided also for wide toxic effects of benzene metabolites with prolonged exposure including: pancytopenia and leucopenia[24–26] and other blood disorders such as leukemia.[26] The gasoline component has a known carcinogen primarily affecting the hematopoietic system. The effects of systemic gasoline exposure can cause acute and chronic clinical disorders of the cardiovascular, respiratory, neurological, gastrointestinal, liver, renal and dermatological local effects, and immunological, metabolic and allergic reactions. While several studies pointed to the risk of occupational exposure to gasoline on hematological profiles and other tests of the above disorders, there are no published studies in this regard in the country, so this study tries to address only the hematological profile of exposed attendants.

Materials and Methods
Study Design and Setting
A comparative cross-sectional study was conducted upon gas station attendants at Mekelle City during the period from January to April 2018. Mekelle is capital city of Tigray Regional State and is located in the Northern part of Ethiopia, at 783 km from the capital city of Ethiopia, Addis Ababa, with an elevation of 2254 meters (7395 feet) above sea level.

Study Population
Study Groups
There were 13 gas stations in Mekelle City and around the city. The study included 43 out of the total 49 gas station attendants, with a response rate of 87.8%. Adult males and females aged 18–60 years, working for at least six months at those gas stations and who volunteered to participate in the study were recruited.

Comparison Group
Seventy-seven age and sex matched apparently healthy non-exposed control participants from Mekelle University, Ayder Comprehensive Specialized Hospital Department of Medical Laboratory staff and graduate students were recruited in this study by using a non-probability convenient sampling method.

Participants with the following data were excluded from the study: history of any acute infection, chronic

diseases, individuals on medication affecting blood cell count and individuals with a blood disorder already.

Data Collection Process

Data was collected over a three-month period by questionnaire interviews focusing on socio-demographic data, years of exposure, working time (hours/day), knowledge of gasoline exposure, utilization of protective methods, health status, smoking habits and medication history.

Specimen Collection and Analysis

About 5 mL of blood was collected in Ethylene a Diamine Tetraacetic Acid (EDTA) test tube from participants who completed the questionnaire and who agreed to give blood. Four workers did not volunteer to participate, and an additional two were excluded due to acute infection and pregnancy cases. Complete blood count (CBC) tests, including total RBC count, total WBC count, Hb and Hct levels, total platelet, MCV, MCH, MCHC, mean platelet volume, and red blood cell distribution width (RDW), absolute and relative counts of lymphocyte and neutrophil, were analyzed using the 3-part hematological auto analyzer (Sysmex XP-300, Sysmex Corporation, Kobe, Japan) within 2 hours of blood collection. Sysmex XP-300 performs rapid and accurate analysis of a 17-parameter CBC, including a 3-part WBC differential.[36]

Data Quality Control

The quality of the collected blood and the participant information were ensured by collecting and processing using a standard operating procedure to address pre-analytical, analytical and post-analytical errors. Blood was collected using a standard operating procedure. The blood sample container was labeled with the participant's unique code to minimize errors. The quality of the collected samples was checked for hemolysis, clot, correct volume, etc. Site assessment and pre-test of data collection were done prior to data collection and the data was checked for completeness, quality and clarity of questionnaire and modified accordingly.

The reliability of the study findings, especially the analytical part, was guaranteed by implementing a quality control (QC) sample for the complete blood count and peripheral morphology through the whole process of laboratory works. The results of the complete blood count (CBC) and peripheral morphology were registered with correct values and units. Data were entered using double-entry method to trace data entry errors.

Specimens were transported and analyzed within 2 hours of collection and, if delayed, refrigerated at 4–8 °C.

Data Analysis

Data were entered and statistically analyzed using the Statistical Package for the Social Sciences (SPSS) version 23. The one-way ANOVA test was used for analysis of variance between quantitative dependent variables and qualitative variables, such as the relationship between hematological parameters by duration of work, age group, level of education and so on. The multiple comparisons were made using the post hoc test for the duration of exposure. The independent samples t-test procedure was used to compare means of quantitative variables between gas station workers' and controls' hematological parameters. Pearson correlation coefficient was used to assess measured parameters with years of exposure, working hours and age. P-values less than 5% (p <0.05) at 95% confidence intervals was taken as statistically significant.

Ethical Considerations

The study was conducted after approval by the research and ethics committee of the Department of Medical Laboratory Sciences, College of Health Science of Addis Ababa University. The research committee is an authorized professional body for giving permission to researchers to conduct their studies with ethical concern in the area. An official letter of request was sent to Mekelle University, Ayder Comprehensive Specialized Hospital to obtain approval to carry out hematological analysis in the central laboratories. Written informed consent to voluntarily participate was obtained from all study participants after explaining the purpose of the study. The confidentiality of the data was also assured. After all, this study was conducted in accordance with the Declaration of Helsinki.

Results

Socio-Demographic Characteristics of the Study Participants

In this study, a total of 120 participants, 43 exposed and 77 controls, was enrolled. There was no statistically significant age and sex difference between the exposed and the control groups (p=0.444, p=0.540), respectively (Table 1).

Table I Socio-Demographic Profile of the Study Group Using *t*-Test and χ^2 Tests at Mekelle City, Tigray Region, Northern Ethiopia, from January to March 2018 (n=120)

Confounding Factors	Gas Station Attendant (n=43)	Control Group (n=77)	Test of Significance

Gender
  Male n (%)
  Female n (%)
	28 (65.1%)
	15 (34.9%)
		49 (63.6%)
		28 (36.4%)
			χ^2 = 0.008, p=0.540

Age in years n (%)
  19–26
  27–34
  35–44
  >45
  Mean±SD
	17 (39.5%)
	16 (37.2%)
	6 (14%)
	4 (9.3%)
	30.09±8.49
		29 (37.7%)
		37 (48.1%)
		9 (11.7%)
		2 (2.6%)
		29.06±6.07
			
			t=0.768, p=0.444

Marital status
  Single n (%)
  Married n (%)
	22 (51.2%)
	21 (48.8%)		

Abbreviation: SD, standard deviation.

Years of Exposure, Working Hours and Awareness Related to Gasoline Exposure

The average exposure time was 5.19±4.38 years (minimum 8 months and maximum 16 years), with an average daily exposure of 11.74±1.89 hours. The working hour was more than 12 hours/day among 58.1% of the participants. The highest number of workers, 17 (39.5%), had worked in the gas station for less than two years, whereas 12 (27.9%) and 14 (32.6%) of them worked 3–7 and for more than 8 years, respectively. All the study population had no history and habit of smoking cigarettes.

Hematological Profile of Gasoline Exposed and Control Groups

The means and standard deviations of hematological indices of exposed and unexposed groups are presented in Table 2. These results show that the hematological markers in both study groups were within the normal

Table 2 Complete Blood Count Picture of the Study Group Using Independent Samples *t*-Test at Mekelle City, Tigray Region, Northern Ethiopia, from January to March 2018 (n=120)

Parameters	Exposed Group(n=43) (mean±SD)	Control Group(n=77) (mean±SD)	95% Confidence Interval of the Difference		
			Lower	Upper	p-value
RBC (×10^12/L)	4.88±0.573	5.35±0.533	−0.674	−0.261	p<0.001
HGB (g/d)	15.04±1.33	15.59±1.25	−1.033	−0.063	p=0.027
HCT (%)	43.29±3.71	44.95±3.10	−2.919	−0.403	p<0.010
MCV (fl)	87.37±4.98	85.87±4.36	0.238	3.226	0.090
MCH (pg)	30.48±2.06	29.52±1.66	0.313	1.686	0.006
MCHC (g/dl)	34.83±0.99	34.32±0.92	0.153	0.873	0.006
RDW (%)	12.87±0.59	12.95±0.84	−0.419	0.253	0.626
WBC (×10^9/L)	6.32±1.73	6.44±1.38	−0.692	0.452	0.679
Lymphocyte (%)	36.02±11.20	34.71±10.04	−2.644	5.254	0.514
Neutrophil (%)	52.14±12.05	54.72±10.44	−6.739	1.589	0.223
Lymphocyte (×10^9/L)	2.16±0.59	2.16±0.58	−0.224	0.219	0.980
Neutrophil (×10^9/L)	3.37±1.46	3.60±1.31	−0.745	0.289	0.384
Platelet (×10^9/L)	248.95±58.19	292.45±62.17	−66.418	−20.583	p<0.001
MPV (fl)	10.74±1.21	10.96±1.78	−3.492	3.054	0.895

Abbreviations: HGB, hemoglobin; HCT, hematocrit; RBC, red blood cell; WBC, white blood cell; RDW, red cell distribution width; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; g/dl, gram per deciliter; fl, femtoliter; pg, picogram.

ranges. The absolute mean number of RBC (10^{12}/L), percentages of hematocrit (%) (p<0.0001), level of hemoglobin (g/dl) (p=0.027) and absolute mean number of platelets (10^9/l) (p<0.0001) were significantly lower among the exposed subjects compared with the control group. The mean cell hemoglobin (pg) and mean cell hemoglobin concentration (g/dl) were significantly higher in the exposed participants compared with the control group, at p=0.006.

Effects of Exposure to Gasoline on Hematological Parameters

The hematological parameters like HCT levels, RBC count, HGB concentration, platelet count, MPV value, lymphocyte percent and neutrophil percent decreased as years of exposure increased. Most participants exposed for longer than eight years had significantly lower values of hemoglobin (g/dl), PCV (%), RBC (10^{12}/L) and platelet (10^9/L) compared to those exposed for ≤2 years as well as 3–7 years. The average values of RBC, HGB, HCT and platelet were similar between individuals exposed for <2 and 3–7 years (Table 3).

Correlation of Hematological Indices with Years of Exposure, Working Hours and Age of the Exposed Group at Mekelle City

As illustrated in Table 4, there was a negative correlation between RBC, HGB, HCT and platelet with years of exposure (r = −0.619, p<0.001, r = −0.581, p<0.001, r = −0.524, p<0.001, r = −0.499, p = 0.001), respectively. Whereas, absolute number of neutrophil was positively correlated with years of exposure (r = 0.337, p = 0.027). There was also a negative correlation of RBC (r = −0.418, p = 0.005), HGB (r = −0.368, p = 0.015) and platelet (r = −0.330, p = 0.030) with age.

Peripheral Blood Morphology Examination Results

Of the examined peripheral blood films from the exposed group, 37 (79.1%) of the results were normal, 4 (9.3%) had macrocytosis with increasing corresponding MCV value, basophilic stippling inclusions and one participant

had microcytic red cells with reduced MCV value (77.1 fl).

Discussion

This study aimed to assess the effect of gasoline and gasoline products exposure on hematological parameters among gas station workers as compared with controls in

Table 3 Comparison of the Hematological Indices with Years of Exposure in Exposed Group Using Post Hoc Analysis at Mekelle City, Tigray Region, Northern Ethiopia, from January to March 2018 (n=43)

Years of Exposure	Parameters	p-value
	RBC(10^{12}/l) (Mean±SD)	
≤2 year vs 3–7 year	5.17±0.40 vs 5.07±0.58	0.587
≤2 year vs ≥8 year	5.17±0.40 vs 4.37±0.40	<0.0001
3–7 year vs ≥8year	5.07±0.58 vs 4.37±0.40	<0.0001
	HGB (g/dl)	
≤2 year vs 3–7 year	15.72±1.08 vs 15.25±1.55	0.271
≤2 year vs ≥8 year	15.72±1.08 vs 14.03±0.70	<0.0001
3–7 year vs ≥8year	15.25±1.55 vs 14.03±0.70	0.010
	HCT (%)	
≤2 year vs 3–7 year	45.15±2.94 vs 43.55±4.86	0.202
≤2 year vs ≥8 year	45.15±2.94 vs 40.80±1.59	<0.001
3–7 year vs ≥8year	43.55±4.86 vs 40.80±1.59	0.041
	WBC (10^9/L)	
≤2 year vs 3–7 year	5.90±1.28 vs 6.11±1.65	0.744
≤2 year vs ≥8 year	5.90±1.28 vs 7.01±2.13	0.078
3–7 year vs ≥8year	6.11±1.65 vs 7.01±2.13	0.187
	LYM (%)	
≤2 year vs 3–7 year	37.47±9.84 vs 41.07±12.32	0.366
≤2 year vs ≥8 year	37.47±9.84 vs 29.91±9.54	0.053
3–7 year vs ≥8year	41.07±12.32 vs 29.91±9.54	0.010
	NUE (%)	
≤2 year vs 3–7 year	50.80±10.47 vs 46.49±13.7	0.318
≤2 year vs ≥8 year	50.80±10.47 vs 58.63±9.83	0.062
3–7 year vs ≥8year	46.49±13.7 vs 58.63±9.83	0.009
	Platelet (10^9/l)	
≤2 year vs 3–7 year	267.05±54.74 vs 280.16±39.07	0.472
≤2 year vs ≥8 year	267.05±54.74 vs 200.21±45.54	<0.0001
3–7 year vs ≥8year	280.16±39.07 vs 200.21±45.54	<0.0001

Abbreviations: HGB, hemoglobin; HCT, hematocrit; RBC, red blood cell; WBC, white blood cell; LYM, lymphocyte; NUETRO, neutrophil; CI, confidence interval; g/dl, gram per deciliter.

Table 4 Correlation of Hematological Indices with Years of Exposure, Working Hours, and Age for the Exposed Group Using ANOVA Statistical Analysis at Mekelle City Tigray Region, Northern Ethiopia, from January to March 2018 (n=43)

Parameters	Years of Exposure		Working Hours		Age of Exposed	
	r	p-value	r	p-value	r	p-value
RBC (10^9/l)	−0.619	**<0.001**	0.292	0.057	−0.418	**0.005**
HGB (g/dl)	−0.581	**<0.001**	0.266	0.085	−0.368	**0.015**
HCT (%)	−0.524	**0.000**	0.332	0.129	−0.270	0.080
MCV (fl)	0.152	0.330	−0.195	0.209	0.172	0.269
MCH (pg)	0.044	0.779	−0.273	0.076	0.050	0.750
WBC (10^9/l)	0.268	0.082	0.017	0.912	0.166	0.289
Lymphocyte (10^9/l)	−0.041	0.794	−0.054	0.730	−0.022	0.890
Neutrophil (10^9/l)	0.337	**0.027**	0.000	1.000	0.154	0.324
Platelet (10^9/l)	−0.499	**0.001**	0.160	0.307	−0.330	**0.030**
MPV (fl)	0.222	0.153	0.242	0.117	0.113	0.470

Note: Bold text is to indicate test is significant at p<0.05.
Abbreviations: HGB, hemoglobin; HCT, hematocrit; RBC, red blood cell; MCV, mean cell volume; MCH, mean cell hemoglobin; WBC, white blood cell; MPV, mean platelet volume; CI, confidence interval; g/dl, gram per deciliter; fl, femtoliter.

Mekelle City, Tigray Region. It also tried to identify risk factors associated with gasoline product exposure in gas station workers and the relationship between duration of gasoline exposure and hematological parameters. The findings are pointing toward the fact that gasoline and its content have adverse effects on the hematological parameters with a longer period of exposure.

The observed statistically significant decrease in the measured parameters of exposed participants, such as red blood cell (RBC), hemoglobin (HGB) concentration, hematocrit (HCT) and platelet (PLT) count, compared to controls may be due to several toxic effects arising from the gasoline and its products. Benzene, one of the main constituents of gasoline, is a well-known systemic toxicant in humans and a cause of aplastic anemia. It is hematotoxic and depresses the bone marrow, leading to pancytopenia (a general depression of erythrocytes (red blood cells), leucocytes (white blood cells) and thrombocytes (platelets).[9] These studies demonstrate that benzene is indeed a hematotoxicant.

The results of the present study showed that the mean hemoglobin concentration, mean RBCs count, mean hematocrit value, platelet count of gas station workers were significantly lower than those of the comparison group. Decreases in hemoglobin content and RBC count could be attributed to shortened life span of RBC as well as impairment of heme synthesis by the metabolic end product of free radicals of benzene and other aliphatic hydrocarbon constituents of gasoline. These free radicals can alter the erythrocyte membrane and heme protein synthesis in bone marrow.[10,11,14] This result is similar with the study

conducted in hematological assessment of gasoline exposure among gas station workers by Schnatter et al,[28] Nair et al,[29,30] and Neghab et al.[27] A decrease in RBC count can lead to a decrease in PCV. But the hematocrit, RBC count, hemoglobin and platelet count were not consistent with those reports which described the hematological changes of individuals occupationally exposed to gasoline as significantly high, such as Saadat et al[31] and Firouzkouhi et al.[32] Reduction in the value of RBC, HCT and HGB content as reported in this study would eventually lead to anemia, a condition which agrees with the report of Uko et al.[33]

In this study, MCV, mean MCH and MCHC value were significantly higher in exposed than comparison group. This finding is in agreement with another study by Firouzkouhi et al[32] and by Nair et al,[29] as they observed statistically significant increases in MCH and MCHC values. Even though the MCV value is not significantly higher in the exposed group, it shows a similar increase to the result reported by Schnatter et al.[28] The increases in MCV, MCH and MCHC in this study can be due to macrocytosis induced by benzene, because benzene is an ingredient of gasoline. It has been established that toxic constituents of petroleum such as benzene and lead are activated in the bone marrow, where the substances exert cytotoxic effects that could be mediated through destruction in DNA function. A defect in DNA synthesis that interferes with cellular proliferation and maturation can lead to large` erythrocytes.[20]

This study also indicates that total RBC count, hemoglobin concentration and HCT value decreased in gas

station workers as the duration of exposure increased from less than 2 years to more than 8 years. These findings were different from the studies conducted by Uzma et al,[34] which showed that, during the early period of exposure (1–5 years and 5–10 years), the average Hb, HCT and RBC counts were unchanged, but as the years of exposure increased to more than 10 years, there was a statistically significant increase in the concentration of Hb, RBC count and HCT value. Platelet counts of the present study significantly decreased as year of exposure increased from two years to eight years and is consistent with a previous study by Uzma et al.[34] Nevertheless, the decrease in total RBC count, hemoglobin concentration and HCT value in workers with longer periods of exposure as the duration of exposure increases from less than 2 year to more than 8 years were consistent to study conducted by Uko et al.[33] In the present study, the reported WBC count was unchanged as year of exposure increased but it was decreased in the study done by Uko et al.[33]

The peripheral blood smear of the study participants showed basophilic stippling and macrocytosis (9.3%). In line with this finding, Firouzkouhi et al[32] and Uko et al[33] reported basophilic stippling and macrocytosis, respectively, from the gasoline exposed attendants. However, this finding is not consistent with Elderdery et al,[35] which reported a microcytic picture in 50% of their participants and a quarter (26%) showed a normocytic picture. This inconsistency might be because of population difference, performance and the gasoline constituent difference in the study.

Finally, this study has some drawbacks. There were no records for baseline and periodic medical examination of workers to identify changes that could be attributed to gasoline exposure. Being a small-scale study with a few gas station attendants and participants limits the generalization of results to the total populations of gas station attendants in other places. However, in the absence of any similar data, this study has also its own strength in that it assesses the effect of occupational exposure to gasoline for the first time, which could attract attention for appropriate intervention.

Conclusion and Recommendation

The present study has shown that occupational exposure to gasoline and its constituent products has a significant effect on some hematological parameters. Moreover, long-term exposure to gasoline-specific components of gasoline, such as benzene, toluene, ethylene, xylene and lead, might have some effects on the human hematopoietic system leading to suppressed bone marrow or might cause ineffective erythropoiesis and thrombopoiesis. It has been observed that chronic ingestion of gasoline might result in a significant reduction in RBC count, Hb concentration and total platelet count. The toxicity of gasoline components usually causes RBC inclusion (basophilic stippling, Heinz's bodies, and pappenheimer bodies) and oxidation of hemoglobin, which decrease the life of RBC and cause hemoglobin synthesis defects, hence decreasing the parameters.

Gas station workers with chronic exposure to gasoline products should have periodic medical examinations including the evaluation of their hematological profile and measurement of blood benzene and blood lead levels. In this regard, gas station owners should provide basic personal protective equipment like face masks and the government should make blood benzene and blood lead level analyzer auto machines available. Further research is recommended to include other gas station workers in the other parts of the country with large sample sizes of these workers occupationally exposed to gasoline.

Abbreviations

AML; acute myeloid leukemia, CBC; complete blood count, CI; confidence interval, DNA; deoxyribo nucleic acid, fl; femtoliter, GST; glutathione S-transferase, HGB; g/dl; gram per deciliter, hemoglobin, HCT; hematocrit, HQ; hydroquinone, K_2EDTA; di-potassium ethylene diamine tetra acetic acid, km, kilometr, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentration, MCV; mean cell volume, MPV; mean platelet volume, PCV; packed cell volume, PPM; parts per million, pg; picogram, QC; quality controls, RBC; red blood cell, RDW; red blood cell distribution width, ROS; reactive oxygen species, SD; standard deviation, SPSS; Statistical Package for Social Sciences, TLC; total leukocyte count, VOCs; volatile organic compounds, WBC; white blood cells.

Data Sharing Statement

Not all data will be made available to protect the participants' confidentiality. But the row data can be obtained from the corresponding author upon reasonable request.

Acknowledgments

Author Contributions

All authors have contributed from inception to shaping of the research design, commenting on the paper and its final approval, acquisition of data, analysis and interpretation of data, drafting the manuscript, revising the manuscript critically for important intellectual content, read and made comments on the manuscript for submission, gave final approval of the manuscript version to be published and agreed to be accountable for every aspect of the work.

Disclosure

The authors declared no conflicts of interest for this work nor regarding the publication of this paper. This manuscript's thesis was uploaded to Addis Ababa University electronic thesis library based on an author's thesis requirement for academic purposes and is available online at: http://etd.aau.edu.et/bitstream/handle/123456789/13806/Gebre%20Teklu.pdf?sequence=1&isAllowed=y.

References

1. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. *Cell Stem Cell*. 2012;10(2):120–136. doi:10.1016/j.stem.2012.01.006
2. Uzma N, Khaja MS, Salar BM, Aziz N, David MA, Reddy VD. Impact of organic solvents and environmental pollutants on the physiological function in petrol filling workers. *Int J Environ Res Public Health*. 2008;5(3):139–146.
3. Khalade A, Jaakkola MS, Pukkala E, Jaakkola JJ. Exposure to benzene at work and the risk of leukemia: a systematic review and meta-analysis. *Environ Health*. 2010;9(1):31. doi:10.1186/1476-069X-9-31
4. Snyder R. Overview of the toxicology of benzene. *J Toxicol Environ Health A*. 2000;61(5–6):339–346. doi:10.1080/00984100050166334
5. Lee SY, Park HJ, Best-Popescu C, Jang S, Park YK. The effects of ethanol on the morphological and biochemical properties of individual human red blood cells. *PLoS One*. 2015;10(12):e0145327.
6. Jasper MN, Martin SA, Oshiro WM, Ford J, Bushnell PJ, El-Masri H. Application of biologically based lumping to investigate the toxicokinetic interactions of a complex gasoline mixture. *Environ Sci Technol*. 2016;50(6):3231–3238. doi:10.1021/acs.est.5b05648
7. Folabi T, Phan T. Evaluation of Volatile Organic Compounds and Polyaromatic Hydrocarbons in Barker Reservoir in Houston, Texas after the 2017 Hurricane Harvey. *Am J Anal Chem*. 2020;11(11):376–388.

8. Al Madhoun WA, Ramli NA, Yahaya AS, Yusof NF, Ghazali NA, Sansuddin NU. A framework for monitoring and modelling of BTEX in various development statuses in Penang Malaysia. *Proceeding of the international conference on environment*. 2008; (ICENV08), Penang, Malaysia.
9. Okonkwo CO, Ehileboh AD, Nwobodo E, Dike CC. The effects of acute gasoline vapour inhalation on some hematological indices of albino Wistar rats. *J Acute Dis*. 2016;5(2):123–125. doi:10.1016/j.joad.2015.11.005
10. Cronkite EP, Drew RT, Inoue T, Hirabayashi Y, Bullis JE. Hematotoxicity and carcinogenicity of inhaled benzene. *Environ Health Perspect*. 1989;82:97–108. doi:10.1289/ehp.898297
11. World Health Organization. Benzene. In: *Air Quality Guidelines for Europe*. 3rd ed. Copenhagen: WHO Regional Office for Europe; 2000:pp 62–66.
12. Kim SY, Choi JK, Cho YH, Chung EJ, Paek D, Chung HW. Chromosomal aberrations in workers exposed to low levels of benzene: association with genetic polymorphisms. *Pharmacogenet Genom*. 2004;14(7):453–463. doi:10.1097/01.fpc.0000114751.08559.7b
13. Rinsky RA, Smith AB, Hornung R, et al. Benzene and leukemia. *New Engl j Med*. 1987;316(17):1044–1050. doi:10.1056/NEJM198704233161702
14. Ross D. The role of metabolism and specific metabolites in benzene-induced toxicity: evidence and issues. *J Toxicol Environ Health Part A*. 2000;61(5–6):357–372. doi:10.1080/00984100050166361
15. Smith MT. The mechanism of benzene-induced leukemia: a hypothesis and speculations on the causes of leukemia. *Environ Health Perspect*. 1996;104(supply 6):1219–1225. doi:10.1289/ehp.961041219
16. Weng MW, Lee HW, Park SH, et al. Aldehydes are the predominant forces inducing DNA damage and inhibiting DNA repair in tobacco smoke carcinogenesis. *Proc Nat Acad Sci USA*. 2018;115(27):E6152–E6161. doi:10.1073/pnas.1804869115
17. Soares M, Woiciechowski AL, Kozliak EI, Paca J, Soccol CR. Biofiltration of gasoline and ethanol-amended gasoline vapors. *J Environ Sci Health, Part A*. 2012;47(7):1008–1016. doi:10.1080/10934529.2012.667316
18. Carbonari D, Chiarella P, Mansi A, Pigini D, Iavicoli S, Tranfo G. Biomarkers of susceptibility following benzene exposure: influence of genetic polymorphisms on benzene metabolism and health effects. *Biomark Med*. 2016;10(2):145–163. doi:10.2217/bmm.15.106
19. Mitri S, Fonseca AS, Otero UB, Tabalipa MM, Moreira JC, Sarcinelli PD. Metabolic polymorphisms and clinical findings related to benzene poisoning detected in exposed brazilian gas-station workers. *Int J Environ Res Public Health*. 2015;12(7):8434–8447. doi:10.3390/ijerph120708434
20. Khisroon M, Gul A, Khan A, Ali N, Zaidi F, Rasheed SB. Comet assay based DNA evaluation of fuel filling stations and automobile workshops workers from Khyber Pakhtunkhwa province, Pakistan. *J Occup Med Toxicol*. 2015;10(1):10–27. doi:10.1186/s12995-015-0069-2
21. Beceren A, Akdemir N, Omurtag GZ, Tatlıpınar ME, Şarda S. DNA damage in gasoline station workers caused by occupational exposure to petrol vapour in turkey. *ACTA PharmaceuticaSciencia*. 2016;54 (1):53–62. doi:10.23893/1307-2080.APS.0545
22. Knutsen JS, Kerger BD, Finley B, Paustenbach DJ. A calibrated human PBPK model for benzene inhalation with urinary bladder and bone marrow compartments. *Risk Analysis*. 2013;33 (7):1237–1251. doi:10.1111/j.1539-6924.2012.01927.x
23. Pemble S, Schroeder KR, Spencer SR, et al. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochemical Journal*. 1994;300 (1):271–276. doi:10.1042/bj3000271
24. Sun R, Zhang J, Yin L, Pu Y. Investigation into variation of endogenous metabolites in bone marrow cells and plasma in C3H/He mice exposed to benzene. *Int J Mol Sci*. 2014;15(3):4994–5010. doi:10.3390/ijms15034994

25. Hayes RB, Zhang L, Yin S, et al. Genotoxic markers among butadiene polymer workers in China. *Carcinogenesis*. 2000;21(1):55–62. doi:10.1093/carcin/21.1.55

26. Li K, Jing Y, Yang C, Liu S, Zhao Y, He X. Increased leukemia associated gene expression in benzene-exposed workers. *Sci Rep*. 2014;4:5369. doi:10.1038/srep05369

27. Neghab M, Hosseinzadeh K, Hassanzadeah J. Hematological study of petrol station workers exposed to unleaded petrol. *Toxicol Environ Chem*. 2014;96(6):951–961. doi:10.1080/02772248.2014.979828

28. Robert Schnatter A, Kerzic PJ, Zhou Y, et al. Peripheral blood effects in benzene-exposed workers. *Chem Biol Interact*. 2010;184(1–2):174–181. doi:10.1016/j.cbi.2009.12.020

29. Nair DS, Bedekar MY, Agrawal MJ. Deleterious effects of petrol fumes on erythrocytes. *IJHSR*. 2015;5(9):237–241.

30. Bedekar MY, Nair DS, Agrawal MJ. Toxic effect of petrol fumes on white blood corpuscles. *Ann Appl Bio-Sci*. 2015;2(3):57–59.

31. Saadat M, Bahaoddini A. Hematological changes due to chronic exposure to natural gasleakage in polluted areas of Masjid-i-Sulaiman (Khozestan province, Iran). *Ecotoxicol Environ Saf*. 2004;58(2):273–276. doi:10.1016/j.ecoenv.2003.07.005

32. Firouzkouhi M, Abdollahimohammad A, Babaiepur-Diveshali M, Firouzkouhi A, Shaikh M. Effects of gasoline on blood, kidney and liver parameters of unregulated gasoline traders. *Der Pharmacia Lettre*. 2016;8(8):58–61.

33. Uko EK, Erhabor O, Bashiru GA, et al. Some hematological indices among petrol station attendants occupationally exposed to premium motor spirit in Sokoto, North Western, Nigeria. *J Int Res Med Pharm Sci*. 2015;4(2):53–58.

34. Uzma N, Salar KM, Kumar S, Aziz N, David MA, Reddy VD. Impact of organic solvents and environmental pollutants on the physiological function in petrol filling workers. *Int J Environ Res Public Health*. 2008;5(3):139–146. doi:10.3390/ijerph5030139

35. Elderdery AY, Elnour AM, Ali NY, et al. Alterations in hematological parameters among workers of fuel stations in White Nile State, Sudan. *Int J Biomed Adv Res*. 2015;6(11):780–784.

36. Sysmex XP-300™ Automated Hematology Analyzer. Sysmex; 2015. Available from: https://www.sysmex.com/us/en/Products/Hematology/3PartDiff/Pages/XP-300-Hematology-Analyzer.aspx. Accessed September 13, 2021.

Taylor & Francis

Distribution of ABO and Rhesus Blood Group Phenotypes Among Blood Donors at Bahir Dar Blood Bank, Amhara, Northwest Ethiopia: A Retrospective Cross-Sectional Study

Biruk Legese, Mikru Shiferaw, Workineh Tamir & Tegenaw Tiruneh

Published online: 16 Sep 2021.

Submit your article to this journal

Article views: 786

View related articles

View Crossmark data

Citing articles: 6 View citing articles

ORIGINAL RESEARCH

Distribution of ABO and Rhesus Blood Group Phenotypes Among Blood Donors at Bahir Dar Blood Bank, Amhara, Northwest Ethiopia: A Retrospective Cross-Sectional Study

Biruk Legese [1],*
Mikru Shiferaw[2],*
Workineh Tamir[3]
Tegenaw Tiruneh [1]

[1]Department of Medical Laboratory Sciences, Debre Tabor University, Debre Tabor, Ethiopia; [2]Bahir Dar Blood Bank Service, Amhara Regional State Health Bureau, Bahir Dar, Ethiopia; [3]Department of Medical Laboratory Sciences, Debre Markos University, Debre Markos, Ethiopia

*These authors contributed equally to this work

Background: Among the blood group antigens identified, ABO and Rhesus are the most important in transfusion medicine. ABO blood group antigens are the most immunogenic followed by Rhesus (D antigen). These blood groups' frequency distribution varies among different regions and races of the world. This study aimed to identifying the frequency distribution of ABO blood group and rhesus factors among blood donors in Ethiopia.

Methods and Materials: A retrospective cross-sectional study was conducted from September 12/2019 to March 18/2021 at Bahir Dar blood bank service. After getting a permission letter from the blood bank, data were collected from the blood bank donor data registration system, and descriptive statistical results were presented in number (frequency) and percentage. A Chi-square test was used to show the difference in the frequency distribution of ABO and Rh blood groups among sex and blood donation site.

Results: From 40,053 blood donors, 67.7% were males and younger donors (within the age range of 18–24 years) account for 63.7%. All donations were from voluntary non-remunerated blood donors. The most common blood group was blood group O (41.5%) followed by A (29.8), B (23.2%), and AB (5.5%). Considering ABO and Rh blood group altogether blood group O positive with 37.9% was the predominant blood group followed by A positive (27.2%), B positive (21.4%), AB positive (5%), O negative (3.6%), A negative (2.6%), B negative (1.8%), and AB negative (0.4%). The majority of study participants were 91.5% Rh (D) positive.

Conclusion: This study showed that blood group O was the predominant followed by A, B, and AB and most of the blood donors' blood groups were Rh-positive (91.5%). About 68.9% of the total donations were from the first time donor.

Keywords: ABO blood group, rhesus factor, frequency distribution

Introduction

Blood donation is a crucial part of worldwide healthcare. It includes blood collection, testing, storage, and transfusion to the patient. Among tests performed on collected blood for transfusion includes blood grouping and screening for transfusion transmissible infectious diseases. The term blood group refers to the entire blood group system comprising red blood cell (RBC) antigens and a series of genes controlled the specificity of the blood group, which can be allelic or linked closely on the same chromosome. Blood type refers to a particular pattern of reaction to

Correspondence: Biruk Legese;
Mikru Shiferaw
Tel +251943592686; +251913257134
Email birukab1979@gmail.com;
mikshif123@gmail.com

849

testing antisera within a given system. About 38 blood group system genes have been recognized and all known alleles sequenced.[1,2]

Among blood group systems identified, ABO (with blood types A, B, AB, and O) and Rhesus (with Rh D-positive or Rh D-negative blood types) are the most important in transfusion medicine. ABO blood group antigens are the most immunogenic of all the blood group antigens followed by Rh (D antigen).[3] The most common cause of death from a blood transfusion is transfusion of incompatible ABO blood type due to a clerical error. These antigens are expressed on the RBC surface and determine an individual's blood group.[4] The Austrian scientist Karl Landsteiner discovered A, B, and O blood types in 1900, and Alfred Von Decastello and Adriano Sturli discovered the fourth type AB, in 1902. Karl Landsteiner and Alexander S. Wiener discovered Rh blood group in the late 1930s.[5]

ABO and Rh blood group frequency distribution varies among different regions and races of the world. Blood group A was the most frequent in Japan, while blood group O was the predominant blood group among Chinese and Americans.[6,7] A study by Liu et al in China showed that the frequency distribution of blood groups O, A, B, and AB was 35.54%, 31.90%, 24.14%, and 8.42%, respectively. The distribution of the Rh (D) negative group was found to be 0.55% among Chinese blood donors.[7,8] A systematic review study in India revealed that O blood group (34.56%) was the predominant followed by B (34.10%), A (23.16%), and AB (8.1%). Based on Rh type, Rh(D) positive and Rh(D) negative population were 94.13% and 5.87%, respectively.[9] In the United States of America, the frequency distribution of blood group O varies from 39.8% in Asian donors to 56.5% in Hispanic donors, and the proportion of Rh (D) negative varies from 1.7% in Asian donors to 17.3% in White non-Hispanic donors.[6] Blood group A (43.8%) was the most frequent and AB the least frequent, and Rh positivity rate was 85% in Turkey.[10]

In Tanzania, the most common blood type was blood group O (52%), followed by blood group A (26%), blood group B (19%) and blood group AB (3%), and 98% (n = 1773) of participants were Rh-positive.[11] Studies in Nigeria and Uganda also revealed blood group O as the most frequent blood group followed by blood group A, B, and AB was the least frequent blood group.[12,13] Studies in Ethiopia showed that blood group O was the dominant blood group followed by A, B, and AB, and the Rh-

positive blood group account for the highest percentage.[14–16]

The objective of this study was to determine the distribution of ABO and Rh blood groups among blood donors at Bahir Dar blood bank. This data is valuable to manage blood availability by blood type since this study showed the ABO and Rh blood distribution by donation site.

Methods and Materials

A retrospective cross-sectional study was conducted on 40,053 blood donor data collected from September 12/2019 to March 18/2021 at Bahir Dar blood bank service. The blood bank is located in the capital city of Amhara regional state, Bahir Dar, Ethiopia. The city is situated in northwest Ethiopia 565 kilometers far from Addis Ababa the capital city of Ethiopia. The blood bank serves more than 28 governmental and private health facilities. It has developed its own blood donor data management system which can store data, perform statistical analysis, send a different text to the blood donor like: "thank you for donating blood", "donor blood type", "reminder for next donation, and post-donation counseling".

Blood was collected from voluntary non-remunerated blood donors, through a mobile campaign and at the blood bank, transported to the blood bank by maintaining the cold chain at 2–10°C with a cold box. After arrival at the blood bank, blood was arranged by blood unit number and stored in a blood bank refrigerator (2–6°C). Forward ABO blood grouping was performed by slid method with known antisera (anti-A and anti B). Rh group was determined by test tube method by using anti-D reagents and Coombs test was performed to detect weak D antigen (Mediclone, Mediclone Biotech, India) and reverse blood grouping was performed with known 5% A and B cell suspension prepared in the laboratory. ABO and Rh blood type of the blood donors were registered into the computerized blood donor data management system. The data were collected after getting a seal of approval from Bahir Dar blood bank. The data registered on an excel sheet on the blood bank blood donor data registration system were checked for completeness and transferred into SPSS version 23 for further analysis.

Descriptive statistical analysis was performed to show the frequency distribution, in number and percentage, of ABO and Rh blood group among blood donors, and a chi-square test was employed to test the absence of ABO and Rh blood group distribution difference by sex, blood

Table I Age and Sex Distribution of Voluntary Non-Remunerated Blood Donors from September 12/2019 to March 18/2021 at Bahir Dar Blood Bank

		Age						Total
		18–24	**25–29**	**30–34**	**35–39**	**40–44**	**>45**	
Sex	Female	10,767	1115	453	270	168	167	12,940 (32.3)
	Male	14,737	4765	3755	1890	1009	957	27,113 (67.7%)
Total		25,504 (63.7%)	5880(14.7%)	4208(10.5%)	2160 (5.4%)	1177 (2.9%)	1124 (2.8%)	40,053 (100%)

donation site, and p-value less than 0.05 were considered as statistically significant. Bonferroni post hoc test was performed to detect which cells from the contingency table are significantly different. The data were collected after getting acquiescence from the blood bank and we used only donor ABO and Rh blood group, age, sex, and site of blood donation based on the code given at the blood bank. Other than these we did not use other voluntary blood donor data like name and phone number. All the data were secured, protected, and accessed only by the investigators.

Result

In this study, which includes 40,053 voluntary non-remunerated blood donors, the majority of blood donors were males (67.7%), and most of them (63.7%) were within the age range of 18–24 years (mean age 24.4 years) (Table 1). Students, from universities and high school, account for 56.6% of the total (Table 2). As to this investigation, most blood donors (60.2%) were from the Bahir Dar city administration and 68.9% of donations were from the first-time donor. A total of 80,567 units of blood were collected from 40,053 blood donors. The most frequent blood group was blood group O (41.5%) followed by A (29.8), B (23.2%), and AB (5.5%). Considering ABO and Rh blood group altogether blood group O positive

Table 2 Occupational Distribution of Blood Donors from September 12/2019 to March 18/2021 at Bahir Dar Blood Bank

Occupation	Frequency	Percent
Civil Servant	7272	18.2
Driver	421	1.1
Private worker	6012	15.0
Student	22,676	56.6
Teacher	459	1.1
Unemploy	2421	6.0
Others	792	2.0
Total	40,053	100.0

Table 3 ABO and Rh Blood Group Distribution of Blood Donors from September 12/2019 to March 18/2021 at Bahir Dar Blood Bank

		Rh blood Group of Blood Donors		Total
		Negative (%)	**Positive (%)**	
ABO	A	1036 (8.7%)	10,897 (91.3%)	11,933 (100.0%)
	AB	179 (8.2%)	2009 (91.8%)	2188 (100.0%)
	B	726 (7.8%)	8570 (92.2%)	9296 (100.0%)
	O	1451 (8.7%)	15,185 (91.3%)	16,636 (100.0%)
Total		3392 (8.5%)	36,661 (91.5%)	40,053 (100.0%)

(37.9%) was the most predominant blood group followed by A positive (27.2%), B positive (21.4%), (91.5%), AB positive (5%), O negative (3.6%), A negative (2.6%), B negative (1.8%), and AB negative (0.4%). About 91.5%of the blood donors were Rh (D) positive and 8.5% were Rh (D) negative (Table 3).

Distribution of ABO and Rh Blood Groups with Sex and Blood Donation Site

ABO and Rh frequency were not significantly different (P= 0.64) between males and females. The proportions of the B, O, and AB blood groups were significantly different across blood donation sites (P = 0.012). As compared with others, Meshenti donation site had a significantly higher (46.9%) and Motta lower (40.4%) proportion of the blood group O, while Merawi (27.0%) and Sekela (7.0%) had a higher proportion of B and AB blood group respectively. Rh-positive blood groups were significantly less frequent among donors around Gimjabet (86.3%) and Durbete (89.6%) as compared to other donation sites (<0.0001) (Table 4).

Discussion

In Ethiopia, even though there are 40 blood banks, blood and blood component supply is in short supply to meet the

Table 4 ABO and Rh Blood Group of Blood Group Distribution by Donation Site

site of Donation	ABO				Rh	
	A (%)	AB (%)	B (%)	O (%)	Negative (%)	Positive (%)
Adet	296 (30.5%)	54 (5.6%)	224 (23.0%)	398(40.9%)	68 (7.0%)	904 (93.0%)
Bahirdar	7150 (29.6%)	1390 (5.8%)	5630 (23.3%)	9953 (41.3%)	1935 (8.0%)	22,188 (92.0%)
Bicolo	42 (26.1%)	9 (5.6%)	38 (23.6%)	72 (44.7%)	7 (4.3%)	154 (95.7%)
Chagni	381 (30.1%)	62 (4.9%)	287 (22.7%)	537 (42.4%)	107 (8.4%)	1160 (91.6%)
Dangila	689 (30.5%)	102 (4.5%)	504 (22.3%)	962 (42.6%)	206 (9.1%)	2051 (90.9%)
Durbete	476 (32.5%)	74 (5.0%)	315 (21.5%)	601(41.0%)	152 (10.4%)	1314 (89.6%)*
Gemejabet	98 (30.5%)	18 (5.6%)	68 (21.2%)	137 (42.7%)	44 (13.7%)	277 (86.3%)*
GendaMoy	382 (29.3%)	61 (4.7%)	325 (24.9%)	536 (41.1%)	114 (8.7%)	1190 (91.3%)
Kosober	975 (28.9%)	163 (4.8%)	786ₐ (23.3%)	1448 (42.9%)	321 (9.5%)	3051 (90.5%)
Merawi	227 (26.7%)	35 (4.1%)*	230 (27.0%)*	359 (42.2%)	79 (9.3%)	772 (90.7%)
Meshenti	80 (25.6%)	15 (4.8%)	71 (22.8%)	146(46.8%)*	31 (9.9%)	281 (90.1%)
Mota	1060 (31.9%)	182 (5.5%)	735 (22.2%)	1341 (40.4%)*	300 (9.0%)	3018 (91.0%)
Sekela	77 (23.4%)	23 (7.0%)*	83 (25.2%)	146 (44.4%)	28 (8.5%)	301 (91.5%)
Total	11,933 (29.8%)	2188 (5.5%)	9296 (23.2%)	16,636 (41.5%)	3392 (8.5%)	36,661 (91.5%)

Notes: *Donation sites with, significantly, higher or lower proportions of B, AB, O, and Rh-positive blood group (P=0.012, and <0.0001 respectively).

national demand. Bahir Dar blood bank is the outstanding blood bank in the country, which satisfies the need for blood and blood component supply for its catchment health facilities. The need for the determination of the distribution of ABO and Rh blood groups arises to maintain the adequacy of safe blood supply.

In this study, which involved 40,053 blood donors at Bahir Dar blood bank, male blood donors accounted for 67.7%, while 32.3% were females. In line with our finding studies done in Ethiopia (Arba Minch Jima and Debre Tabor) and Tanzania indicated that males were more involved in blood donation than females.[11,14,16] This might be due to female donors are more likely to be different than male donors due to medical grounds, such as low hemoglobin levels, low body weight, pregnancy, and breastfeeding. Of the total blood donors in our investigation, the younger population (18–24 years) and students were the most common blood donors. The reason behind might be due to this portion of the population are dynamic and easily convinced.

In this investigation, the most common blood group was O (41.5%) followed by A (29.8), B (23.2%), and AB (5.5%) which was in line with other findings showed that blood group O as the most common followed by A, B and AB.[13,14,17,18] However, a research conducted in Turkey and Pakistan indicated that blood group A was the predominant,[19,20] while others revealed B was the most common blood group.[21,22] This difference in the frequency distribution of ABO blood group might be due to genetic variations of the study participant.

As to our finding Rh-positive blood group was the most predominant which covered 91.5% and the rest (8.5%) was Rh-negative. Similarly, a study in Debre Tabor, Ethiopia indicated that Rh positive blood group was the most frequent with 92.7% and the remaining 7.3% was Rh-negative.[14] However, a study in Gambela, Ethiopia reported a higher (19.37%) proportion of Rh-negative.[23] Considering ABO and Rh blood group altogether, blood group O positive (37.9%) was the most predominant blood group whereas AB negative (0.4%) was the rarest. Similarly, a study in Ethiopia and Uganda showed O positive as the most frequent and AB negative the least blood group,[13,14] but a study by Jahanpour et al in Tanzania showed A negative (3%) followed by B negative and O negative 2% each.[11] This study also showed that ABO and Rh blood group distribution varies among different blood donation sites so that the local blood bank will make use of this finding to adjust bloodstock by blood type.

Conclusion

In this study, blood group O was the most common followed by A, B, AB. Most of (91.5%) of the blood donors' blood group were Rh-positive. About 68.9% of the total donations were from the first-time donor. To make these donors regular non remunerated blood donors strong promotion activities are required. Our study also showed that the distribution of ABO and Rh blood groups varies among blood donation sites and it will be help full for the blood bank to adjust bloodstock by blood type.

Abbreviations

°C, Degree Celsius; RBC, Red Blood Cells; RH, rhesus factor.

Data Sharing Statement

All relevant data are included in this document.

Ethical Considerations

We obtained a permission letter from Bahir Dar blood bank management bodies. Since we used secondary data, written informed consent was not obtained from each study participant.

Consent for Publication

Consent for publication of this work was found from Bahir Dar blood bank.

Acknowledgments

We would like to thank Bahir Dar blood bank laboratory staff for their cooperation during the data extraction process.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted, agree to be accountable for all aspects of the work.

Funding

The authors declared that there is no funding was obtained for this work.

Disclosure

The authors have declared that no competing interests exist.

References

1. Smart E, Armstrong B. Blood group systems. *ISBT Sci Series*. 2020;15:123–150. doi:10.1111/voxs.12593
2. Mitra R, Mishra N, Rath GP. Blood groups systems. *Indian J Anaesth*. 2014;58(5):524. doi:10.4103/0019-5049.144645
3. Westhoff CM. The Rh blood group system in review: a new face for the next decade. *Transfusion*. 2004;44(11):1663–1673. doi:10.1111/j.0041-1132.2004.04237.x
4. Dean L, Dean L. *Blood Groups and Red Cell Antigens*. Vol. 2. Bethesda, Md, USA: NCBI; 2005.
5. Patel P. Frequency and distribution of blood groups in blood donors in western Ahmedabad–a hospital based study. *Natl J Med Res*. 2012;2(2):202–206.
6. Garratty G, Glynn SA, McEntire R, et al. ABO and Rh (D) phenotype frequencies of different racial/ethnic groups in the United States. *Transfusion*. 2004;44(5):703–706. doi:10.1111/j.1537-2995.2004.03338.x
7. Liao H, Li J. Distribution characteristics of ABO and RhD blood groups among the voluntary blood donors in Chongqing: a retrospective study. *Medicine*. 2020;99(42):e22689. doi:10.1097/MD.0000000000022689
8. Liu J, Zhang S, Wang Q, et al. Frequencies and ethnic distribution of ABO and RhD blood groups in China: a population-based cross-sectional study. *BMJ Open*. 2017;7(12):e018476. doi:10.1136/bmjopen-2017-018476
9. Patidar GK, Dhiman Y. Distribution of ABO and Rh (D) blood groups in India: a systematic review. *ISBT Sci Series*. 2020;16(1):37–48. doi:10.1111/voxs.12576
10. Dilek İ, Demir C, Ali BA, Akdeniz H. ABO and Rh blood groups frequency in men and women living in eastern Turkey. *Int J Hematol Oncol*. 2006;30(4):023–026.
11. Jahanpour O, Pyuza JJ, Ntiyakunze EO, et al. ABO and Rhesus blood group distribution and frequency among blood donors at Kilimanjaro Christian Medical Center, Moshi, Tanzania. *BMC Res Notes*. 2017;10(1):1–5. doi:10.1186/s13104-017-3037-3
12. Egesie U, Egesie OJ, Usar I, Johnbull TO. Distribution of ABO, Rhesus blood groups and haemoglobin electrophoresis among the undergraduate students of Niger Delta University Nigeria. *Niger J Physiol Sci*. 2008;23(1–2):5–8.
13. Apecu RO, Mulogo EM, Bagenda F, Byamungu A. ABO and Rhesus (D) blood group distribution among blood donors in rural south western Uganda: a retrospective study. *BMC Res Notes*. 2016;9(1):1–4.
14. Tiruneh A, Yetneberk T, Gelaw M, et al. Frequency of ABO and Rh blood group distribution at Debre Tabor Blood Bank, Amhara Region, North-Central Ethiopia. A six-year retrospective survey. *J Blood Med*. 2020;11:357. doi:10.2147/JBM.S266624
15. Tesfaye K, Petros Y, Andargie M. Frequency distribution of ABO and Rh (D) blood group alleles in Silte Zone, Ethiopia. *Egypt J Med Human Genet*. 2015;16(1):71–76. doi:10.1016/j.ejmhg.2014.09.002
16. Alemu G, Mama M. Assessing ABO/Rh blood group frequency and association with asymptomatic malaria among blood donors attending Arba Minch blood bank, South Ethiopia. *Malar Res Treat*. 2016;2016:1–7. doi:10.1155/2016/8043768
17. Negash W. ABO and RH blood group type frequencies among students from different ethnic groups at Enchini Secondary School, West Shewa, Oromia Region, Ethiopia. *Ethiop J Nat Computat Sci*. 2021;1(1):1–12.
18. Andalibi M, Dehnavi Z, Afshari A, et al. Prevalence of ABO and Rh blood groups and their association with demographic and anthropometric factors in an Iranian population: Mashad study. *East Mediterran Health J*. 2020;26(8):916–922. doi:10.26719/emhj.20.048
19. Torun YA, Kaynar LG, Karakükcü Ç, et al. ABO and Rh blood group distribution in Kayseri Province, Turkey. *Turk J Hematol*. 2012;29(1):97.
20. Ullah S, Ahmad T. Distribution of ABO and Rh (D) blood groups in the population of District Dir Lower, Khyber Pakhtunkhwa Pakistan. *World Appl Sci J*. 2015;33(1):123–135.
21. Kaur D, Doda V, Kandwal M, et al. ABO Rh (D) blood group distribution among whole blood donors at two different setups of tertiary care hospitals in North India. *Int J Commun Med Public Health*. 2016;3(10):2806–2811. doi:10.18203/2394-6040.ijcmph20163365
22. John S. Prevalence of ABO and Rhesus blood groups in blood donors: a study from a tertiary care centre in South Kerala. *Int J Contemp Med Res*. 2017;4(11):2314–2316.
23. Golassa L, Tsegaye A, Erko B, et al. High rhesus (Rh (D)) negative frequency and ethnic-group based ABO blood group distribution in Ethiopia. *BMC Res Notes*. 2017;10(1):1–5. doi:10.1186/s13104-017-2644-3

Journal of Blood Medicine

Dovepress

Publish your work in this journal

The Journal of Blood Medicine is an international, peer-reviewed, open access, online journal publishing laboratory, experimental and clinical aspects of all aspect pertaining to blood based medicine including but not limited to: Transfusion Medicine; Blood collection, Donor issues, Transmittable diseases, and Blood banking logistics; Immunohematology; Artificial and alternative blood based therapeutics; Hematology; Biotechnology/nanotechnology of blood related medicine; Legal aspects of blood medicine; Historical perspectives. The manuscript management system is completely online and includes a very quick and fair peer-review system. Visit http://www.dovepress.com/testimonials.php to read real quotes from published authors.

Submit your manuscript here: http://www.dovepress.com/journal-of-blood-medicine-journal

Catha edulis Forsk and Ascorbic Acid Effects on Hematological Indices in Rat

Abebaye Aragaw Leminie, Tesfaye Tolessa Dugul, Eyasu Makonnen Eshetu & Daniel Seyifu Melka

Published online: 25 Sep 2021.

Submit your article to this journal 🗗

Article views: 138

View related articles 🗗

View Crossmark data 🗗

ORIGINAL RESEARCH

Catha edulis Forsk and Ascorbic Acid Effects on Hematological Indices in Rat

Abebaye Aragaw Leminie[1]
Tesfaye Tolessa Dugul [iD][1]
Eyasu Makonnen Eshetu[2]
Daniel Seyifu Melka[3]

[1]Department of Physiology, Addis Ababa University, College of Health Sciences, Addis Ababa, Ethiopia; [2]Department of Pharmacology, Addis Ababa University, College of Health Sciences, Addis Ababa, Ethiopia; [3]Department of Biochemistry, Addis Ababa University, College of Health Sciences, Addis Ababa, Ethiopia

Background: The prevalence of chewing *Catha edulis Forsk* and the use of ascorbic acid is increasing from time to time. Their subchronic effects on hematological indices are not well examined. The present study was aimed to investigate their subchronic effects on hematological indices in rats.

Materials and Methods: A total of 36 adult (7–8 weeks) wild-type rats weighing between 213 and 229g were used in this study. They received *Catha edulis Forsk* extract (Ce) (100 milligrams/kilogram, 200 milligram/kilogram and 300 milligram/kilogram b.w), *Catha edulis Forsk* juice (2.5 mL/kg), ascorbic acid (AA 200 milligram/kilogram), and 2% tween 80 in distilled water (T80W- v/v) for twelve weeks. Hematological indices were measured with Sysmex KX-21. Data were analyzed by SPSS version 21.0 and Microsoft Excel.

Results: Neutrocytes ($p < 0.01$), lymphocytes ($p < 0.05$), plateletcrit ($p < 0.05$), average size of platelets ($p < 0.05$), platelet size variability ($p < 0.01$), platelet–large cell ratio ($p < 0.05$) and neutrocytes/lymphocytes ratio ($p < 0.001$) were significantly greater, while hemoglobin concentration per red blood cell ($p < 0.05$) and hemoglobin concentration per volume of red blood cells were significantly reduced ($p < 0.05$) in rats received khat. The red cell distribution width ($p < 0.05$), platelet size variability ($p < 0.05$) and platelet–large cell ratio ($p < 0.01$) were significantly greater in rats received ascorbic acid.

Conclusion: Crude *Catha edulis Forsk* extract and juice changed some hematological indices and increased platelet activities. The platelet activity was also increased by ascorbic acid. The mechanisms for these changes need to be investigated.

Keywords: *Catha edulis Forsk*, ascorbic acid, hematological indices

Introduction

Catha Edulis Forsk (CEF) is one of the stimulants chewed by the people of Ethiopia.[1] It is extensively chewed regardless of its adverse effects. Anemia, cancer, schizophrenia, anxiety, depression, diabetes mellitus, and inflammation are more common among *CEF* chewers.[2] Hematological changes are observed in these diseases.[3] It also affects the therapeutic effects of drugs.[4,5] Certain disease conditions are aggravated by *CEF*.[6,7] Cathinone, Cathine, tannins, ascorbic acid (AA), and electrolytes are some of the composites in it.[8,9] The compounds found in this psychostimulant are expected to affect hematological indices.

Its subacute effects on hematologic indices have been evaluated in an animal model. Most of the findings are controversial and incomplete.[10] AA has been taken as a protective agent against different diseases effects.[11–14] The purpose of taking AA is to regulate hematological and other changes in disease conditions. Acute administration of AA reduces oxidative stress and increases blood cell counts.[15]

Correspondence: Abebaye Aragaw Leminie
Email abebaye.aragaw@aau.edu.et

855

Yet, no studies have been conducted on subchronic hematological effects of *CEF* and AA.

Investigate the subchronic effects of *CEF* and AA on hematological indices in rats is the aim of the current study.

Materials and Methods

Diethyl ether, chloroform (Sigma-Aldrich, Germany), Tween 80, AA, and 70% ethanol were chemicals used in this study. These chemicals were purchased from local suppliers in Addis Ababa, Ethiopia.

Plant Materials Collection

Bundles of fresh *CEF* leaves (9kg) were collected from Aweday, Eastern Ethiopia. The plant specimen was identified and a voucher number (October 16, 2018, AA002) was given. The leaves have been deposited at the National Herbarium of Ethiopia, Addis Ababa University.

Plant Material Extraction

After the edible parts of the leaves were separated and washed with tap water, the leaves were freeze-dried at $-20^{\mathrm{O}}C$[16] for 2 days and crushed using mortar and pestle. Two hundred grams of freeze-dried crushed leaves were placed into a conical flask wrapped with aluminum foil. A total of 400 mL organic solvents, ie, 300 mL diethyl ether and 100 mL chloroform (3:1v/v ratio) were added into the flask. The mixture was shaken under dark conditions for 48hrs using a rotary shaker (New Brunswick Scientific Co, USA) at 120 rpm and $20^{\mathrm{O}}C$. It was then filtered initially using cotton gauze followed with grade I Whatman filter paper (Cat No 1001 150). The organic solvents were then removed through evaporation using Rota-vapor under a controlled temperature of 36°C, rotation of 120 rev/min, and 240 Pascal negative pressure. The water in the extract was removed through lyophilization and the dry residue was weighed using an analytical balance and stored in a desiccator till used. The *CEF* juice (ChJ) was prepared from 12g/kg b.w of fresh leaves using 2% tween 80 in distilled water (v/v). In this study, 12 grams of fresh *CEF* leaves for a rat weighing 1 kilogram was taken as a selected dose. The leaf extract for each rat was calculated according to the weight of each rat (calculated dose = Weight of the rat (g) * the selected dose (g)/ 1000 (g)). The fresh leaves with tween 80 in distilled water (T80W) were crushed using a blender machine. The juice was then squeezed and filtered using the gauze and grade I Whatman filter paper. The T80W used to extract the given weight of the leaves was determined based on the total weight of each rat and vehicle volume used (2.5 mL/kg b.w).

Animal

A total of 36 adult wild-type male white albino rats aged between 7 and 8 weeks were used. Their weight was ranged between 213 and 229g. They were purchased from Laboratory Animal Breeding Section of the Ethiopian Public Health Institution. Three rats per plastic cage under natural light and dark (12:12hrs) cycles at room temperature were housed. Pellet and water were available ad libitum throughout the experimental period. Rats were weighed twice a week to ensure appropriate dosing based on body weight changes. Department of Medical Physiology and Institutional Review Board (IRB) Committee of the College of Health Sciences approved the study. Rules in animal care and use[17] have been used during animal handling.

Grouping and Dosing

Rats were randomly assigned into six groups (n= 6/group) and received T80W, *CEF* extract (Ce) 100 milligram/kilogram, 200 milligram/kilogram and 300 milligrams/kilogram, AA (200 milligram/kilogram), and *CEF* juice (ChJ 2.5 mL/ kg). They were administered for twelve weeks. T80W was used as a vehicle and the quantity for *CEF* extract and AA were selected based on previous reports.[18,19]

Test Substances and Volume Determination

Fresh *CEF* extract, AA, ChJ, and vehicle were prepared every day. *CEF* extract was dissolved in T80W. AA was powdered and dissolved in T80W to make a stock solution of 80 mg/mL. Dose of the extract administered in each rat was calculated based on the total body weight (b.w) of each rat. Appropriate standard vehicle volume (2.5 mL/kg b.w) was used to determine how much volume was used to dissolve the calculated dose of *CEF* extract and AA. Each rat in its respective group received a single daily oral vehicle, *CEF* extract, ChJ, and AA. 1 mL was the final volume and all these were administered orally.

Blood Collection

The procedure used by Ketema et al[20] was used during blood withdrawal. Three milliliters of whole blood were collected from each rat through cardiac puncture after they were

anesthetized using sodium pentobarbital (1mL/kg b.w). Blood was collected at 9:am and put into a tube containing ethylenediaminetetraacetic acid (EDTA) 24 hours after the last administration of test substances. Total leukocytes, Neutrocytes (NEUT), lymphocytes (LYMPH), monocytes (MONO), Eosinophils (EO), basophils (BASO), Hemoglobin (Hg), Hematocrit (HCT), Red blood cell size (RBCs), and red blood cell size variability (RsV) were analyzed by CBC machine (Sysmex KX-21). Hemoglobin concentration per red blood cell (HgpRBC), Hemoglobin concentration per volume of red blood cells (HgpvRBCs), platelet, Platelet size variability (PsV), plateletcrit (PCT), and platelet-large cell ratio (P-LCR) were also analyzed. NEUT to LYMPH ratio (NLR) was determined from quantified cells.

Data Analysis

The statistical analysis was done using SPSS version 21.0 and graphs were plotted using Microsoft Excel. Mean ±S. E.M have been used to express the values. Differences in hematological indices between three and more groups were analyzed using one-way ANOVA followed by Tukey's post hoc analysis. An independent t-test was also used in this study to compare values obtained from only two groups such as the test versus control groups.

Results

Significant differences in total leukocyte count ($F_{(5, 30)}$ =1.61, $p > 0.05$), MONO (F $_{(5, 30)}$ = 1.37, $p > 0.05$), EO (F $_{(5, 30)}$ = 1.64, $p > 0.05$) and BASO (f $_{(5, 30)}$ = 1.39, $p > 0.05$) were not observed between groups. Significant difference in NEUT (F $_{(5, 30)}$ = 6.42, $P < 0.001$), LYMPH (F $_{(5, 30)}$ = 3.27, $p < 0.05$) and NLR (F $_{(5, 30)}$ = 6.97, $p < 0.001$) was observed between groups.

NEUT and NLR in rats receiving *CEF* extract were significantly higher when compared with the T80W (t $_{(26.07)}$ =−3.72, $p < 0.01$, 95% CI [−11.69,-3.37] and t $_{(24.72)}$ = −3.22, $p < 0.01$, 95% CI [−20.28,-4.44]) and AA (t $_{(7.44)}$ = −2.34, $p < 0.05$; 95% CI [−9.25,-0.11] and t $_{(27.99)}$ = −2.06, $p < 0.05$; 95% CI [−17.06,-0.06], respectively) as shown in Figure 1.

Rats received Ce100 milligram/kilogram had significantly higher NEUT ($p < 0.001$, 95% CI [7.09, 29.21]) and NLR ($p < 0.001$, 95% CI [13.46, 54.49]) compared with rats received T80W (Table 1). NEUT and NLR in rats received Ce100 milligram/kilogram were also significantly higher when compared with rats received AA ($p < 0.01$, 95% CI [4.25, 26.37] and $p < 0.01$, 95% CI [9.65, 50.69], respectively). However, LYMP was significantly less in this group of rats when compared with rats received T80W ($p < 0.05$, 95% CI [−31.12,-0.89]) (Table 1).

Significant differences in RBC count (F $_{(5, 30)}$ = 2.75, $p < 0.05$), HGB (F $_{(5, 30)}$ = 3.69, p-value less than 0.05), HCT (F $_{(5, 30)}$ = 3.60, $p < 0.05$) and RBCS (F $_{(5, 30)}$ = 4.07, P value less than 0.01) were observed between groups. Post hoc analysis results indicated no significant differences in RBC count, Hg concentration, and HCT (Table 1).

HgpRBC in rats received the Ce was significantly less than in those received T80W (t $_{(24.27)}$ = 3.57, $p < 0.05$; 95% CI [0.41, 1.52]) as shown in Figure 1. Rats received Ce 100 milligram/kilogram ($p < 0.05$, 95% CI [0.07, 11.63]), Ce 200 milligram/kilogram (p value less than 0.05, 95% CI [−11.97,-0.41]) and Ce 300 milligram/kilogram ($p < 0.05$, 95% CI [−12.73,-1.17]) had significantly less RBCS than received AA 200 milligram/kilogram.
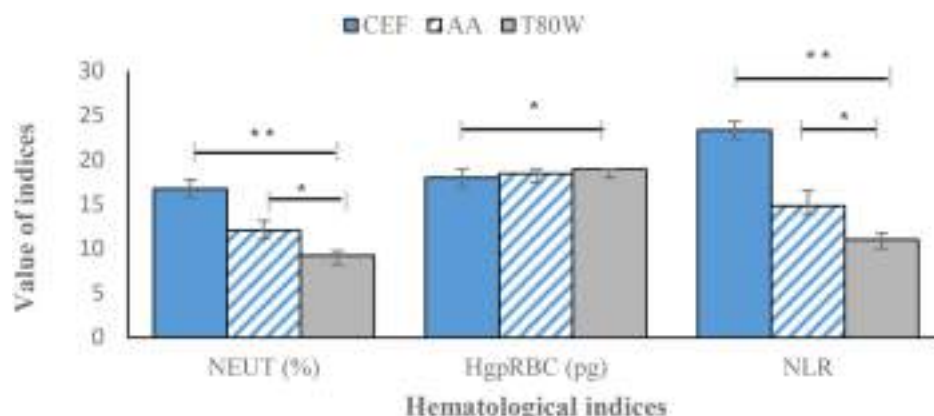
Figure 1 Effects of *Catha edulis Forsk* on HgpRBC, NEUT, and NLR in Rats. Each bar represents the mean ± SEM of these indices in rats received AA, T80W, and khat. **Statistical difference at p < 0.01 and *Statistical difference at p < 0.05 when rats received *CEF*, AA and T80W were compared to each other.
Abbreviations: *CEF, Catha edulis Forsk*; AA, ascorbic acid; HgpRBC, hemoglobin concentration per red blood cell; NEUT, neutrocyte; NLR, neutrocyte to lymphocyte ratio.

Table I Effect of *Catha edulis Forsk* and Ascorbic Acid on Hematological Indices

Parameter	Group					
	T80W	**AA200 mg/kg**	**Ce100 mg/kg**	**Ce200 mg/kg**	**Ce300 mg/kg**	**ChJ2.5 mL/kg**
WBC(10^3/μL)	9.06±0.56	10.57±1.83	7.02±0.68	6.91±0.82	8.20±1.01	8.25±1.03
MONO (%)	4.07±0.43	4.53±0.83	3.88±0.49	3.00±0.46	2.97±0.10	3.33±0.64
EO (%)	0.89±0.21	1.30±0.54	0.50±0.05	1.12±0.07	0.63±0.09	0.67±0.06
BASO (%)	0.30±0.04	0.10±0.04	0.17±0.06	0.18±0.04	0.25±0.12	0.15±0.03
NEUT	9.23±0.55	12.06±1.08	27.37±5.60***ee	12.87±1.50	11.42±1.99	15.32±0.78
LYMP (%)	84.42±3.21	82.65±2.35	68.42±6.40*	82.48±1.76	86.20±2.97	80.28±2.31
NLR (%)	10.99±0.76	14.79±1.74	44.96±10.92***ee	15.79±2.14	13.39±2.67	19.25±1.41
RBC (10^6/μL)	8.75±0.16	6.66±1.21	9.28±0.47	8.84±0.218	9.17±0.48	6.55±1.21
Hg (g/dL)	16.95±0.26	12.22±2.08	16.93±0.48	15.80±.52	17.10±0.51	11.53±2.30
RBCs (%)	48.46±0.59	35.07±5.78	48.88±1.11	45.77±0.71	48.28±0.72	34.88±6.19
sRBCs (fL)	54.13±0.49	59.24±2.48	53.38±0.42 e	53.05±0.98 e	52.28±0.48e	57.08±1.76
HgpRBC (pg)	18.94±0.05	18.47±0.46	18.42±0.46	18.37±0.48	18.67±0.47	17.78±0.58
HgpRBCs (g/dL)	35.13±0.24	33.01±0.99	34.53±0.75	34.50±0.94	35.40±0.49	31.42±1.30*
RsV(fL)	31.73±0.56	34.11±0.55*	33.38±0.56	31.31±0.32	34.02±0.75*	30.92±0.29
PLT(10^3/μL)	476.83±22.	381.83±102.6	509.00±111.7	543.17±90.9	607.33±126.3	379.17±119.1
PCT (%)	0.35±0.03	0.38±0.08	0.65±0.03*e	0.43±0.07	0.65±0.03*e	0.37±0.08
aPs(fL)	8.39±0.24	8.90±0.47	8.29±0.08	8.11±0.06	11.37±1.36*	8.90±0.92
PsV(fL)	9.28±0.26	10.28±0.21*	9.30±0.10	8.95±0.28	10.89±0.51**	9.33±0.08
P-LCR (%)	13.48±0.87	18.79±0.8**	13.28±0.91e	12.48±0.74e	13.47±0.86e	18.01±1.15*

Notes: Hematological indices in rats (n= 6/group) receivedT80W, AA 200mg/kg, Ce(100 mg/kg, 200 mg/kg, and 300 mg/kg) and ChJ 2.5 mL/kg represented as mean ± SEM of. ***Statistical difference at $p < 0.001$; **Statistical difference at $p < 0.01$ and *Statistical difference at $p < 0.05$ when rats received Ce100mg/kg, Ce200mg/kg, Ce300mg/kg, ChJ 2.5 mL/kg and AA 200mg/kg were compared with rats received T80W. eestatistical difference at $p < 0.01$ and estatistical difference at $p < 0.05$ when rats received Ce100 mg/kg, Ce200 mg/kg, Ce300 mg/kg, ChJ 2.5 mL/kg were compared with rats received AA 200mg/kg.
Abbreviations: Ce, *Catha edulis Forsk* extract; μL, microliter; sRBCs, size of red blood cells; dL, deciliter; fL, femtoliter; NEUT, Neutrocytes; pg, picogram; NLR, Neut to Lymph ratio; Hg, hemoglobin; HgpRBC, hemoglobin concentration per red blood cell; HgpvRBCs, hemoglobin concentration per volume of red blood cells; PCT, plateletcrit; RsV, red blood cell size variability; PsV, platelet size variability; aPs, average platelet size; P-LCR, platelet-large cell ratio; T80W, tween 80 in distilled water; mg/kg, milligram/kilogram; AA, ascorbic acid; khJ, *Catha edulis Forsk* juice.

RsV in rats received Ce300 milligram/kilogram ($p < 0.05$, 95% CI [0.01, 4.57]) and AA ($p < 0.05$, 95% CI [0.10, 4.66]) was significantly higher when compared with rats received T80W (Table 1).

Significant differences were observed in aPs (F $_{(5, 30)}$ = 3.82, $p < 0.01$), PCT (F $_{(5, 30)}$ = 5.51, P-value less than 0.01), PsV(F $_{(5, 30)}$ = 7.14, $p < 0.001$) and P-LCR (F $_{(5, 30)}$ = 8.95, $p < 0.001$) between groups. But, no significant difference in platelet count F $_{(5, 30)}$ =0.79, $p > 0.05$) among the groups was observed. PCT was higher in rats received Ce100 milligram/kilogram and Ce300 milligram/kilogram compared to rats received T80W (P-value less than 0.05, 95 CI [0.05, 0.57] and $p < 0.05$, 95% CI [0.05, 0.57], respectively). PCT in these groups was also significantly higher when compared to rats received AA ($p < 0.05$, 95% CI [0.01, 0.53] and $p < 0.05$, 95% CI [0.01, 0.53], respectively). PsV in rats received Ce300 milligram/kilogram was significantly greater than in received T80W ($p < 0$ 0.01, 95% CI [0.31, 2.80]). Rats received Ce100 milligram/

kilogram ($p < 0.01$, 95% CI [−9.43, −1.57]), Ce200 milligram/kilogram (p-value less than 0.001, 95% CI [−10.23, −2.37]) and Ce300 milligram/kilogram ($p < 0.01$, 95% CI [−9.25, −1.39]) had significantly less P-LCR than rats received ascorbic acid. As shown in the table, rats receiving ChJ 2.5 mL/kg had higher P-LCR than in rats received T80W (p- vale was less 0.05, 95% CI [0.60, 8.46].

Discussion

In this study, total leukocytes, monocytes, basophils, and eosinophils were not affected by crude *CEF* extract and juice (Table 1). Alele et al[21] also revealed that total leukocyte was not affected by this extract. However, Bin-Jaliah et al[10] and Ketema et al[20] revealed that total leukocyte was reduced by *CEF* extract indicating that this extract suppressed immunity. Disagreement between these findings might be because of variation in extraction solvent, duration of administration, and animal species used to see the effects.

Even though the statistical difference in the total leukocytes did not observe between rats receiving the vehicle and AA in this study, previous studies indicated that white cell count was significantly increased by vitamin C.[19,22] In the previous study, Vitamin C (30 milligrams/kilogram and 70 milligrams/kilogram) was given for four weeks twice per day. However, it was administered (200 milligrams/kilogram) for twelve weeks per day in the current evaluation. Discrepancy between these findings might be attributed to the dose and duration of administration. These findings showed that vitamin C administered for a short period with a minimum dose increases white blood cells. In our study, neutrocytes and NLR were increased significantly while lymphocytes count was reduced, particularly by the lower dose. The tolerance effects of *CEF* might be less at the lower dose. Although much has not been done on the tolerance effects of *CEF*, a previous study showed that *CEF* responses are gradually reduced, indicating that it has a tolerance effect.[23]

An increase in the Neutrocytes count by extract in this study might be because of its inflammatory,[24,25] oxidative stress,[26,27] adrenocortical function, and sleep physiology[28] effects. *CEF* increases adrenocortical function and cortisol secretion.[6,29] In turn, cortisol increased neutrophil count.[30] On the other hand, stress increases neutrophil count[31] and cathinone in *CEF* causes oxidative stress.[26,32] *CEF*-induced sleep restriction increases neutrophil count.[33,34] Like the previous study,[10] the lower dose of *CEF* extract reduced lymphocyte count in our study. However, another study revealed that lymphocyte count was increased in schizophrenic patients.[35] *CEF* increased dopamine levels in healthy and schizophrenic patients.[7] Bogale et al[36] reported that schizophrenic-like symptoms were observed in mice received *CEF* extract. In this way, *CEF* should have increased lymphocyte count, but opposite result was obtained in our study that might be because of variation in the duration of administration.

Reduction in lymphocyte count observed in the current study might be through *ECF* hematopoietic effects. A previous study indicated that the destruction of dopaminergic cells affected blood cell synthesis.[37,38] On the other hand, this stimulant affects bioavailability of dopamine in body fluid.[7] Adrenalin level that affects hematopoiesis could be affected by khat. Another study indicated that sympathetic fibers innervating bone marrow released adrenalin and dopamine affect stem cell activities.[28] This indicates that dysregulation of adrenergic and dopaminergic fibers might be associated with hematopoietic disturbances.

The higher neutrophil-to-lymphocytes ratio observed in this study could be associated with depression effects of the *CEF*.[2,26] This ratio is high in patients with severe depression disorder.[3] Psychiatric disorders associated with subchronic inflammation increased NLR and *CEF* showed inflammatory responses.[3,24,25] *CEF* extract and juice did not affect red blood cell count, hemoglobin concentration, size of red blood cells (sRBCs), and Hematocrit (HCT) in this study (Table 1). However, when the comparison was made between rats received *CEF* and AA, sRBCs were less in rats at all doses of the extract (Table 1). Nevertheless, Owu et al[39] showed that AA (vitamin C, 200 milligrams/kilogram) administered in rats for 28 days reduced sRBCs indicating that the duration of vitamin C administration could contribute to the differences.

Rats received higher dose of the extract and AA had greater RsV compared with rats received vehicle. *Catha edulis Forsk* juice also reduced MCHC when compared with vehicle (Table 1). However, in the study conducted before, the PCV and hemoglobin concentration were significantly reduced, while RBCS was increased.[10] The discrepancy could be attributed to the dose of the extract, duration of administration, and solvent used to extract the plant material. The dose of the *Catha edulis Forsk* extract used by Bin-Jaliah et al[10] was 500 milligram/kilogram and administered for only one week, while the higher dose in our study was 300 milligrams/kilogram administered for twelve weeks. The solvent used in this previous study was hydro-ethanol while diethyl/chloroform (v/v; 3:1) was used in our study. Like in our study, Bin-Jaliah et al[10] also revealed that RsV was increased by the crude extract of *CEF*.

In this study, the increase in RsV in rats received *CEF* might be the result of the adverse effects of *Catha edulis Forsk* on the liver. Previous studies indicated that RsV significantly increased in patients with progressive liver fibrosis and inflammation.[40,41] On the other hand, *CEF* chewing was associated with acute liver injury, subchronic inflammation and cirrhosis.[24,42]

In this study, *CEF* juice reduced HgpvRBC (Table 1). Another study also showed *CEF* reduced HgpvRBCs.[24] However, Ketema et al[20] showed that statistical difference in HgpvRBCs was not observed between mice received the extract and vehicle.

The solvent, animal model and duration of administration difference used by the previous and current study might be the reason for variation in the results. Ketema et al[20] used methanol to extract *CEF* and was administered for four weeks in mice while we used diethyl ether and

chloroform to extract the leaves and administered for twelve weeks in rats.

Hemoglobin reduction in each RBC observed in this study might be resulted from its effect on iron absorption problems across the gastrointestinal tract. Previous studies indicated that tannins reduced absorption of iron and vitamins; involved in the synthesis of red blood cells.[43,44] One of the components found in the leaves of CEF is tannin,[8,9] suggesting that CEF affects iron and vitamin absorption. Another study also showed that CEF reduced level of iron and vitamin B12 in the serum.[45] Gastrointestinal problems such as esophagitis, gastritis, delaying gastric empty and impaired intestinal absorption, some of the effects of the leaves.[2] Liver problems induced by CEF[24] could also be attributed to bleeding problems and iron deficiency among people who are chewing the leaves of CEF.[46]

The average HgpRBC reduction in rats received CEF (Figure 1) in our study might be because of its effect on the renal system. Erythropoietin is one of the proteins involved in the synthesis of RBCs and this could be affected by CEF extract. However, Ketema et al[20] and Bin-Jaliah et al[10] also could not see any significant differences in HgpRBC in mice and rats, respectively.

Statistical difference in the total number of platelet has not been seen in our study (Table 1). Alsalahi et al[24] and Alele et al[21] also found analogous outcomes. But Ketema et al[20] reported that its methanolic extract (100 and 200 milligrams/kilogram) administered for four weeks reduced platelet number in mice. Contrarily, a study conducted by Bin-Jaliah et al[10] revealed that its hydro-ethanolic extract (500 milligrams/kilogram) administered for one week increased platelet count in the rat model. The dissimilarity of these findings might be because of administration duration and extraction solvent variation.

In this study, lower and higher doses of CEF extract increased PCT, while the higher dose increased PsV and aPs. Its juice significantly increased the P-LCR. These findings indicated that CEF increases platelet activities. Strong platelet activity is represented by higher PCT, PsV, and P-LCR.[47] platelet activities are increased when there is organ inflammation,[48] anxiety and depression disorders.[49] On the other hand, an increase in platelet activities and oxidative stress are among the risk factors for thrombosis.[50,51] This, in turn, indicated that CEF might aggravate organ inflammation, anxiety disorders, and thrombosis. Previous studies indicated that organ inflammation is more prevalent among people who are using CEF.[25,52]

AA also increased P-LCR and PsV. Studies indicated that a higher dose of ascorbic acid contributed to oxidative stress,[53] the release of thromboxane A_2 and prostaglandin E_2 in subjects with depression and thrombosis.[54] Platelet activities were higher in patients with depression, schizophrenia and other psychiatric problems and contributed to the secretion of serotonin.[55]

In conclusion, CEF extract, CEF juice, and AA altered some of the hematological indices. AA and CEF increased platelet activities and size variability of red blood cells. The mechanisms of action for these changes need to be studied.

Acknowledgment

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation and preparing the manuscript. They have involved in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Tesfaye G, Derese A, Hambsa MT. Substance use and associated factors among university students in Ethiopia: a cross-sectional study. *J Addict*. 2014;2014:1–8. doi:10.1155/2014/969837
2. Al-Habori M. The potential adverse effects of habitual use of Catha edulis (khat). *Expert Opin Drug Saf*. 2005;4(6):1145–1154. doi:10.1517/14740338.4.6.1145
3. Sunbul EA, Sunbul M, Yanartas O, et al. Increased neutrophil/lymphocyte ratio in patients with depression is correlated with the severity of depression and cardiovascular risk factors. *Psychiatry Investig*. 2016;13(1):121–126. doi:10.4306/pi.2016.13.1.121
4. Odenwald M, Neuner F, Schauer M, et al. Catha edulis forskuse as risk factor for psychotic disorders: a cross-sectional and case-control study in Somalia. *BMC Med*. 2005;3(5):1–10. doi:10.1186/1741-7015-3-5.2
5. Issa FH, Al-Habori M, Chance ML. Effect of Catha edulis forsk (Catha edulis) use on the bioavailability, plasma levels and antimalarial activity of chloroquine. *Med J*. 2016;16(2):182–188.

6. El-Sayed MK, Amin HK. Effect of Catha edulis on insulin, resistin and cortisol levels in type-2 diabetics and nondiabetics. *Am J Biochem Biotech*. 2012;8(3):157–163. doi:10.3844/ajbbsp.2012.157.163

7. El-Sayed MK, Amin HK. Catha edulis chewing effects on the treatment of paranoid schizophrenic patients. *Neuropsychiatr Dis Treat*. 2015;11:1067–1076. doi:10.2147/NDT.S81011

8. Algabr M, Dunia AM, Aissaoui H, et al. Flavonoids glycosides from leaves of Catha edulis (Celastraceae). *Der Pharma Chem*. 2015;7 (11):193–196.

9. Al-Alimi KR, Razak A, Saub R, Alabsi AM. Tannins acid, ascorbic acid and fluoride from Catha edulis forskChewing plant. *Int J Dent Oral Health*. 2017;3(4):1–4. doi:10.16966/2378-7090.240

10. Bin-Jaliah I, Dallak MA, Al-Hashem FH, et al. Derangement of hemopoiesis and hematological indices in Catha edulis forsk(Catha edulis) - treated rats. *Afr J Biotech*. 2014;13(2):349–355. doi:10.5897/AJB2013.13373

11. Oliveira CP, Kassab P, Lopasso FP, et al. Protective effect of ascorbic acid in experimental gastric cancer: reduction of oxidative stress. *World J Gastroenterol*. 2003;9(3):446–448. doi:10.3748/wjg.v9.i3.446

12. Padayatty SJ, Riordan HD, Hewitt SM, Arie Katz L, Hoffer J, Mark L. Intravenously administered vitamin C as cancer therapy: three cases. *CMA*. 2006;174(7):937–942. doi:10.1503/cmaj.050346

13. Klimant E, Wright H, Rubin D, Seely D, Markman M. Intravenous vitamin C in the supportive care of cancer patients: a review and rational approach. *Curr Oncol*. 2018;25(2):139–148. doi:10.3747/co.25.3790

14. Aburawi SM, Fathia A, Ghambirlou F, Attumi AA, Altubuly RA, Kara AA. Effect of ascorbic acid on mental depression drug therapy: clinical study. *Clin Study J*. 2014;4(1):1–8. doi:10.4172/2161-0487.1000131

15. Moretti M, Budni J, Santos D, et al. Protective effects of ascorbic acid on behavior and oxidative status of restraint-stressed mice. *J Mol Neurosci*. 2013;49(1):68–79. doi:10.1007/s12031-012-9892-4

16. Alfaifi H, Abdelwahab SI, Mohan S, et al. *Catha edulis forsk* (Khat): evaluation of its antidepressant-like activity. *Pharmacogn Mag*. 2017;13(50):354–358. doi:10.4103/pm.pm_442_16

17. National Institution of Health. *Guide for the Care and Use of Laboratory Animals*. 8th ed. National Academic Press (US); 2011.

18. Mohammed F, Engidawork E, Nedi T. The effect of acute and sub-chronic administration of Crude Catha edulis forskExtract (Catha Edulis F.) on weight in mice. *ASRJETS*. 2015;14(1):132–141.

19. Foster MN, Carr AC, Alina Antony A, Selene PS, Fitzpatric MG. Intravenous vitamin C administration improved blood cell counts and health-related quality of life of patient with history of relapsed acute myeloid leukaemia. *Antioxidants*. 2018;7(7):92. doi:10.3390/antiox7070092

20. Ketema T, Yohannes M, Alemayehu E, Ambelu A. Effect of chronic Catha edulis forsk(Catha edulis, Forsk) use on outcome of Plasmodium berghei ANKA infection in Swiss albino mice. *BMC Infect Dis*. 2015;15(1):170. doi:10.1186/s12879-015-0911-2

21. Alele PE, Abayomi M, Ajayi AM, Imaniramp L. Chronic Catha edulis forsk(Catha edulis) and alcohol marginally alter complete blood counts, clinical chemistry, and testosterone in male rats. *J Exp Pharmacol*. 2013;5:33–44. doi:10.2147/JEP.S46635

22. Sharaf A, Farrag AR, Fahmy H. Protective effects of vitamin C on hematological and biochemical parameters of intoxicated male albino rats with lead and cadmium. *MEJAST*. 2017;7(1):57–67.

23. Nencini P, Ahmed AM, Amiconi G, Elmi AS. Tolerance develops to sympathetic effects of *Catha edulis forsk*in humans. *Pharmacology*. 1984;28(3):150–154. doi:10.1159/000137956

24. Alsalahi A, Abdulla MA, Al-Mamary M, et al. Toxicological features of Catha edulis (Khat) on livers and kidneys of male and female Sprague-Dawley rats: a subchronic study. *Evid Based Complement Alternat Med*. 2012;2012:1–11. doi:10.1155/2012/829401

25. Al-Mamary H, El-Shaibany A, Al-Habori M, Al-Meeri A, Al-Zubairi AS, Al-Mamary M. Effect of *Catha edulis* on the activities of enzyme markers of carcinogenicity in chemically-induced hepatocellular carcinoma in rabbits. *Int J Cancer Res*. 2014;10(1):1–13. doi:10.3923/ijcr.2014.1.13

26. Safhi MM, Alam MF, Hussain S, et al. Cathinone, an active principle of Catha edulis, accelerates oxidative stress in the limbic area of Swiss albino mice. *J Ethnopharmacol*. 2014;156:102–106. doi:10.1016/j.jep.2014.08.004

27. Abdelwahab SI, Alsanosy R, Taha E, Syam MS. Catha edulis forskInduced toxicity: role on its modulating effects on inflammation and oxidative stability. *Biomed Res Int*. 2018;2018:1–8. doi:10.1155/2018/5896041

28. Manzar MD, Salahuddin M, Sony P, et al. Sleep disturbances and memory impairment among pregnant women consuming khat: an under-recognized problem. *Ann Thorac Med*. 2018;12:247–251.

29. Ahmed MB, El-Qirbi AB. Biochemical effects of *Catha edulis*, cathine and cathinone on adrenocortical functions. *J Ethnopharmacol*. 1993;39(30):213–216. doi:10.1016/0378-8741(93)90039-8

30. Deutsch V, Lerner-Geva L, Reches A, Boyko V, Limor R, Grisaru D. Sustained leukocyte count during rising cortisol level. *Acta Haematol*. 2007;118(2):73–76. doi:10.1159/000103216

31. Nishitani N, Hisataka Sakakibara H. Association of psychological stress response of fatigue with white blood cell count in male daytime workers. *Ind Health*. 2014;52(6):531–534. doi:10.2486/indhealth.2013-0045

32. Al-Zubairi A, Al-Habori M, Al-Geiry A. Effect of Catha edulis (khat) chewing on plasma lipid peroxidation. *J Ethnopharmacol*. 2003;87 (1):3–9. doi:10.1016/S0378-8741(03)00101-6

33. Boudjeltia KZ, Faraut B, Stenuit P, et al. Sleep restriction increases white blood cells, mainly neutrophil count, in young healthy men: a pilot study. *Vasc Health Risk Manag*. 2008;4(6):1467–1470. doi:10.2147/VHRM.S3934

34. Rosales C. Neutrophil: a cell with many roles in inflammation or several cell types. *Front Physiol*. 2018;9(113):1–17. doi:10.3389/fphys.2018.00113

35. Boneberg EM, Seydlitz EV, Propster K, Watzl H, Rock-stroh B, Illges H. D3 dopamine receptor mRNA is elevated in T cells of schizophrenic patients whereas D4 dopamine receptor mRNA is reduced in CD4+ -T cells. *J Neuroimmunol*. 2006;173(2):180–187. doi:10.1016/j.jneuroim.2005.11.018

36. Bogale T, Engidawork E, Yisma E. Subchronic oral administration of crude Catha edulis forskextract (Catha edulis forsk) induces schizophernic-like symptoms in mice. *BMC Complement Altern Med*. 2016;16(153):1–12. doi:10.1186/s12906-016-1145-6

37. Hritcu L. Hematological disorders in 6-hydroxydopamine induced rat model of Parkinson's disease. *Turk J Hematol*. 2008;25:140–144.

38. Cosentino M, Marino F, Maestroni GJ. Sympathoadrenergic modulation of hematopoiesis: a review of available evidence and of therapeutic perspectives. *Front Cell Neurosci*. 2015;9(302):1–12. doi:10.3389/fncel.2015.00302

39. Owu DU, Nwokocha CR, Ikpi DE, Ogar EI. Effect of vitamin C supplementation on platelet aggregation and serum electrolytes levels in streptozotocin-induced diabetes mellitus in rats. *Niger J Physiol Sci*. 2016;31(1):55–61. Available from: www.njps.com.ng.

40. Cengiz M, Candır BA, Yılmaz G, Akyol G, Ozenirler S. Is increased red cell distribution width an indicating marker of nonalcoholic steatohepatitis and fibrotic stage? *World J Gastroenterol*. 2013;19 (42):7412–7418. doi:10.3748/wjg.v19.i42.7412

41. Xu WS, Qiu XM, Ou QS, et al. Red blood cell distribution width levels correlate with liver fibrosis and inflammation A noninvasive serum marker panel to predict the severity of fibrosis and inflammation in patients with hepatitis B. *Medicine*. 2015;94(10):1–7.

42. Alhaddad OM, Elsabaawy MM, Waked IA. Catha edulis forskIntertwined liver damage. *J Infect Dis Ther*. 2017;5(5):1–3. doi:10.4172/2332-0877.1000337

43. Carrera G, Mitjavila S, Derache R. Effect of tannic acid on vitamin B 12 absorption in rats. *Sci Hebd Seances*. 1973;276(2):239–242.

44. Afsana K, Shiga K, Ishizuka S, Hara H. Reducing effects of ingesting tannic acid on the absorption of iron but not zinc, copper and magnesium by rats. *Biosci Biotec Biochem*. 2004;68(3):584–592. doi:10.1271/bbb.68.584

45. Al-Dubai W, Al-Ghazaly J, Al-Habori M, Saif-Ali R, Mohammed A. Catha Edulis (Khat) chewing decreases serum levels of iron, ferritin and vitamin B12 and increases folic acid. *Int J Vit Nutr Res*. 2014;84 (5–6):223–228. doi:10.1024/0300-9831/a000208

46. Gkamprela E, Deutsch M, Pectasides D. Iron deficiency anemia in chronic liver disease: etiopathogenesis, diagnosis and treatment. *Ann Gastroenterol*. 2017;30(4):405–413.

47. Swaminathan A, Amitkumar K, Ganapathy S, Ayyavoo S. Evaluation of the impact of cigarette smoking on platelet parameters. *Natl J Physiol Pharm Pharmacol*. 2015;5(5):426–430. doi:10.5455/njppp. 2015.5.1009201570

48. Budak YU, Polat M, Huysal K. The use of platelet indices, platelet-crit, average size of platelets and Platelet size variability in emergency non-traumatic abdominal surgery: a systematic review. *Biochem Med*. 2016;26(2):178–193. doi:10.11613/BM.2016.020

49. Almis BH, Aksoy I. Average size of platelets level in patients with generalized anxiety disorder. *Psychiatry Clin Psychol*. 2018;28 (1):43–47. doi:10.1080/24750573.2017.1385210

50. Zhang X, Cui -M-M, Fu S, et al. Platelet size variability correlates with the prognosis of gastric cancer. *Oncotarget*. 2017;8(12):20 13–20219.

51. Gawlita M, Wasilewski J, Osadnik T, Reguła R, Bujak K, Gonera M. Average size of platelets and platelet-large cell ratio as prognostic factors for coronary artery disease and myocardial infarction. *Folia Cardiol*. 2016;10(6):418–422. doi:10.5603/FC. 2015.0079

52. Nigussie T, Gobena T, Mossie A. Association between Catha edulis forskchewing and gastrointestinal disorders: a cross-sectional study. *Ethiop J Health Sci*. 2013;23(2):123–130.

53. Sadighara P, Salar-Amoli J. High concentrations of vitamin C induce oxidative stress in blood. *Biharean Biol*. 2009;3(1):33–35.

54. Mohammed BM, Sanford KW, Fisher BJ, et al. Impact of high dose vitamin C on platelet function. *World J Crit Care Med*. 2017;6 (1):37–47. doi:10.5492/wjccm.v6.i1.37

55. Ehrlich D, Humpe C, Platelets in psychiatric disorders. *World J Psychiatr*. 2012;2(6):91–94. doi:10.5498/wjp.v2.i6.91

Usage and Acceptability of the Wateen Application Among the Population of Saudi Arabia

Noot AlOtaibi, Sarah Alsaleebi, Fahad Alanezi, Hala Alhodaib, Bashair AlThani, Duaa Aljabri, Demah Alsalman, Asma Al-Fayez, Amjad Saadah, Sumaiah Alrawiai, Norah Alyousif & Turki Alanzi

Published online: 01 Oct 2021.

Submit your article to this journal 🗗

View related articles 🗗

View Crossmark data 🗗

ORIGINAL RESEARCH

Usage and Acceptability of the Wateen Application Among the Population of Saudi Arabia

Noot AlOtaibi [ID][1]
Sarah Alsaleebi[2]
Fahad Alanezi [ID][3]
Hala Alhodaib [ID][4]
Bashair AlThani [ID][5]
Duaa Aljabri [ID][1]
Demah Alsalman [ID][1]
Asma Al-Fayez[1]
Amjad Saadah[1]
Sumaiah Alrawiai [ID][1]
Norah Alyousif[6]
Turki Alanzi [ID][1]

[1]Department of Health Information Management and Technology, College of Public Health, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [2]Blood Bank, King Fahad Specialist Hospital, Dammam, Saudi Arabia; [3]Community College, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [4]Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyad, Saudi Arabia; [5]College of Business Administration, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [6]Information Technology Department, Saudi Aramco, Dhahran, Saudi Arabia

Purpose: The main objective of this research was to investigate the opinion of the population of Saudi Arabia on the use and acceptability of the Wateen application.

Methods: This research was a quantitative cross-sectional study in which an online questionnaire was distributed among the target population who were healthy people over 18 years of age that had used or known about the Wateen application. A total of 352 participants responded to the questionnaire. The data collection was carried out between November and December 2020. A basic descriptive statistical analysis was used to analyze the data.

Results: The participants had used the Wateen app to follow the donation record, as a reminder of donation dates, to find lists and blood bank locations, to request blood donation, and to find donation requests on social media. After receiving the blood donation request from the Wateen app, majority of the respondents felt encouraged, motivated, and educated to donate blood, and about blood donation, respectively. Participants also indicated that the application helped them to find donors. Besides, majority of the respondents stated that the Wateen app had reduced the gap between blood donors and the need for blood. However, few participants expressed that they had not received or observed a post about the Wateen application on social media networks.

Conclusion: The results of this research indicated that there is a general acceptance of the use of the Wateen app among the participants of this study. However, the importance of the Wateen app needs to be further advertised so that it can be used in the blood donation process in all regions of Saudi Arabia. In this way, the people of Saudi Arabia will be more aware of the importance of the Wateen app in the blood donation process, and the goal of the Ministry of Health of bridging the gap between donation centers and blood donors could be achieved.

Keywords: Wateen application, blood donation, m-health, Saudi Arabia, user acceptability

Introduction

Blood donation is a humanitarian, altruist, noble, and solidary act supported by all health organizations in the world.[1–3]

> Blood transfusion saves lives and improves health … Providing safe and adequate blood should be an integral part of every country's national health care policy and infrastructure.[2]

In this regard, statistics from the World Health Organization have indicated that 118.5 million blood donations were collected worldwide in 2020.[2] Forty percent of these donations were collected in high-income countries where 16% of the world's population lives.[2]

Correspondence: Turki Alanzi
Department of Health Information Management and Technology, College of Public Health, Imam Abdulrahman Bin Faisal University, King Faisal Road, Dammam, 31441, Saudi Arabia
Tel +966 133331211
Email talanzi@iau.edu.sa

With the advancement of information and communication technologies, new technological tools such as social networks and applications have been used for the blood donation process on a global scale.[1,3–12] However, finding blood donors is a challenging problem for blood banks in almost all countries.[13] In this sense, some specific applications have been developed such as the Blood application of the Red Cross, the Blood Donor Finder application of Neologix, BLOODR, and others.[4,7,11,13] The Red Cross Blood app has been used to locate and make appointments with blood banks and donation centers, the Blood Donor Finder allows users to find the closest donors when they need a blood donation, and BLOODR provides proper communication between patients and donor regarding the donation requirements.[4,7,13] Also, Ouhbi et al carried out a systematic search of blood donation applications available in Apple apps, Google Play, BlackBerry App World, and Windows Mobile stores.[1]

In Saudi Arabia, some studies have been carried out to investigate the blood donor system in this country that relies mainly on voluntary and involuntary donors.[14–17] One of these studies conducted in the central region of Saudi Arabia revealed that blood donation was not satisfactory, probably due to misconceptions, lack of education, and an unfavourable attitude towards the donation.[15] Another study conducted with Saudi male students from King Saud University found that 98% of participants considered donating blood to be important and suggested that campaigns were needed to raise awareness and motivate students about the importance of blood donation.[17] Similarly, Al-Johar et al found that in Saudi Arabia the attitude of female students towards donating blood was positive and their contribution to the donation system could be increased by conducting awareness and education campaigns.[16] Also, in a survey conducted by Statista in Saudi Arabia in 2018, it was observed that 58% of respondents agreed to donate blood to help others.[18] In a different context, Abdel Gader et al investigated the attitudes, feelings, beliefs, and motivations of a sample of blood donors from Saudi Arabia.[14] The results indicated that 91% of the participants thought that it was not necessary to pay for the blood donation.[14] Furthermore, 34% of the participants did not complain about donating blood routinely, and 67% of them did not mind going to the donation center to donate blood.[14] Also, 91% of the participants considered donating blood to be a religious duty.[14] About this last result, religion is strongly rooted in the Saudi society where the Muslim religion is prevalent.[14] It is worth mentioning that this religion motivates the donation of blood in safe conditions for both the donor and the recipient.[14] Concerning this topic some studies have pointed out that the religious beliefs can have a positive or negative impact on the blood donation process.[19,20] In this sense, a study carried out in Nigeria revealed that 20.3% of the participants did not donate blood and did not accept blood transfusions due to their religious beliefs.[21]

In this regard, to reduce the gap between donors and blood banks in the Kingdom of Saudi Arabia and facilitate the blood donation process, the Minister of Health launched the Wateen app in 2019 (March).[22] The objective of this initiative was to provide blood to all blood banks in the Kingdom, promote donation and awareness campaigns on voluntary blood donation, facilitate the procedures of the blood donation process, and improve the experience of voluntary blood donors.[23] The application was called "Wateen" to relate it to the aorta, which is the major artery in the human body.[23] The name also refers to the fact that giving blood means giving life.[23] The Wateen app has become the official blood donation app in Saudi Arabia.[23] This application offers multiple uses for blood donors, non-donors, or representatives of blood banks.[24] Through the Wateen application, the user can request a blood donation from the Wateen community, share donation requests on social networks, know the list and locations of blood banks, follow donation records, and receive reminders about campaigns and donation dates.[24] The Health Ministry hopes that using this technology, blood banks will have a sufficient supply of blood and its components in the future.[23]

According to the literature review, there have been no studies on the use of the Wateen app in Saudi Arabia. And, to the best of the authors' knowledge, there are no published studies on the acceptability of blood donation apps by blood donors and requesters. In this sense, the main objective of this research was to investigate the opinion of the population of Saudi Arabia on the use and acceptability of the Wateen application.

Methods
Study Settings and Participants

This research was a quantitative cross-sectional study aimed at determining the use and acceptability of the Wateen app among the population of Saudi Arabia. In achieving the research aim, the different tasks including planning, data collection, data analysis, preparing the

report were divided among the research team in order to simplify the process of achieving research tasks and minimize the risks. In this investigation, an online questionnaire was distributed among the target population who were healthy people over 18 years of age who had used or known about the Wateen application. The participants were informed about the objective of the study with a brief introduction to the Wateen application. In addition, their consent to participate in the study was requested and it was clearly stated to them that participation was completely voluntary and without obligation to answer the questionnaire. The Institutional Review Board at Imam Abdulrahman Bin Faisal University in Dammam, Saudi Arabia, approved the ethical protocol of this research and it was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from the participants.

Inclusion and Exclusion Criteria

The population included in the study were healthy people over 18 years of age who had used or known about the Wateen application. The rest of the population was excluded.

Description of the Questionnaire

The questionnaire was designed and revised by the research team constituted by an assistant professor, a master student, and a specialist who worked in a blood bank laboratory of a Saudi Arabian hospital. The questionnaire consisted of two sections and twelve questions. The questions were closed-ended and multiple choice. Question 10 had an open entry to allow the participant to write a comment. Also, question 12 had the option "other". The questionnaire is shown in Appendix 1.

The first section had 3 questions about the demographic information of the participants: What is your gender? (male, female); What is your age group in years? (18–25, 26–30, 31–35, 36–40, 41–45, 46–51, >52); Where do you live? (East province, West province, Central province, North province, South province).

The second section had 9 questions about the usage and acceptability of the Wateen application: Did you use the Wateen application before? (yes, no); Do you know anyone who used the Wateen application? (yes, no); What did you use the Wateen application for? (blood donation request from the Wateen community, donation requests in social networks, list and locations of blood banks, follow the donation record, all of the above); Did the Wateen

application motivate you to donate blood? (yes, no); Did the Wateen application educate you about the donation process? (yes, no); From your personal experiences, did the Wateen application help you to find blood donors for your patient/family member/beloved one? (yes, no); Do you believe that the Wateen application reduced the gap between blood donors and the need for blood in hospitals? (yes, no; if no, why … ?); Did you ever receive broadcasts or see a post or campaign requesting blood donation through the Wateen application on social media platforms? (yes, no); What was your most likely response when you received the blood donation request on the Wateen application? (nothing, share it with others, it encouraged me to donate blood, other).

Validity and Reliability of the Questionnaire

The questionnaire was validated by 3 experts in the field of blood donation. In addition, a pilot test was conducted with 10 participants to test the reliability of the questionnaire. The responses were consistent, indicating that the questionnaire was reliable. Furthermore, Cronbach's alpha was used to test the reliability of the items. All the relevant items in the questionnaire achieved alpha values greater than 0.80 indicating good reliability.

Data Collection

As the objective of this study was to identify and evaluate the usability and acceptance of Wateen application for blood donation process, the need to include a diverse group with large sample was realised. To calculate the sample size (S), Cochran's formula[28] was used.

$$ S = \frac{Z^2 pq}{e^2} $$

- e is the desired level of precision (ie, the margin of error),
- p is the (estimated) proportion of the population which has the attribute in question,
- q is 1 – p.

At p = 0.5, and 95% confidence interval, and at least 5%—plus or minus—precision; a 95% confidence level gives us Z values of 1.96, per the normal tables, so the sample size was calculated to be 385.

S = ((1.96)^2 (0.5) (0.5))/(0.05)^2 = 385

To achieve the estimated sample, the research team realized the need to use various online platforms for

Table 1 Demographic Information of the Participants (n=352)

Demographic Characteristics	n (%)
Gender	
Male	181 (51.4)
Female	171 (48.6)
Living location	
East province	171 (48.6)
West province	52 (14.8)
Central province	112 (31.8)
North province	5 (1.4)
South province	12 (3.4)
Age (years)	
18–25	137 (38.9)
26–30	62 (17.6)
31–35	45 (12.8)
36–40	32 (9.1)
41–45	33 (9.4)
46–51	25 (7.1)
> 52	18 (5.1)

distributing the survey, and random sampling technique was adopted to reach maximum population, as it ensures that results obtained from your sample should approximate what would have been obtained if the entire population had been measured.[29] Accordingly, the questionnaire was developed using QuestionPro application,[30] to which a survey link was generated. The survey link was distributed through WhatsApp, Twitter, Instagram, and other social media platforms. Data collection was carried out between November and December 2020.

Data Analysis

A basic descriptive statistical analysis was used to analyze the data collected from the questionnaire. The results were presented in percentages in tables and figures.

Results

The demographic information of the 352 participants of this study is shown in Table 1. This table indicates that 51.4% of the respondents were male. Moreover, the majority of the participants (69.3%) were under 35 years of age. Also, almost half of the respondents (48.6%) were from the East province of Saudi Arabia.

Regarding the use of the Wateen application, Figure 1 reveals that (57.1%, 201/352) of the participants had used the Wateen application before. Similarly, Figure 2 displays that (45.2%, 159/352) of the respondents knew people who had used the Wateen app previously. The t-tests were conducted considering all the participants (N=352) to identify if there is any difference in the findings between the different participant groups (Table 2). Focusing on the usage of Wateen application, significant difference was observed among the male (N=181, Mean=0.66) and female (N=171, Mean=0.47) participants with t-value = 8.438 and p-value < 0.0001 ($p<0.05$) at 95% confidence interval. Furthermore, the usage of the application was analyzed between two
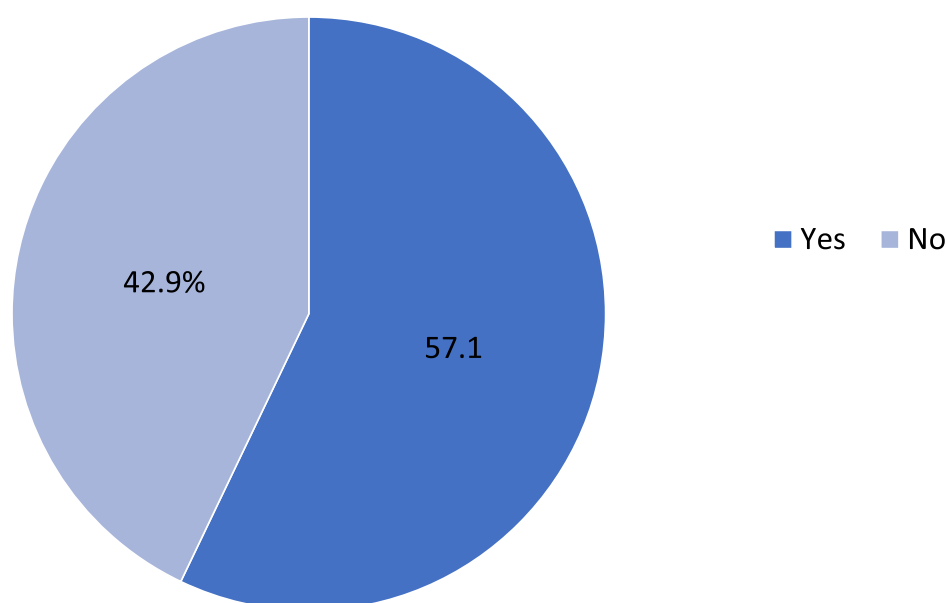
Figure 1 Usage of the Wateen App (n=201).

Figure 2 Knew people who used the Wateen App (n=159).

groups: participants aged 30 or less; and participants aged more than 30 years. Significant difference was observed among the participants aged 30 years or less (N=199, Mean=0.84) and the participants aged more than 30 years (N=153, Mean=0.57) participants with t-value = 12.4585 and p-value < 0.0001 (p<0.05) at 95% confidence interval.

Also, according to Table 3, the participants had used the Wateen app to follow the donation record (35.8%, 72/201), as a reminder of dona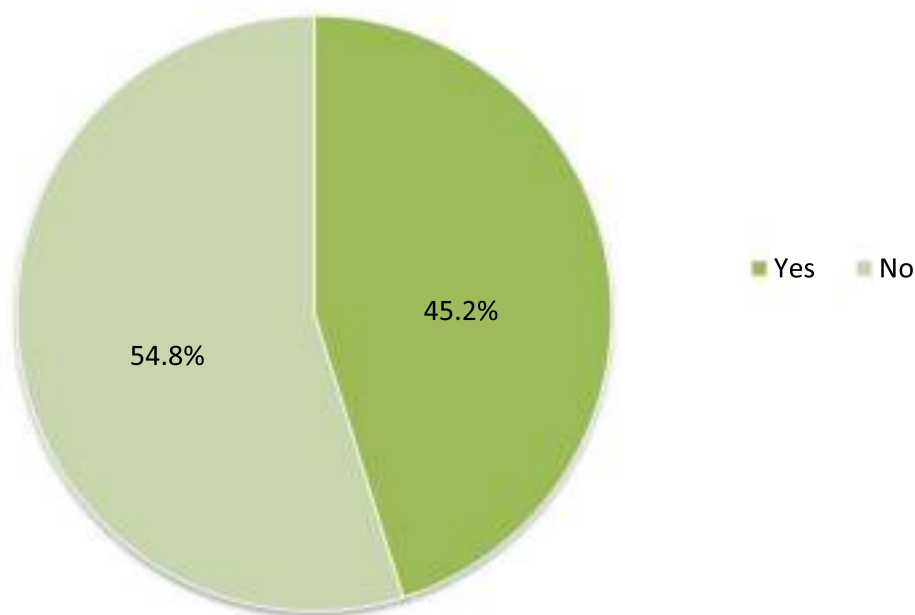tion dates and campaigns (24.4%, 49/201), find lists and blood bank locations (16.9%, 34/201), request blood donation from the Wateen community (10.0%, 20/201), find donation requests on social media (6.0%, 12/201), and all of the above objectives (7.0%, 14/201).

In addition, Table 4 indicates that after receiving the blood donation request from the Wateen app, 85.6% (172/201) of the respondents felt encouraged to donate blood, 10.0% (20/201) of them shared the information with others, 2.5% (5/201) answered "nothing", and 2.0% (4/201) replied "other".

Similarly, Figure 3 shows that (94.0%, 189/201) of the participants were motivated to donate blood by the Wateen app. Alike, Figure 4 indicates that 81.6% (164/201) of the respondents were educated about the donation process by the Wateen app. As well, Figure 5 illustrates that 30.8% (62/201) of the participants who used the Wateen app indicated that the application helped them to find donors. T-tests were conducted considering all the participants who used Wateen

application (N=201) to identify if there are and difference in the findings between the different participant groups (Table 2). Focusing on the motivation of Wateen application for engaging in blood donation, significant difference was observed among the male (N=104, Mean=0.58) and female (N=97, Mean=0.72) participants with t-value = 6.1319 and p-value < 0.0001 (p<0.05) at 95% confidence interval. Furthermore, the results were analyzed between two groups: participants aged 30 or less; and participants aged more than 30 years. Significant difference was observed among the participants aged 30 years or less (N=112, Mean=0.72) and the participants aged more than 30 years (N=89, Mean=0.54) participants with t-value = 4.1819 and p-value < 0.0001 (p<0.05) at 95% confidence interval.

Related to the reduction of the gap between blood donors and the need for blood, Figure 6 shows that 89.1% (179/201) of the respondents stated that the Wateen app had reduced this gap. T-tests were conducted considering all the participants who used Wateen application (N=201) to identify if there are and difference in the findings between the different participant groups (Table 2). Focusing on the education/learning enabled by Wateen application about blood donation, no significant difference was observed among the male (N=104, Mean=0.64) and female (N=97, Mean=0.68) participants with t-value = 0.9318 and p-value =0.3526 (p>0.05) at 95% confidence interval. Furthermore, the results were analyzed between

Table 2 T-Tests Results

	N	Mean	Standard Deviation	*df*	*t*-value	*p*-value
	Usability: By gender					
Male Female	181 171	0.66 0.47	0.25 0.16	350	8.438	<0.00001*
	Usability: By age					
≤ 30 years > 30 years	199 153	0.84 0.57	0.21 0.19	350	12.4585	<0.00001*
	Motivation: By gender					
Male Female	104 97	0.58 0.72	0.13 0.19	199	6.1319	<0.00001*
	Motivation: By age					
≤ 30 years > 30 years	112 89	0.72 0.54	0.26 0.35	199	4.1819	<0.00001*
	Education: By gender					
Male Female	104 97	0.64 0.68	0.21 0.38	199	0.9318	0.3526 (*p*>0.05)
	Education: By age					
≤ 30 years > 30 years	112 89	0.74 0.69	0.18 0.23	199	1.7292	0.0853 (*p*>0.05)

Note: *Statistically significant.

two groups: participants aged 30 or less; and participants aged more than 30 years. No significant difference was observed among the participants aged 30 years or less (N=112, Mean=0.74) and the participants aged more than 30 years (N=89, Mean=0.69) participants with *t*-value = 1.7292 and *p*-value = 0.853 (*p*>0.05) at 95% confidence interval.

On the other hand, Figure 7 suggests that 48.9% (172/ 352) of the participants expressed that they had not

Table 3 Use of the Wateen App for (N= 201)

Participants' Responses	n (%)
A blood donation request from the Wateen community	20 (10.0)
Donation requests in social networks	12 (6.0)
List and locations of blood banks	34 (16.9)
Follow the donation record	72 (35.8)
Reminders of donation dates and donation campaigns around you	49 (24.4)
All of the above	14 (7.0)

received or observed a post about the Wateen application on social media networks. Concerning this issue, most of the respondents expressed the following weaknesses of the blood process using the Wateen app:

1. The Wateen application needs more advertising on the most powerful social media platforms to increase awareness of the population.
2. In the application, there is no way to communicate between the donor and the person who needs the blood.
3. Unfortunately, Wateen App does not connect with all hospitals in the Kingdom.

Table 4 Response When the Blood Donation Request from the Wateen App Was Received (n=201)

Participants' Responses	n (%)
Nothing	4 (2.0)
Share it with others.	20 (10.0)
It encouraged me to donate blood.	172 (85.6)
Other	5 (2.5)
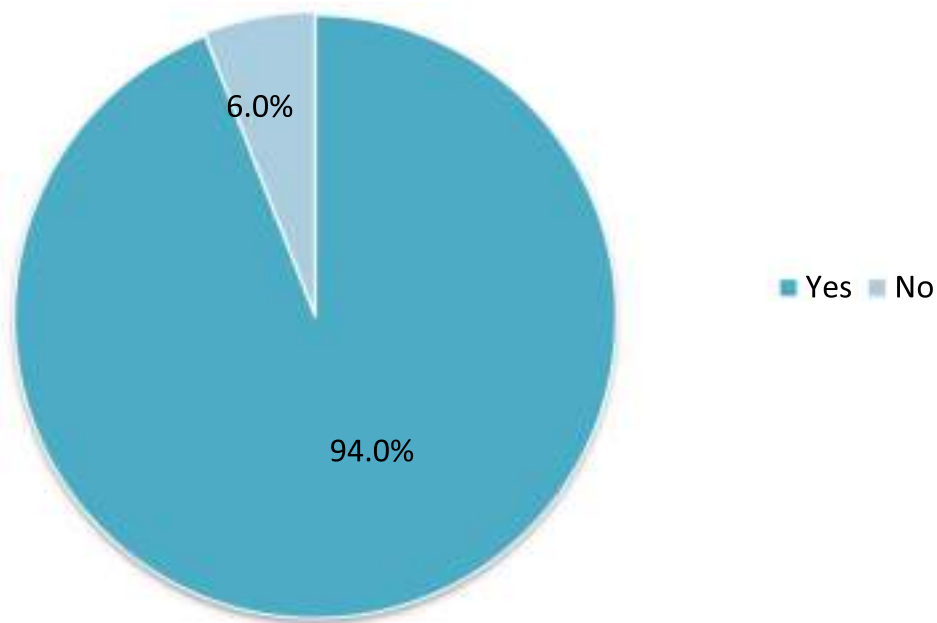
Figure 3 Wateen App motivation to donate blood (n=201).
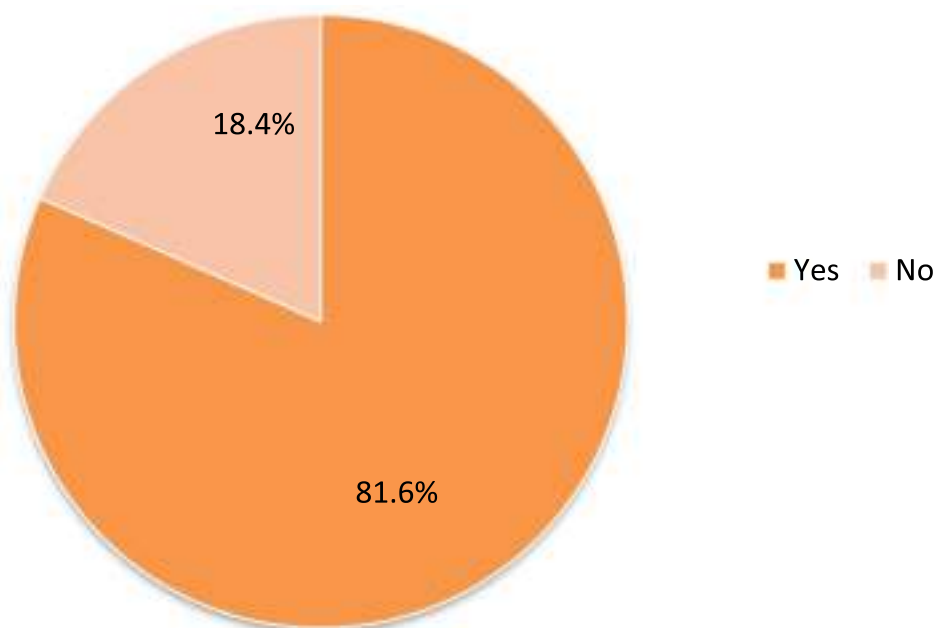
Figure 4 Wateen App education about donation (n=201).

Discussion

Regarding the outcomes of this research on the use of the Wateen app in the blood donation process in Saudi Arabia, it was observed that more than half of the respondents (57.1%) had used the Wateen app. Also, the Wateen app motivated and encouraged most of the participants to donate blood when they received the request for blood donation.

It is argued[31] that acceptability of digital health interventions may usefully be considered an emergent property of a complex, adaptive system of interacting components (eg, affective attitude, beliefs), which in turn influences
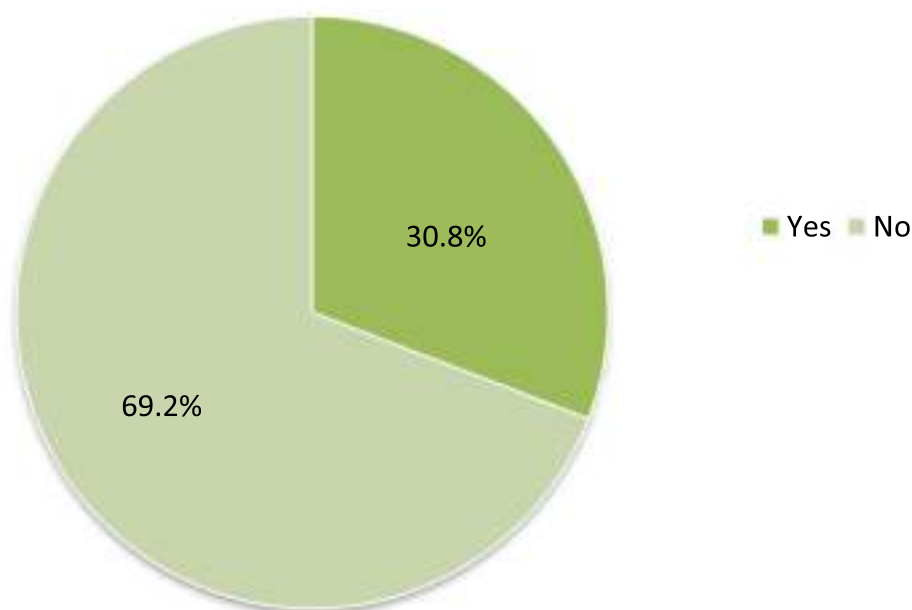
Figure 5 Help of the Wateen App to find blood donors (n=201).
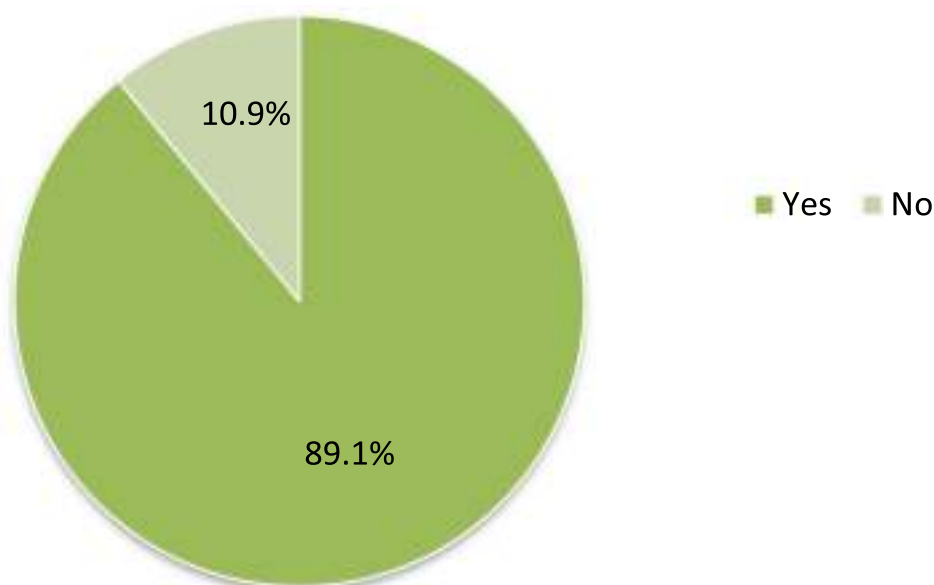
Figure 6 Wateen App reduced gap between blood donors and the need for blood (n=201).

(and is influenced by) user engagement. Accordingly, this study identified that usability and motivation among the participants regarding the Wateen application for blood donation were identified to be satisfactory, though significant differences existed among both genders and different age groups. Young participants were identified to be more motivated and found good usability of the application

compared to older patients (Table 2). While male participants found it to have high usability, female participants identified it to be more motivating. Secondly, acceptability is important due to its ability to predict and explain key outcomes of interest, including user engagement and intervention effectiveness. Findings revealed that the participants found the application to be effective for learning
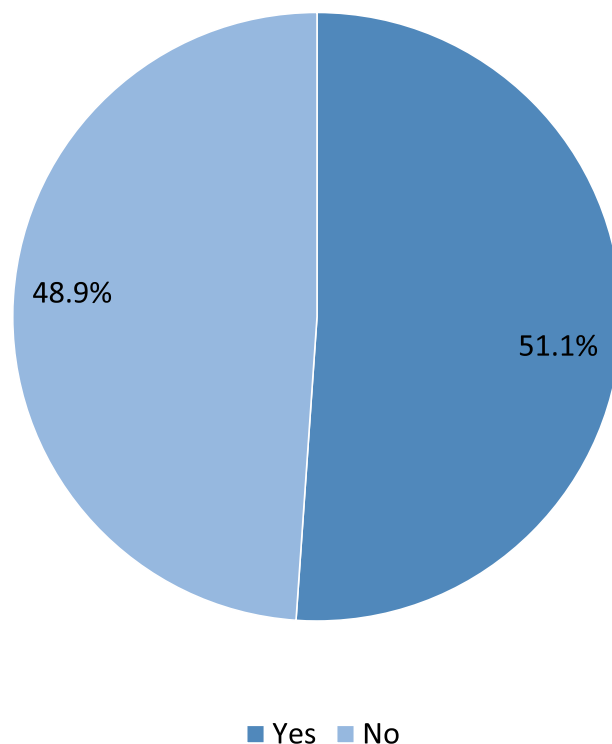
about blood donation, which can raise interest and engagement. Finally, precisely what people find acceptable is deeply contextualized and interlinked with prevailing social and cultural norms. The Saudi culture reflects a positive approach for blood donation, indicating the influence of culture on the acceptability of the application.

Since the Wateen app was recently launched by the Ministry of Health (March 2019), the aforementioned results suggested that there was a relatively high acceptance and use of the Wateen app among the participants in Saudi Arabia.[23] These results are aligned with the fact that applications can be valuable tools for the blood donation process and are accepted nationally and internationally for this purpose.[4–7,11,13,25] Furthermore, we also think that the high acceptance of the Wateen application is because the Arab people consider that blood donation is a humanitarian, noble, and religious activity.[14] It is pertinent to mention that, in general, the Arab people have a positive attitude towards blood donation.[16–18]

Another relevant part of this study was determining what the participants used the Wateen app for. The results described in Table 2, insinuate that a culture about blood donation is being implemented in Saudi Arabia. This culture would increase if the chain of the donation process

was smooth and made it easier for donors to follow the donation record, remind them of the date of donation campaigns, list the blood available in nearby donation centers, and facilitate other functions available in the application. It can be suggested that the creation of a blood donation culture in Saudi Arabia is feasible because the Saudi population has a motivating positive approach towards donating blood.[14,16–18] Until now, the majority of respondents thought that the Wateen app had narrowed the gap between blood donors and the need for blood.

Based on the results of this study, it would be very helpful to powerfully advertise the Wateen app to a wider audience via social media platforms and other sources. In connection with this comment, Alanzi et al have pointed out that social media platforms can help implement a robust blood donation program in Saudi Arabia that would encourage and motivate people to donate blood.[3] Similarly, Abdelgader et al have indicated that there is a high potential for volunteer donors in Saudi Arabia, but proper administrative planning is necessary to ensure that blood banks rely on voluntary donations and fill gaps in the donation system of the Kingdom.[26] In the same context, if the Wateen app were linked or connected to government apps like Absher, Saudi citizens who are eligible to donate blood would be more aware of the potential of this app for donation purposes.[27] Furthermore, studies[31,32] observed that eHealth interventions can be adopted in times of health emergency, as a convenient, safe, scalable, effective, and green method of providing clinical care. In this regard, since the Wateen app is a national app endorsed and launched by the Saudi Arabian Ministry of Health, it must be connected to all public and private hospitals. By incorporating the above-mentioned requirements into the blood donation process in Saudi Arabia, the Wateen app would be a potentially valuable tool in promoting blood donation to a high proportion of the population of this country. However, there are few issue identified in this study, which include poor advertising, communication issues on the application, and lack of connectivity of the application with all hospitals. Accordingly, the reviews in the play store[33] indicated few negative reviews and poor rating (2.1/5) for the Wateen application. Considering these factors, it is important for the developers (Ministry of Health) to address the technical issues in the application and improve its usability by linking the application with all major hospitals across the country to simplify the blood donation process.

Conclusion

The results of this research indicated that there is a general acceptance of the use of the Wateen app among the participants in this study. Based on the findings, this study can have various practical implications. Firstly, it allows the decision-makers (Ministry of Health) in evaluating the application and making necessary improvements to it. Secondly, in evaluating the participants' usability aspects analyzed by the demographic characteristics, necessary decisions can be made in order to increase the usability among the targeted population. Furthermore, the findings also help the decision-makers in understanding the various issues associated with the application such as technical/usability issues and address them for increasing the usability.

The main limitations of this study were related to the fact that the questionnaire was conducted online, which resulted in a limited number of participants. In addition, limited time and resources also posed a challenge. Furthermore, no studies related to the acceptability of blood donation applications by blood donors and requesters were found in the literature. This fact restricted our discussion because we were unable to compare the results of this research with other studies related to the acceptability of blood donation apps.

While the stated limitations have no impact in terms of nullifying the results observed in this investigation, future studies will be addressed to minimize these limitations. Also, it would be interesting to study the barriers to using the Wateen app. Furthermore, it would be important to compare the Wateen app with other apps available around the world, including apps accessible on Google Play and Apple app stores. Future research may focus on addressing these limitations, develop strategies for increasing the use of eHealth interventions such as Wateen for various healthcare needs and evaluate them.

Acknowledgments

We thank Mr. Aqeel Al Otaibi of John Hopkins Aramco Healthcare, the blood bank laboratory at King Fahad Specialist Hospital, and Ms. Atheer Al Otaibi for their help in facilitating the data collection.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Ouhbi S, Fernández-Alemán J, Toval A, et al. Free blood donation mobile applications. *J Med Syst*. 2015;39:52. doi:10.1007/s10916-015-0228-0
2. World Health Organization. Blood safety and availability. Available from: https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability. Accessed February 12, 2021.
3. Alanzi T, Alsaeed B. Use of social media in the blood donation process in Saudi Arabia. *J Blood Med*. 2019;10:417–423. doi:10.2147/JBM.S217950
4. Blood App. American red cross; 2016. Available from: http://www.redcrossblood.org/bloodapp. Accessed February 12, 2021.
5. Fahim M, Cebe H, Rasheed J, et al. MHealth: blood donation application using android smartphone. 2016 6th Int. Conf. Digit. Inf. Commun. Technol. Its Appl. DICTAP; 2016:35–38. doi:10.1109/DICTAP.2016.7543997.
6. Jenipha T, Backiyalakshmi R. Android blood donor life saving application in cloud computing. *Biom Bioinf*. 2014;6(1):34–36.
7. Google. Blood donor finder; 2016. Available from: https://play.google.com/store/apps/details?id=com.Neologix.BloodDonorFinder&hl=en. Accessed February 12, 2021.
8. Siromani U, Thasian T, Isaac R, et al. WhatsApp: a new tool for recruitment and retention of voluntary blood donors. *Int J Adv Med Health Res*. 2015;2(1):72. doi:10.4103/2349-4220.159176
9. Yuan S, Chang S, Uyeno K, et al. Blood donation mobile applications: are donors ready? *Transfusion*. 2016;56(3):614–621. doi:10.1111/trf.13387
10. Abbasi R, Maqbool O, Mubashar M. Saving lives using social media: analysis of the role of twitter for personal blood donation requests and dissemination. *Telematics Inf*. 2020;35:892–912.
11. The NHS Give Blood App. Available from: https://www.blood.co.uk/the-donation-process/the-nhs-give-blood-app/. Accessed February 14, 2021.
12. Sümnig A, Feig M, Greinacher A, et al. The role of social media for blood donor motivation and recruitment. *Transfusion*. 2018;58 (10):2257–2259. doi:10.1111/trf.14823
13. Tatikonda V, El-Ocla H. BLOODR: blood donor and requester mobile application. *mHealth*. 2017;3:40. doi:10.21037/mhealth.2017.08.08
14. Abdel Gader A, Osman A, Al Gahtani F, et al. Attitude to blood donation in Saudi Arabia. *Asian J Transfus Sci*. 2011;5(2):121–126. doi:10.4103/0973-6247.83235
15. Abolfotouh M, Al-Assiri M, Al-Omani M. Public awareness of blood donation in Central Saudi Arabia. *Int J Gen Med*. 2014;7:401–410. doi:10.2147/IJGM.S67187
16. Al-Johar A, Al-Saud A, Abalkhail Y, et al. Why do Saudi women refrain donating their blood? A study on the attitude, belief and motivation of Saudi female university students towards blood donation. *Clin Lab*. 2016;62:771–779. doi:10.7754/Clin.Lab.2015.150718
17. Al-Faris A, Bahabri A, Abdullah A, et al. Attitude to blood donation among male students at King Saud University. *J Appl Hematol*. 2013;4:70–77.
18. Share of people who agree that they frequently give blood to help others in Saudi Arabia as of May 2018. Available from: https://www.statista.com/statistics/917703/saudi-arabia-attitudes-towards-blood-donation/. Accessed February 11, 2021.
19. Mann M, Votto J, Kambe J, et al. Management of the severely anemic patient who refuses transfusion: lessons learned during the care of a Jehovah's Witness. *Ann Intern Med*. 1992;117(12):1042–1048. doi:10.7326/0003-4819-117-12-1042
20. Rosengart T, Helm R, DeBoiss W, et al. Open heart operations without transfusion using a multimodality blood conservation strategy in 50 Jehovah's Witness patients: implications for a "bloodless" surgical technique. *J Am Coll Surg*. 1997;184(6):618–629.

21. Okpara R. Attitudes of Nigerians towards blood donation and blood transfusion. *Trop Geogr Med*. 1989;41(1):89–93.

22. SaudiGazette. Health Ministry launches blood donation app. SaudiGazette. Available from: http://saudigazette.com.sa/article/561386/SAUDI-ARABIA/Health-Ministry-launches-blood-donation-app. Accessed May 1, 2019.

23. MOH. MOH launches «Wateen» blood donation app; 2019. Available from: https://www.moh.gov.sa/en/Ministry/MediaCenter/News/Pages/news-2019-03-14-001.aspx.Accessed May 1, 2019.

24. Kfshrc. Wateen application; 2019. Available from: https://www.kfshrc.edu.sa/en/home/patientcare/patientsvisitorsinformation/4990. Accessed May 1, 2019.

25. Ahmed A, Christensen L. Improving and supporting blood donation practices in Khartoum Sudan blood banks through android mobile app and web application system. *Int J Comput Sci Trends Technol*. 2019;7(2):83–92.

26. Abdelgader A, Al Ghumkas A. The future of voluntary blood donation in the Kingdom of Saudi Arabia. *Transfusion*. 2020;60(1):28–34. doi:10.1111/trf.15683

27. 18m used Saudi Arabia's Absher services last year. Available from: https://www.arabnews.com/node/1774491/saudi-arabia. Accessed February 15, 2021.

28. Woolson R, Bean J, Rojas P. Sample size for case-control studies using cochran's statistic. *Biometrics*. 1986;42(4):927–932. doi:10.2307/2530706

29. Shadish WR, Cook TD, Campbell DT. Experimental and quasi-experimental designs for generalized causal inference. Cengage Learning: Boston, MA; 2002.

30. QuestionPro. Free online survey software and tools | questionPro® [Internet]. Questionpro.com; 2021 [cited September 6, 2021]. Available from: https://www.questionpro.com/. Accessed September 23, 2021.

31. Anthony B Jnr. Implications of telehealth and digital care solutions during COVID-19 pandemic: a qualitative literature review. *Inform Health Soc Care*. 2020;46(1):68–83. doi:10.1080/17538157.2020.1839467

32. Bokolo A. Application of telemedicine and eHealth technology for clinical services in response to COVID-19 pandemic. *Health Technol*. 2021;11(2):359–366. doi:10.1007/s12553-020-00516-4

33. Google Inc. Wateen [Internet]. Play.google.com; 2021 [cited September 6, 2021]. Available from: https://play.google.com/store/apps/details?id=com.wateen.rc&hl=en_IN&gl=US. Accessed September 23, 2021.

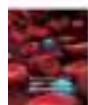# Successful Treatment of Factor X Deficiency in a Patient with Lymphoplasmacytic Lymphoma with Bendamustine Plus Rituximab Regimen: A Case Report and Literature Review

Tarinee Rungjirajittranon, Yingyong Chinthammitr & Chattree Hantaweepant

CASE REPORT

Successful Treatment of Factor X Deficiency in a Patient with Lymphoplasmacytic Lymphoma with Bendamustine Plus Rituximab Regimen: A Case Report and Literature Review

Tarinee Rungjirajittranon
Yingyong Chinthammitr
Chattree Hantaweepant

Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Background: Acquired factor X deficiency is an uncommon condition, and affected individuals have severe and spontaneous bleeding. The associated conditions include malignancy, infection, burn, and inflammatory bowel disease. Many previous studies reported association between lymphoproliferative disease and factor X disappearance. Amyloid deposition causing factor X absorption was the most common mechanism. Here, we report a case of stage IV lymphoplasmacytic lymphoma (LPL) with factor X deficiency who was successfully treated with bendamustine plus rituximab (BR) regimen.

Case Presentation: A 52-year-old Thai woman presented with heavy menorrhea, hoarseness, and widespread ecchymosis at her extremities. On physical examination, the patient had bilateral periorbital purpura and vocal cord hematoma. Coagulation testing showed prolonged prothrombin time (PT) and prolonged activated thromboplastin time (aPTT); however, after mixing with 1:1 normal pooled plasma, PT and aPTT were both corrected to normal levels. Factor assays demonstrated markedly decreased factor X levels, but no presence of factor X inhibitor. Bone marrow examination revealed numerous abnormal lymphoplasmacytoid lymphocytes with kappa light chain expression. Serum free light chain assay also showed kappa light chain restriction [kappa 716.16 mg/L, lambda 16.96 mg/L, ratio 42.23 (0.26–1.65)]. The patient was diagnosed as lymphoplasmacytic lymphoma with factor X deficiency. She received chemotherapy with 6 cycles of bendamustine plus rituximab (BR) regimen. The patient responded favorably to treatment, she remains in lymphoma remission at one year after diagnosis, and her factor X level was more than 20%.

Conclusion: We performed a literature review to identify previous case reports about lymphoma-associated factor X deficiency or inhibitor to determine a possible explanation in our patient. It is important to emphasize that when patients present with acquired factor deficiency, including factor X, lymphoproliferative disease is commonly one of the underlying conditions. Furthermore, the recovery of coagulation factor deficiency is possible if successful remission of lymphoma can be achieved.

Keywords: factor X, lymphoproliferative disorder, lymphoplasmacytic lymphoma, amyloidosis

Correspondence: Chattree Hantaweepant
Clinical Lecturer of Hematology, Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkoknoi, Bangkok, 10700, Thailand
Tel +66 2-419-4448
Email chattree.hantaweepant@gmail.com

Introduction

Factor X, which is a vitamin k-dependent coagulation factor, is synthesized by hepatocytes.[1] It is activated by tissue factor and FVIIa from the extrinsic pathway.[2] Factor IXa and factor VIIIa from the intrinsic pathway work concomitantly to change

factor X to active form.[2] The resulting effect is the formation of thrombin and fibrin clots.[2] Bleeding manifestation in deficient patients is well correlated with the amount of residual factor X activity.[3] Patients with a factor X level less than 10–20 IU/dL usually experience severe and spontaneous bleeding.[4–6] The estimated prevalence of congenital factor X deficiency (Stuart-Prower disease) is approximately 1 in 1 million; however, data specific to factor X acquired deficiency/inhibitor remains scarce.[3,6] Being different from other coagulation factors, the presence of factor X auto-inhibitor is less common than factor X deficiency.[7] Previous studies reported factor X inhibitor to be associated with respiratory tract infection, leprosy, gastrointestinal lymphoma, burn, and inflammatory bowel disease.[8–11] In contrast, the most common cause of acquired factor X deficiency is light chain amyloidosis, which is a plasma cell dyscrasias that is characterized by deposition of amyloid fibrils from abnormal monoclonal immunoglobulins.[12] The proposed mechanism of factor X deficiency in systemic amyloidosis is rapid clearance of factor X by amyloid fibril absorption and probably splenic sequestration.[13,14] Here, we report a case of an adult Thai female patient who was recently diagnosed as lymphoplasmacytic lymphoma with factor X deficiency due to suspected amyloid deposition.

Case Presentation

A 52-year-old Thai woman presented with heavy menorrhea for 4 months. Two months later, she felt fatigue, anorexia, and noticed spontaneous hematoma along her extremities. Two weeks ago, she had a low-grade fever and odynophagia, so she visited a primary hospital and physical examination showed a cachectic woman with an 8 cm hematoma at her left forearm. Throat exam showed ecchymosis at the epiglottis and vallecula. Non-blanchable erythematous patches were noticed at both upper eyelids (Figure 1). Other organ systems were within normal limits. During admission at that primary hospital, her laboratory tests showed prolonged prothrombin time (PT) and prolonged activated thromboplastin time (aPTT) with no recovery after plasma and vitamin K replacement (Figure 2). However, her hematoma was improved without new bleeding symptoms. She was then transferred to our hospital, which is a national tertiary referral center, for further investigation. Her initial complete blood count showed hemoglobin of 8.5 g/dl, mean corpuscular volume of 80 femtolitres, a white blood cell count of 6.9×10^9/L (neutrophil 65.4%, lymphocyte 62%, monocyte 5%), and increased platelet count of 800×10^9/L. Blood chemistry showed LDH level of 235 U/L (normal 135–214 U/L), normal liver function tests, and normal creatinine level. Coagulation study showed prolonged PT and prolonged aPTT (65.7 seconds (10.5–12.5) and 85.1 seconds (22.0–31.0), respectively). After mixing with 1:1 normal pooled plasma, PT and aPTT was 13.8 seconds and 28.8 seconds, respectively. The fibrinogen level was 410 mg/dl (200–400). The levels from coagulation factor II, V, and X assay were 117.7% (50–150%), 125.5% (50–150%), and 2.1% (50–150%), respectively. The Bethesda assay of factor X inhibitor was negative. The result of the mixing study suggested that this patient had no functional auto-antibodies. However, our patient's PT and aPTT levels did not improve after plasma or vitamin K replacement. Bone marrow Wright's stain and pathological examination showed 50–60% infiltrations of kappa+/CD20+ small lympho-plasmacytoid lymphoid cells (Figure 3). Congo red stain was negative. Serum protein electrophoresis and immunofixation were normal. Free kappa light chain was markedly elevated [Kappa 716.16 mg/L, Lambda 16.96 mg/L, ratio 42.23 (0.26–1.65)]. A diagnosis of stage IV lymphoplasmacytic lymphoma was made. Based on the presence of typical skin changes and light chain monoclonal gammopathy, factor X deficiency from systemic

Figure 1 Periorbital purpura; skin changes in this patient.
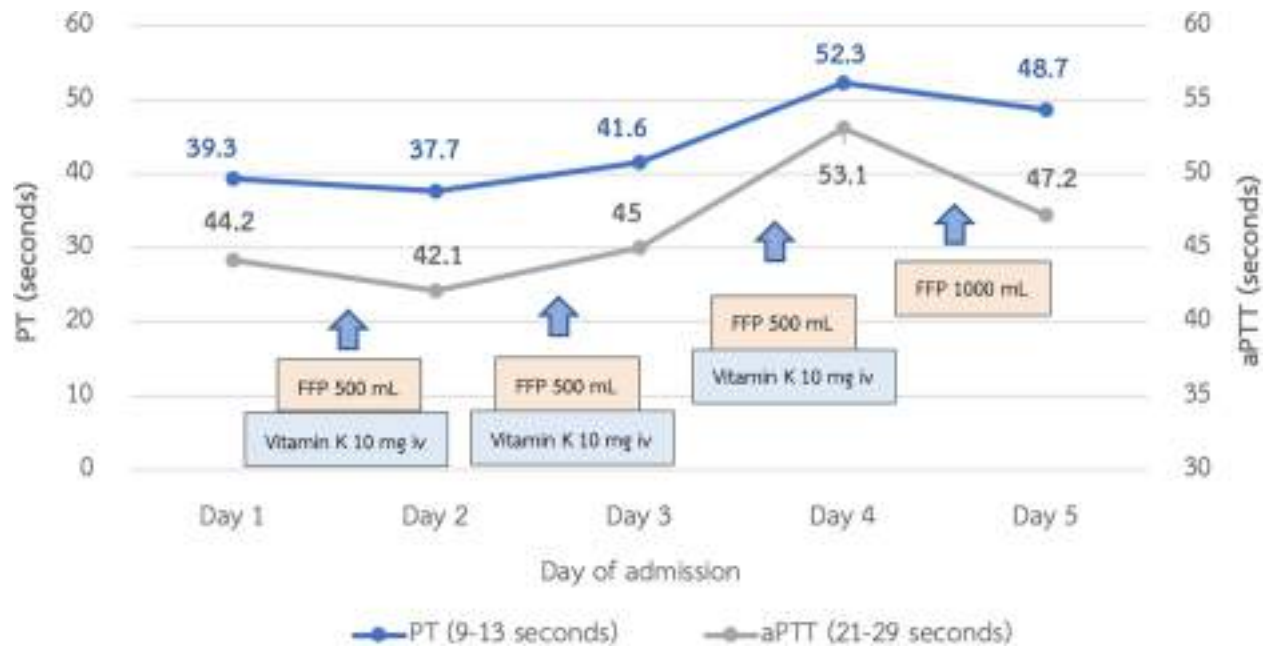
Figure 2 Treatment and coagulogram at previous hospital.

amyloidosis was suspected. To demonstrate amyloid deposition, we carefully performed an abdominal fat pad biopsy immediately after fresh frozen plasma replacement. No bleeding consequence developed after the procedure, and the result was negative. More extensive biopsy was not performed due to a concern about bleeding complication. Echocardiography showed no specific pattern of cardiac amyloidosis. Computed tomography scan of neck, chest, and abdomen revealed multiple enhancing mediastinal lymph nodes, the largest of which was 1.5 cm in diameter.

Systemic chemotherapy of bendamustine plus rituximab (BR) for 6 cycles was the chosen regimen to minimize toxicities. A good correlation between factor X normalization and disease remission was demonstrated. Improvement in abnormal laboratory results after treatment are shown in Table 1. Since our patient's residual
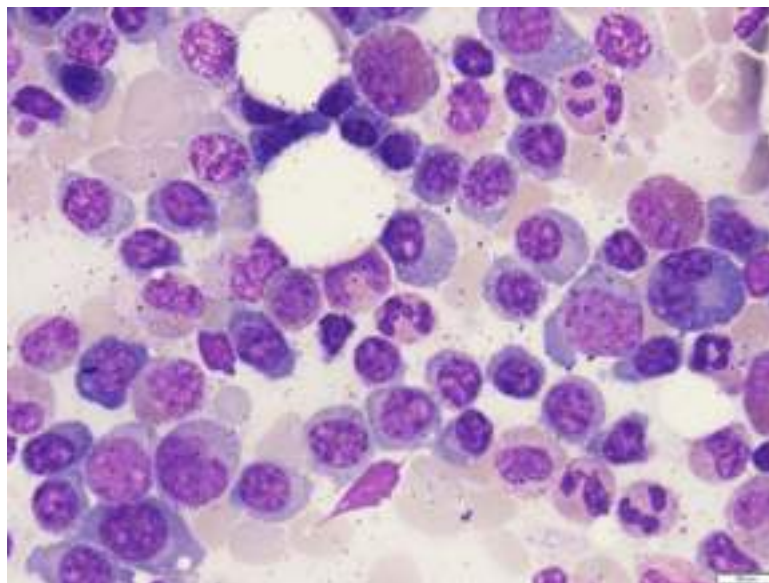
Figure 3 Bone marrow aspirate smear.

Table 1 Follow-Up Laboratory Investigations After Treatment

Lab	Normal	At Diagnosis	After 1st BR	After 2nd BR	After 3rd BR	After 4th BR	After 5th BR	After 6th BR
PT	10.5–12.5 s	65.7	67.6	47.7	N/A	25.4	22.5	18.7
APTT	22.0–31.0 s	85.1	64.6	66.2	N/A	44.1	38.8	40.7
Fibrinogen	200–400 mg/dl	410.1	279.0	N/A	N/A	N/A	N/A	N/A
Factor X assay	50–150%	2.1	1.1	1.5	N/A	5.4	9.8	13.9
Serum free light chain (mg/L)	κ: 3.30–19.40; λ: 5.71–26.30 κ:λ ratio: 0.26–1.65	κ 716.16; λ 16.96 (Ratio 42.23)	κ 131.22; λ 15.95 (Ratio 8.23)	N/A	κ 20.47; λ 7.51 (Ratio 2.73)	N/A	N/A	κ 12.07; λ 5.53 (Ratio 2.18)
Bone marrow study		Small B-cell lymphoid neoplasm with plasmacytic differentiation, favor lympho-plasmacytic lymphoma (LPL) 50–60% of total nucleated marrow cells	N/A	N/A	A small number of scattered PAX5+ small and medium-sized B cells 2–3% of total nucleated marrow cells	N/A	N/A	No abnormal lymphoid cells seen
CT neck, chest, abdomen		Multiple mediastinal nodes at left upper paratracheal, both lower paratracheal, and subcarinal regions, the largest one is measured about 1.5 cm in diameter			No significant intrathoracic and intraabdominal lymph node enlargement			No significant intrathoracic and intraabdominal lymph node enlargement

Abbreviations: aPTT, activated partial thromboplastin time; BR, bendamustine-rituximab; N/A, not available; PT, prothrombin time.

factor X increased to more than 10 IU/dL, she had no spontaneous bleeding. At 12 months after diagnosis, she maintained a good response after BR regimen with a factor X level of approximately 22%.

Discussion

We have described a case that well represents lymphoproliferative disorders associated with coagulation factor deficiency. Many previously published studies reported acquired coagulation inhibitors accompanying lymphoma, mostly acquired hemophilia A or factor VIII inhibitors.[15–20] Similarly, acquired factor X deficiencies or inhibitors showed association with lymphoproliferative diseases via three mechanisms.[7,21] The most prevalent mechanism was amyloid fibril absorption.[22] The second most commonly reported mechanism was non-functional antibody that enhanced factor X clearance from circulation.[23] The third and least common mechanism involves a pathologic functional antibody that was difficult to detect by citrate-based assays because it is a calcium-ion dependent anti-FX IgG antibody.[8,24,25] All of these cases required complicated treatments not only to manage severe bleeding, but also to control the primary disease (Table 2).

In our case, the in vitro mixing study demonstrated correctable aPTT to normal value, and PT to near-normal value by normal pool plasma. It showed factor deficiency pattern. However, both prolonged values were uncorrectable

after in vivo plasma and vitamin k replacement even though bleeding was clinically improved. The cause of factor X disappearance could be from either non-functional antibody or amyloid substances deposition. We hypothesized that the cause of factor X deficiency in this patient was from amyloid deposition. The evidence to support our hypothesis is that periorbital purpura, which was the pathognomonic skin sign, indicated weakening vascular integrity by amyloid deposition even though it occurred in only 15% of patients.[26] Moreover, the persistent low level of factor X after immunosuppressive treatment implied that it was not the natural course of autoimmune disease.[23] However, the level was high enough to prevent spontaneous bleeding. Due to concerns about patient safety, we decided not to perform any further biopsy to confirm our presumption of amyloidosis. A previous study by TashiroH[22] also could not demonstrate amyloid deposition in bone marrow or skin from antemortem biopsy; however, autopsy showed amyloid depositions in multiple organs, including liver, spleen, kidney, bone marrow, lymph nodes, adrenal glands, lungs, and heart. Those authors identified factor X accompanied by amyloid fibrils. Similarly, their patient's mixing study was correctable, but the coagulogram was not improved after replacement with substantial amounts of plasma and anti-inhibitor coagulant complex. The mechanism may be rapid clearance of factor X from circulation with subsequent rapid distribution of amyloid throughout the body.[13]

Table 2 Previous Case Reports of Lymphoma with Factor X Deficiency/Inhibitor

Name of Author and Year of Publication	Age and Sex of Patients	Type of Lymphoma	Bleeding Manifestation	Factor X Level at Diagnosis (%)	Treatment	Outcome After Treatment (Follow-Up Time)	Etiology of Decreased Factor X Level
Meenhuis A et al (2015)[23]	81-year-old man	Nodal marginal zone lymphoma	Soft tissue and intra-muscular hematoma	3–4%	Chlorambucil and rituximab	Normalization of factor X level (1 year after diagnosis)	Non-inhibitory antibody
Tashiro H et al (2018)[22]	37-year-old woman	Lympho-plasmacytic lymphoma	Not mentioned	2%	-Steroids - Cyclophosphamide - Rituximab - Fludarabine - Cladribine - Bortezomib - Thalidomide -Allogeneic SCT with matched sibling donor (RIC conditioning regimen)	Not improved	Sequestration by amyloid fibrils

Abbreviations: SCT, stem cell transplantation; RIC, reduced intensity conditioning regimen; CR, complete remission.

Our case demonstrates successful treatment of lympho-plasmacytic lymphoma and bleeding complication by BR regimen. However, a strategy to prolong our patient's survival poses an additional and more complex challenge. Autologous and allogeneic stem cell transplantation should be carefully considered due to the high rate of non-relapse mortality in lymphoplasmacytic lymphoma.[27] The benefits and drawbacks of this treatment alternative will be discussed with our patient so that a plan can be in place when and if our patient relapses.

Conclusion

Acquired factor deficiency is an acquired bleeding disorder that frequently presents with occult primary cause, especially hematologic malignancies. We presented an interesting case of factor X deficiency associated with lymphoproliferative disorder. The patient was treated with 6 cycles of BR regimen, she remains in lymphoma remission at one year after diagnosis, and her factor X level has increased to more than 20%.

Abbreviations

aPTT, activated thromboplastin time; PT, prothrombin time.

Data Sharing Statement

The datasets used in the current study are available upon reasonable request from the corresponding author.

Ethics Approval and Informed Consent

In Thailand, a case report does not require ethics approval. The patient gave written permission to publish her personal data.

Consent for Publication

Written informed consent was obtained from the patient for publication of the report and accompanying images. A copy of that consent form is available for review via a request from the Editor-in-Chief.

Author Contributions

All authors contributed to data analysis, drafting, or revising the article, gave final approval of the version to be published, agreed to the submitted journal, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests in this work.

References

1. Bajaj SP, Mann KG. Simultaneous purification of bovine prothrombin and factor X. Activation of prothrombin by trypsin-activated factor X. *J Biol Chem*. 1973;248(22):7729–7741. doi:10.1016/S0021-9258(19)43250-X

2. Sucker C, Zotz RB. The Cell-Based Coagulation Model. In: Marcucci CE, Schoettker P, editors. *Perioperative Hemostasis: Coagulation for Anesthesiologists*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2015:3–11.

3. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. *Blood*. 2015;125(13):2052–2061. doi:10.1182/blood-2014-08-532820

4. Peyvandi F, Palla R, Menegatti M, et al. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European Network of Rare Bleeding Disorders. *J Thromb Haemost*. 2012;10(4):615–621. doi:10.1111/j.1538-7836.2012.04653.x

5. Menegatti M, Peyvandi F. Treatment of rare factor deficiencies other than hemophilia. *Blood*. 2019;133(5):415–424. doi:10.1182/blood-2018-06-820738

6. Peyvandi F, Auerswald G, Austin SK. et al. Diagnosis, therapeutic advances, and key recommendations for the management of factor X deficiency. *Blood Rev*;2021. 100833. doi:10.1016/j.blre.2021.100833

7. Menegatti M, Biguzzi E, Peyvandi F. Management of rare acquired bleeding disorders. *Hematology Am Soc Hematol Educ Program*. 2019;2019(1):80–87. doi:10.1182/hematology.2019000066

8. Chan IS, Ogunsile FJ. An Acquired Factor X Inhibitor: the Importance of Understanding Coagulation. *Am J Med*. 2017;130(7): e307–e8. doi:10.1016/j.amjmed.2017.01.046

9. Hsia CC, Keeney M, Bosco AA, Xenocostas A. Treatment of acquired factor X inhibitor by plasma exchange with concomitant intravenous immunoglobulin and corticosteroids. *Am J Hematol*. 2008;83(4):318–320. doi:10.1002/ajh.21105

10. Lankiewicz MW, Bell WR. A unique circulating inhibitor with specificity for coagulation factor X. *Am J Med*. 1992;93(3):343–346. doi:10.1016/0002-9343(92)90244-6

11. Ness PM, Hymas PG, Gesme D, Perkins HA. An unusual factor-X inhibitor in leprosy. *Am J Hematol*. 1980;8(4):397–402. doi:10.1002/ajh.2830080408

12. Patel G, Hari P, Szabo A, et al. Acquired factor X deficiency in light-chain (AL) amyloidosis is rare and associated with advanced disease. *Hematol Oncol Stem Cell Ther*. 2019;12(1):10–14. doi:10.1016/j.hemonc.2018.05.002

13. Furie B, Greene E, Furie BC. Syndrome of acquired factor X deficiency and systemic amyloidosis in vivo studies of the metabolic fate of factor X. *N Engl J Med*. 1977;297(2):81–85. doi:10.1056/NEJM197707142970203

14. Rosenstein ED, Itzkowitz SH, Penziner AS, Cohen JI, Mornaghi RA. Resolution of factor X deficiency in primary amyloidosis following splenectomy. *Arch Intern Med*. 1983;143(3):597–599. doi:10.1001/archinte.1983.00350030211041

15. Nixon CP, Prsic EH, Guertin CA, Stevenson RL, Sweeney JD. Acquired Factor XIII inhibitor associated with mantle cell lymphoma. *Transfusion*. 2017;57(3):694–699. doi:10.1111/trf.13947

16. Gesierich W, Munker R, Geiersberger U, Pohlmann H, Brack N, Hartenstein R. Spontaneous Bleeding in a Patient with Malignant Lymphoma: a Case of Acquired Hemophilia. *Onkologie*. 2000;23(6):584–588.

17. Aljohani NI, Matthews JH. Acquired factor V inhibitor in a patient with mantle cell lymphoma presenting with hematuria followed by thrombosis: a case report. *Int Med Case Rep J*. 2014;7:27–30. doi:10.2147/IMCRJ.S59236

18. Regina S, Colombat P, Fimbel B, Guerois C, Gruel Y. Acquired inhibitor to factor VIII in a patient with Hodgkin's disease following treatment with interferon-alpha. *Haemophilia*. 2001;7(5):526–527. doi:10.1046/j.1365-2516.2001.00555.x

19. Kelsey PR, Leyland MJ. Acquired inhibitor to human factor VIII associated with paraproteinaemia and subsequent development of chronic lymphatic leukaemia. *Br Med J*. 1982;285(6336):174–175. doi:10.1136/bmj.285.6336.174

20. Lee ES, Hibsman BK, Liebman HA. Acquired bleeding disorder in a patient with malignant lymphoma: antibody-mediated prothrombin deficiency. *Cancer*. 2001;91(4):636–641. doi:10.1002/1097-0142-(20010215)91:4<636::AID-CNCR1046>3.0.CO;2-V

21. Lee G, Duan-Porter W, Metjian AD. Acquired, non-amyloid related factor X deficiency: review of the literature. *Haemophilia*. 2012;18 (5):655–663. doi:10.1111/j.1365-2516.2012.02773.x

22. Tashiro H, Shirasaki R, Watanabe M, Kawasugi K, Takahashi Y, Direct Factor SN. X sequestration by systemic amyloid light-chain amyloidosis. *Clin Case Rep*. 2018;6(3):513–515. doi:10.1002/ccr3.1398

23. Meenhuis A, van Vliet R, Hudig F, Ypma PF, Schipperus MR, Hollestelle MJ. Successful treatment of a noninhibitory antibody-mediated acquired factor X deficiency in a patient with marginal-zone lymphoma. *Clin Case Rep*. 2015;3(7):587–593. doi:10.1002/ccr3.294

24. Broze GJ Jr. An acquired, calcium-dependent, factor X inhibitor. *Blood Cells Mol Dis*. 2014;52(2–3):116–120. doi:10.1016/j.bcmd.2013.09.001

25. Kyriakou DS, Alexandrakis MG, Passam FH, et al. Acquired inhibitors to coagulation factors in patients with gastrointestinal diseases. *Eur J Gastroenterol Hepatol*. 2002;14(12):1383–1387. doi:10.1097/00042737-200212000-00016

26. Merlini G, Dispenzieri A, Sanchorawala V, et al. Systemic immunoglobulin light chain amyloidosis. *Nat Rev Dis Primers*. 2018;4(1):38. doi:10.1038/s41572-018-0034-3

27. Parrondo RD, Reljic T, Iqbal M, et al. Efficacy of Autologous and Allogeneic Hematopoietic Cell Transplantation in Waldenström Macroglobulinemia: a Systematic Review and Meta-analysis. *Clin Lymphoma Myeloma Leuk*. 2020;20(10):e694–e711. doi:10.1016/j.clml.2020.05.021

A Prospective Observational Study of Antihemophilic Factor (Recombinant) Prophylaxis Related to Physical Activity Levels in Patients with Hemophilia A in the United States (SPACE)

Barbara A Konkle, Doris V Quon, Leslie Raffini, Michael Recht, Vlad C Radulescu, Shannon L Carpenter, Amy L Dunn, Mei Lu & Maureen Watt

ORIGINAL RESEARCH

A Prospective Observational Study of Antihemophilic Factor (Recombinant) Prophylaxis Related to Physical Activity Levels in Patients with Hemophilia A in the United States (SPACE)

Barbara A Konkle[1]
Doris V Quon[2]
Leslie Raffini[3]
Michael Recht [4]
Vlad C Radulescu[5]
Shannon L Carpenter[6]
Amy L Dunn[7]
Mei Lu[8]
Maureen Watt[9]

[1]Department of Medicine, The University of Washington and Bloodworks Northwest, Seattle, WA, USA; [2]Orthopaedic Hemophilia Treatment Center, Orthopaedic Institute for Children, Los Angeles, CA, USA; [3]Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, PA, USA; [4]The Hemophilia Center, Oregon Health & Science University, Portland, OR, USA; [5]Department of Pediatric Hematology and Oncology, University of Kentucky Medical Center, Lexington, KY, USA; [6]Department of Hematology/ Oncology, Children's Mercy Hospital, Kansas City, MO, USA; [7]Hemophilia Treatment Center, Nationwide Children's Hospital and The Ohio State University College of Medicine, Columbus, OH, USA; [8]Takeda Development Center Americas, Inc, Lexington, MA, USA; [9]Outcomes Research & Epidemiology, Takeda Pharmaceuticals International AG, Zurich, Switzerland

Correspondence: Maureen Watt
Takeda Pharmaceuticals International AG, Thurgauerstrasse 130, Glattpark-Opfikon, Zurich, 8152, Switzerland
Tel +41 44 555 10 00
Fax +41 44 555 10 01
Email maureen.watt@takeda.com

Introduction: High collision-risk physical activity can increase bleeding risk in people with hemophilia A, as can increasing the time between factor VIII (FVIII) administration and physical activity. FVIII prophylaxis may be tailored to planned activities to prevent activity-related bleeding.
Aim: To explore the relationship between physical activity levels, FVIII infusion timing, and occurrence of bleeding in patients with severe/moderately severe hemophilia A without FVIII inhibitors receiving antihemophilic factor (recombinant) (rAHF; ADVATE®; Baxalta US Inc., a Takeda company, Lexington, MA, USA).
Methods: SPACE was a 6-month, prospective, multicenter, observational outcomes study (NCT02190149). Enrolled patients received an eDiary application and a wearable activity tracker, which recorded physical activity, rAHF infusion, and occurrence of bleeding. Physical activity risks were ranked using National Hemophilia Foundation criteria.
Results: Fifty-four patients aged 11–58 years (n = 47 prophylaxis, n = 7 on-demand) were included in the analysis. Patients had a mean (SD) 8.14 (10.94) annualized bleeding rate, and recorded 4980 intervals between an rAHF infusion and physical activity; 1759 (35.3%) of these intervals were ≤24 hours. Analysis of recorded eDiary data showed that the risk of activity-related bleeding did not significantly increase with time between last infusion and activity, but did increase with higher-risk physical activities. Analysis of activity tracker recorded data showed that the risk of bleeding reported by patients as spontaneous increased with prolonging time (≤24 to >24 hours) from last infusion to physical activity start (odds ratio 2.65, $p < 0.05$). Joint health data collected at baseline were not included in the regression analysis because of small sample size; therefore the study could not assess whether patients with more joint disease at baseline were at higher risk of injury-related and reported spontaneous occurrence of bleeding.
Conclusion: These results show that activities with a high risk of collision lead to an increased risk of bleeding. Further investigation is warranted to explore potential benefits of FVIII infusion timing to reduce the risks of activity-related occurrence of bleeding.
Keywords: hemophilia A, recombinant factor VIII, physical activity, post-authorization study, prophylaxis, bleeding

Introduction

In people with hemophilia A (PwHA), prophylactic factor VIII (FVIII) replacement therapy has been the standard of care for bleeding prevention by maintaining plasma FVIII activity levels of ≥1%.[1,2] The World Federation of Hemophilia

(WFH) 2020 guidelines recommend FVIII activity levels of ≥3%.[3] FVIII prophylaxis reduces frequency of bleeding with the goal of improving quality of life in PwHA.[4–6] Individualized prophylactic regimens that aim to tailor FVIII levels to lifestyle and physical activity requirements are likely to offer further beneficial effects in minimizing bleeding risk while facilitating the physical and psychosocial benefits of exercise.[7,8]

Limited data exist on the relationship between FVIII levels and bleeding risk in PwHA and physically active lifestyles. Participation in vigorous, high-risk collision sports has been associated with a transient increase in bleeding risk, with most activity-related episodes manifesting within 1 hour of physical activity.[9] While tailoring FVIII replacement therapy to a patient's physical activity pattern is likely to be beneficial, it has only recently become part of routine practice.[10] The evolution of hemophilia treatment in recent years, including the development of extended half-life FVIII products and non-factor replacement therapies,[11] has meant that personalization of treatment is now becoming more widespread. Indeed, personalization of therapy based on each individual's activities and lifestyle is recommended in the WFH 2020 guidelines.[3]

Patient questionnaires have typically been used to monitor physical activity in relation to occurrence of bleeding in PwHA. The use of commercial activity trackers in hemophilia research is limited, but continuous and long-term monitoring of activity with activity trackers was suitable in PwHA in an observational prospective study.[12] Mobile applications for recording and monitoring occurrence of bleeding and dosing regimens offer an innovative method of data collection in clinical studies, with applications in telehealth for promoting appropriate use of clotting factors in patients with hemophilia.[13]

The prospective non-interventional Study of Prophylaxis, ACtivity and Effectiveness (SPACE) explored the effect of physical activity levels and timing of antihemophilic factor (recombinant) (rAHF; ADVATE®; Baxalta US Inc., a Takeda company, Lexington, MA, USA) infusion on occurrence of bleeding in patients with hemophilia A.

Materials and Methods
Study Design and Conduct
This prospective, multicenter, post-authorization, 6-month, observational outcomes study (NCT02190149) was conducted at 21 centers in the United States from June 2014 to June 2016. The study was approved by independent ethics committees for all participating sites and conducted in accordance with standards of good clinical practice in effect at the time of the study and the Declaration of Helsinki. All patients (or their legally authorized representatives) were provided with information about the purpose of the study and gave written consent before enrolment.

Patients
Inclusion criteria were: patients aged 13–65 years with severe or moderately severe hemophilia A (FVIII ≤2%), receiving rAHF and with a history of plasma-derived or recombinant FVIII replacement therapy for ≥150 exposure days. Patients with hemophilia B (factor IX [FIX] ≤2%) receiving recombinant factor IX, nonacog gamma (BAX 326, Rixubis®; Baxalta US Inc., a Takeda company, Lexington, MA, USA) were also eligible, but those results are not reported here. Patients with an inhibitor titer of ≥0.6 Bethesda units, or being treated for an inhibitor, or who had elective surgery planned within 6 months after enrolment that might interfere with daily activities, or those continuously requiring walking assistance devices were not eligible for enrolment.

Procedures
At baseline, investigators recorded treatment regimens (prophylaxis vs on-demand) and infusion timing schedules for patients receiving prophylactic rAHF (routine prophylaxis: 20–40 IU/kg every other/every third day; dosage targeted to maintain FVIII trough levels ≥1%).[13] Patients remained on rAHF treatment as directed by their physician throughout this non-interventional study (12-month recruitment period, 6-month observation period). Patients received a customized eDiary smartphone application (Exco InTouch, an ERT company, Nottingham, United Kingdom) and a wearable activity tracker (Fitbit®; Fitbit, Inc., San Francisco, CA, USA), which recorded data during the observation period (ie, period between eDiary/ activity tracker start and end dates) and at study completion (Figure 1A). The eDiary application was downloaded onto the patient's smartphone and served as the primary data collection tool for patient physical activity, infusion timing, and occurrence of bleeds; if the patient did not have access to a suitable phone, one was provided for the study duration. During the observation period, upon occurrence of a bleeding episode, patients recorded in the

A
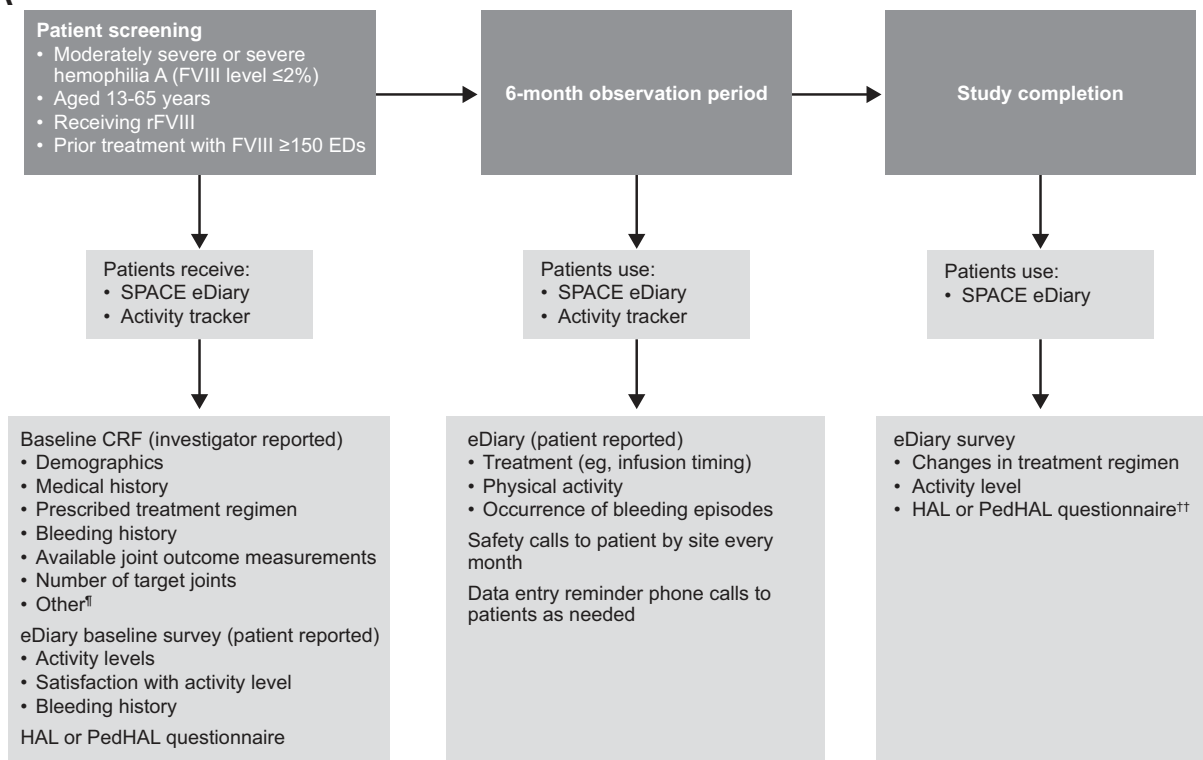
B
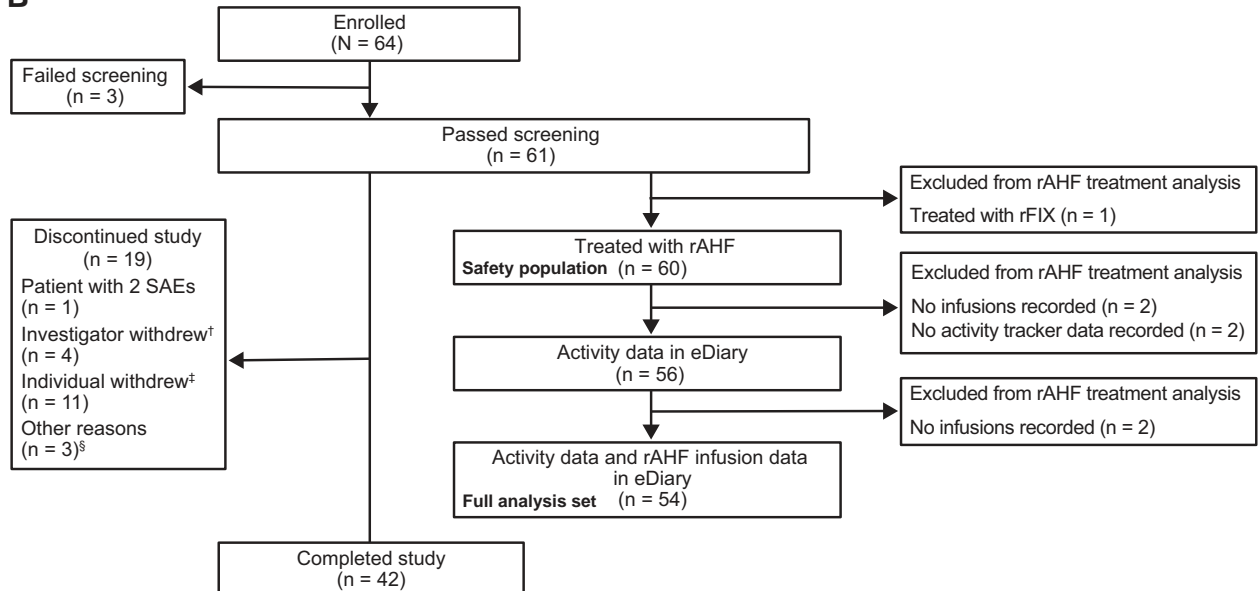
Figure 1 (**A**) SPACE study design and timing of assessments. (**B**) Patient disposition. [†]For inactivity (n = 1), loss to follow-up (n = 1), non-compliance with required reporting (n = 1), or non-compliance with data entry (n = 1). [‡]Technical problems, difficulty committing time (n = 6); non-compliance, withdrew consent (n = 5). [§]Non-compliance (n = 1) and technical difficulties (withdrawn per protocol; n = 2). [¶]Other data includes number of concomitant medications, non-drug therapies and available pharmacokinetic data history. [††]Completed 1 week before study end.
Abbreviations: CRF, case report form; ED, exposure day; FVIII, factor VIII; HAL, Hemophilia Activities List; PedHAL, Paediatric Hemophilia Activities List; rFIX, recombinant factor IX; rAHF, antihemophilic factor (recombinant); SAE, serious adverse event.

eDiary the doses and reasons for infusions (routine prophylaxis or on-demand [ie, treatment for new occurrence of bleeding]). Lastly, the patients captured changes in treatment regimen at the time of study completion. Data collected via the activity tracker included steps taken and active minutes. Additionally, upon activity-related occurrence of bleeding, patients indicated what type of activity was associated with the occurrence of bleeding.

Objectives

The primary study objective was to explore the relationship between physical activity level, rAHF infusion timing, and occurrence of bleeding episodes in PwHA during a 6-month observation period. Secondary objectives included collecting physical activity data (type, frequency) using a patient self-reported eDiary and a consumer-based activity tracker.

Data Collection and Analyses

Estimation of Time Since Last Infusion to Start of Activity

To estimate the time (ie, number of hours) since the last rAHF infusion to the start of physical activity, each day was divided into the following four 6-hour cycles: Morning, 06:00 to 12:00; afternoon, >12:00 to 18:00; evening, >18:00 to sleep; and night, 24:00 to <06:00.

Physical Activity

At baseline, patients documented in the eDiary the physical activity type, average number of days per week spent participating at each risk level, barriers to participation, and information on FVIII infusions before planned activities. Patients rated their activities in the eDiary according to the National Hemophilia Foundation "Playing it safe" activity listing, which identifies a risk collision score associated with various activities; if an activity was not listed, patients were asked to estimate the risk level.[14] Activities were thus classified into one of the following three categories based on the associated risk: (1) significant collisions are not expected; (2) significant collisions might occur, and (3) significant collisions are inevitable. During the observation period, patients recorded type, duration, and timing of activities undertaken daily in the eDiary. Timing of physical activity was recorded as a time range (ie, not the exact time); for the purposes of the analysis, the "morning" time range was set to start at 06:00 (morning), the "afternoon" time range at 12:00 (noon) and the

"night" range at 18:00 (night). The activity tracker recorded daily totals for steps taken and active minutes.

Occurrence of Bleeding

At baseline, patients recalled from memory the number and location of occurrences of bleeding (eg, total and joint bleeds) during the 6 months before the date of informed consent, as well as the number and location of target joints, which were recorded on the case report form. Information on bleed history was also compiled from patient medical records. During the study's 6-month observation period, patients recorded location, cause (any, activity-related, spontaneous, or other), type (joint or non-joint), and duration of occurrence of bleeding in the eDiary (retrospective entry for previous 2 days of data was permitted). If no end date was recorded for a bleeding episode, the study site followed up with the individual for bleed status. If the patient did not record/report bleed or infusion timing data during the allowed window of time, the data were considered missing. Only observed data were used in the analysis.

The number of bleeding episodes that happened during the study period was summarized by treatment regimen (prophylaxis or on-demand) vs type of bleed and by age group vs bleed type. Bleed frequency, annualized bleeding rate (ABR), and annualized joint bleeding rate (AJBR) were calculated for patient-level data.

Safety

From the time of informed consent to study completion, patients reported serious adverse events (SAEs) directly to study sites (ie, not in the eDiary); additional safety information was collected during follow-up calls every other month. SAEs included medical occurrences where the outcome was fatal, life-threatening, required inpatient hospitalization, or was a medically important event. AEs were categorized according to the *Medical Dictionary for Regulatory Activities* (version 19.0). The potential causal relationship between rAHF treatment and an SAE was also evaluated.

Treatment Adherence

Measures of adherence to prophylactic treatment during the 6-month observation period included dose adherence (proportion of total prescribed dose actually administered) and infusion timing schedule adherence vs baseline regimen. Infusion schedule was calculated both as a proportion of planned infusions administered and a proportion of planned infusions administered on

schedule (within a 1-day window). All parameters were reported as means (SDs) of proportions.

Compliance to Data Entry

Data entry compliance was calculated as proportion of days during the observation period with complete entries. Patients received a motivational phone call every other month to ensure data entry compliance. Individuals with no recorded data for 2 consecutive weeks or for a total of 3 weeks were withdrawn from the study. Compliance was categorized by quintiles.

Data Collection Procedures

Patients were trained to use an integrated web portal (Exco InTouch database), which linked to the eDiary and activity tracker data. Data from the eDiary were automatically exported to the clinical database. Patients had to sync their activity tracker data to the web portal daily if their smartphone did not have the required capabilities for automatic syncing. A reminder message was sent to patients after a few days if they forgot to connect their activity tracker.

Statistics

To obtain 50 evaluable patients, the enrolment target was 60 patients. Because study objectives were exploratory, no formal power calculations were performed to determine sample size. Results were summarized by treatment regimen using descriptive statistics (Table S1 includes variables and subgroup definitions used in the analysis). The safety population comprised all patients who were treated with ≥1 rAHF dose. The full analysis set (FAS) population comprised patients in the SAS with valid eDiary activity data recorded.

Longitudinal logistic regression models were used to evaluate the dynamic association between physical activity levels, time since last infusion to start of physical activity cycle, and activity-related bleeding risk of patients in the FAS, regardless of treatment discontinuation. eDiary and activity tracker data were analyzed separately. Covariates are described in Table S1. Occurrence of bleeding episodes in each cycle was modelled as a binary dependent variable, and outcomes were expressed as the odds of bleeding in a given situation (odds ratio [OR] [95% confidence interval (CI)]) vs in absence of the situation (activity vs no activity). For safety data, *p*-values were calculated using chi-square or Fisher's exact tests for categorical variables.

Results

Patients

Of 64 patients enrolled, 60 with hemophilia A receiving rAHF were eligible and 54 had both activity and rAHF infusion data recorded in the eDiary (FAS). Forty-two patients completed the study; 3 failed screening and 19 discontinued prematurely (Figure 1B). Information on data entry compliance for both eDiary and activity tracker entries was available for 53/54 patients, of whom 16 (30.2%) had entries for ≥60% of observation days (Table S2).

Mean (SD) age at screening was 23.7 (12.1) (range 11.0–58.0) years (Table 1). One patient in the FAS was younger than the lower age limit for study eligibility (13 years) and received a waiver to permit enrolment. Patients had a mean (SD) 5.0 (9.0) bleeding episodes (all bleeds) in the 6 months before providing informed consent. The majority of patients (47/54) received prophylaxis. The most common prophylactic dosing schedule at baseline and during the observation period was three infusions per week (n = 26, 55.3%), then infusions every second day (n = 8, 17.0%), then twice per week (n = 7, 14.9%). One patient in each regimen group at baseline switched regimens during the study.

Physical Activity

Physical activity intensity data recorded via the eDiary were available for 54 patients at baseline and 34 at study completion. In the 6-month pre-observation period, mean (SD) number of days per week spent on mild, moderate, and strenuous activity was 2.74 (2.29), 2.30 (1.70), and 1.65 (1.75), respectively. At study completion, mean (SD) number of days spent on each activity per week was 2.50 (2.29), 2.50 (1.80), and 2.35 (1.95) days, respectively (Table 2). Of 34 evaluable patients with available physical activity data at study completion, 29 (85.3%) patients reported that they infused prior to activity (27/31 patients on prophylaxis; 2/3 on-demand regimen).

Among all 54 patients (prophylaxis or on-demand treatment) during the 6-month observation period, there were 4980 total intervals recorded between an rAHF infusion and the next recorded physical activity (morning, noon, or night). Overall, patients administered most infusions >24 hours before initiating activity (64.7%, 3221/4980 intervals); 479 (9.6%) intervals were ≥5 days. Overall, 27.8% of patients infused 1–2 days before starting an activity; the proportion was consistent across the

Table 1 Patient Baseline Characteristics

| Characteristic | Total N = 54 | Treatment Regimen | |
		On-Demand n = 7[†]	Prophylaxis n = 47[†]
Age, y, mean ± SD	23.7 ± 12.1	26.6 ± 17.9	23.3 ± 11.3

Race, n (%)
  White
  Black
  Asian
	
	44 (81.5)
	7 (13.0)
	3 (5.6)
		
		6 (85.7)
		0 (0)
		1 (14.3)
			
			38 (80.9)
			7 (14.9)
			2 (4.3)

Body mass index, kg/m^2 mean ± SD[‡]	25.0 ± 7.3	20.2 ± 5.8	25.7 ± 7.3
No. of bleeding events per patient during the 6 months before enrollment, median (min–max)			
All bleeding events	2 (0.0–60.0)	3 (0.0–60.0)	2 (0.0–16.0)

Type[‡]
    Joint
    Non-joint
	
	1 (0.0–30.0)
	0 (0.0–30.0)
		
		3 (0.0–30.0)
		1 (0.0–30.0)
			
			1 (0.0–14.0)
			0 (0.0–15.0)

Cause
    Spontaneous
    Activity-related
    Unknown
	
	0 (0.0–15.0)
	0 (0.0–60.0)
	0 (0.0–3.0)
		
		0 (0.0–7.0)
		1 (0.0–60.0)
		0 (0.0–1.0)
			
			0 (0.0–15.0)
			0 (0.0–16.0)
			0 (0.0–3.0)

Total no. of target joints per patient at screening, mean ± SD	0.6 ± 1.4	0.6 ± 1.5	0.6 ± 1.4

Notes: [†]One patient, who was treated with prophylaxis at baseline, received only one on-demand treatment during the observation period and was included in the on-demand group. One patient who was treated on-demand at baseline changed to prophylaxis treatment. [‡]Based on a total of 53 patients (on-demand, n = 7; prophylaxis, n = 46).

activity risk level categories. Of 4980 total intervals, 3761 (75.5%) were associated with low-risk activities and 196 (3.9%) with high-risk activities (Table 3).

Occurrence of Bleeding
During the 6-month observation period, 17/54 (31.5%) patients reported no bleeding episodes (Table S3). The

Table 2 Physical Activity Duration and Intensity at Baseline and Study End (eDiary Data)

| | Total | Treatment Regimen | |
		On-Demand	Prophylaxis
Baseline (during the 6 months before the study)			
Patients, n	54	7	47

Days per week by intensity level, mean ± SD
  Strenuous
  Moderate
  Mild
	
	1.65 ± 1.75
	2.30 ± 1.70
	2.74 ± 2.29
		
		2.57 ± 2.44
		2.57 ± 1.90
		1.71 ± 2.06
			
			1.51 ± 1.61
			2.26 ± 1.69
			2.89 ± 2.31

Study end			
Patients, n	34	3	31

Days per week by intensity level, mean ± SD
  Strenuous
  Moderate
  Mild
	
	2.35 ± 1.95
	2.50 ± 1.80
	2.50 ± 2.29
		
		1.67 ± 0.58
		2.33 ± 0.58
		0.33 ± 0.58
			
			2.42 ± 2.03
			2.52 ± 1.88
			2.71 ± 2.28

Table 3 rAHF Infusions by Time from Most Recent Infusion Before Start of Physical Activity Based on NHF Activity Risk Level (eDiary Data)

		NHF Activity Risk Level				
	Total N = 54	**1 (Low)**	**1.5 (Low to Moderate)**	**2 (Moderate)**	**2.5 (Moderate to High)**	**3 (High)**
Infusion intervals included in analysis, n[†]	4980	3761	488	935	330	196
Time between infusion and start of activity, n (%)						
≤8 h	560 (11.2)	412 (11.0)	79 (16.2)	122 (13.0)	32 (9.7)	26 (13.3)
8–12 h	310 (6.2)	246 (6.5)	28 (5.7)	61 (6.5)	27 (8.2)	13 (6.6)
12–24 h	889 (17.9)	710 (18.9)	55 (11.3)	148 (15.8)	76 (23.0)	44 (22.4)
1–2 d	1382 (27.8)	1047 (27.8)	130 (26.6)	283 (30.3)	92 (27.9)	52 (26.5)
2–3 d	802 (16.1)	632 (16.8)	74 (15.2)	124 (13.3)	37 (11.2)	33 (16.8)
3–5 d	558 (11.2)	411 (10.9)	57 (11.7)	98 (10.5)	31 (9.4)	19 (9.7)
≥5 d	479 (9.6)	303 (8.1)	65 (13.3)	99 (10.6)	35 (10.6)	9 (4.6)

Notes: [†]eDiary recorded time of infusions but not activity time. It was assumed that the activity occurred in the morning at 6 AM, afternoon at 12 PM, and night at 6 PM.
Abbreviations: NHF, National Hemophilia Foundation; rAHF, antihemophilic factor (recombinant).

remaining 37 patients reported 185 bleeding episodes in total. Overall (n = 54), mean (SD) number of bleeds per person was 3.43 (5.06) for all bleeds, of which 1.52 (2.94) were reported by patients as spontaneous and 1.39 (2.11) as related to physical activity. For 31 patients receiving prophylaxis, there were 3.02 (4.72) bleeds per person (1.21 [2.41] reported by patients as spontaneous, and 1.26 [2.03]

reported as activity-related); for six patients receiving on-demand treatment, there were 6.14 (6.77) bleeds per person (3.57 [5.13] reported as spontaneous and 2.29 [2.63] reported as activity-related).

At study end, mean (SD) ABR for all bleeds was 8.14 (10.94) among patients with ≥5 months of data during the observation period. ABR for all bleeds was higher for

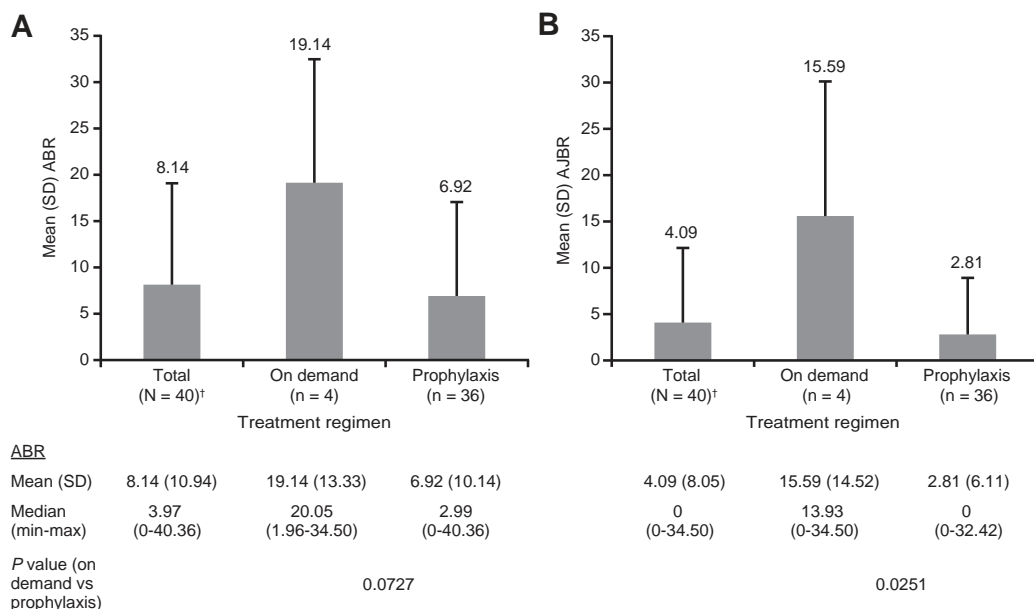| ABR						
Mean (SD)	8.14 (10.94)	19.14 (13.33)	6.92 (10.14)	4.09 (8.05)	15.59 (14.52)	2.81 (6.11)
Median (min-max)	3.97 (0–40.36)	20.05 (1.96-34.50)	2.99 (0–40.36)	0 (0–34.50)	13.93 (0–34.50)	0 (0-32.42)
P value (on demand vs prophylaxis)	0.0727			0.0251		

Figure 2 Annualized bleeding rates (ABR) at study end by treatment regimen. (**A**) All bleeds. (**B**) Joint bleeds. [†]Patients with ≥5 months of follow-up data.
Abbreviation: AJBR, annualized joint bleeding rate.

Table 4 Treatment Adherence to Prophylaxis

| Parameter | Total | Age Group | | p-value |
		Age <18 y	Age ≥18 y	
Adherence to dose: Proportion of total prescribed dose administered				
Patients, n	43[†]	19	24	
Mean ± SD	0.65 ± 0.31	0.55 ± 0.26	0.73 ± 0.33	0.0547
Adherence to schedule (gap between infusions): Proportion of planned infusion administered according to schedule within a 1-day window				
Patients, n	47	21	26	
Mean ± SD	0.48 ± 0.32	0.36 ± 0.27	0.57 ± 0.33	0.0351
Adherence to schedule (number of infusions): Proportion of planned infusions administered according to schedule within a 1-day window				
Patients, n	47	21	26	
Mean ± SD	0.64 ± 0.28	0.53 ± 0.21	0.73 ± 0.29	0.013

Note: [†]Five patients were not included due to missing data.

patients receiving on-demand therapy vs prophylaxis (Figure 2A). AJBR was 4.09 (8.05) overall, with a higher AJBR observed for patients receiving on-demand treatment vs prophylaxis (Figure 2B).

Safety

Overall, 10 AEs, of which 7 were SAEs, were reported in 7 patients (all on prophylaxis); none of these AEs or SAEs were considered related to rAHF. The proportion of patients experiencing AEs did not differ significantly between age groups (Table S4).

Treatment Adherence and rAHF Consumption

For dose adherence assessment, the mean (SD) proportion of total rAHF dose received vs dose prescribed was 0.65 (0.31). For schedule adherence, the mean (SD) proportion of infusions received on schedule vs infusions planned was 0.48 (0.32) based on gap between infusions and 0.64 (0.28) based on number of infusions (Table 4); 19.1% (9/47) of patients received 80–100% of the prescribed prophylactic rAHF dose and 19.1% (9/47) received 80–100% of planned infusions (based on gap between infusions). Individuals aged ≥18 years were more adherent to prophylactic rAHF than those aged <18 years, with statistically significant differences for schedule adherence but not dose adherence (Table 4).

Risk of Physical Activity-Related and Spontaneous Bleeds

According to data recorded in the eDiary, activity-related bleeding risk tended to increase commensurately with physical activities classified as higher risk (odds of activity-related bleeding for level 3 risk activity were 5 times that for no activity in those receiving prophylaxis; OR [95% CI] 5.06 [1.33–19.27], $p = 0.0173$; Table 5A). There was no significant correlation between bleeds recorded in the eDiary as activity-related and the time since last rAHF infusion.

Regression analysis of activity tracker data showed no significant relationship between odds of physical activity-related occurrence of bleeding with either physical activity type or time between last rAHF infusion and start of physical activity (Table 5B), although prolonging time from last rAHF infusion to start of physical activity from ≤24 to >24 hours was significantly associated with higher risk of bleeding reported by patients as spontaneous (OR range, 2.61–2.65; all $p < 0.05$; Table 6).

Discussion

This study was designed to elucidate the temporal relationship between physical activity levels, FVIII infusion timing, and occurrence of bleeding episodes in adolescents and adults with hemophilia A who were prescribed rAHF in the United States. As expected, a higher-risk physical activity correlated with greater probability of bleeding reported as physical activity-related. However, eDiary and activity

Table 5 Effect of Physical Activity and Time Since Last rAHF Infusion to Start of Activity on Risk of Bleeding Events Reported as Activity-Related by Patients with Hemophilia A Receiving Prophylactic rAHF: Logistic Regression Model Based on Data from the (A) eDiary and (B) Activity Tracker

A. eDiary data (N = 47)[†,‡]			
Parameter	**Odds ratio**	**95% CI**	**p-value**
Model 1: Activity vs no activity			
Activity: Yes vs no	1.74	0.81–3.77	0.1571
Time since last infusion to activity start[§]:			
12–24 vs ≤12 h	1.17	0.53–2.59	0.6966
>24 vs ≤12 h	1.00	0.36–2.77	0.9928
Last infusion dose, IU/kg	1.00	0.98–1.02	0.9030
Model 2: Physical activity by risk level			
Activity level[¶]			
Risk levels 1 and 1.5 vs no activity	1.65	0.77–3.56	0.2006
Risk levels 2 and 2.5 vs no activity	1.92	0.83–4.44	0.1255
Risk levels 3 vs no activity	5.06	1.33–19.27	0.0173
Time since last infusion to activity start[§]:			
12–24 vs ≤12 h	1.13	0.51–2.51	0.7696
>24 vs ≤12 h	1.01	0.37–2.75	0.9892
Last infusion dose, IU/kg	1.00	0.97–1.02	0.8697
Model 3: Time spent on physical activities			
1–30 min of activity vs no activity	1.47	0.56–3.82	0.4331
31–60 min of activity vs no activity	1.95	0.71–5.36	0.1938
>60 min of activity vs no activity	2.92	0.94–9.14	0.0650
Time since last infusion to activity start[§]:			
12–24 vs ≤12 h	1.15	0.53–2.51	0.7265
>24 vs ≤12 h	1.02	0.37–2.77	0.9757
Last infusion dose, IU/kg	1.00	0.97–1.02	0.8794
Age	0.95	0.89–1.01	0.1152
Model 4: Time spent on the physical activity by risk level			
Every 30 min spent on activity risk levels 1 and 1.5	1.03	1.01–1.04	0.0043
Every 30 min spent on activity risk levels 2 and 2.5	1.05	0.93–1.18	0.4673
Every 30 min spent on activity risk level 3	1.23	1.18–1.29	<0.0001
Time since last infusion to activity start[§]:			
12–24 vs ≤12 h	1.20	0.55–2.62	0.6423
>24 vs ≤12 h	1.02	0.38–2.72	0.9648
Last infusion dose, IU/kg	1.00	0.97–1.02	0.8039
Age	0.96	0.91–1.01	0.1415
Model 5: Time spent on common physical activities			
Every 30 min spent on common activities			
Walking (risk level 1)	1.19	1.08–1.30	0.0002
Body sculpting (risk level 1.5)	1.61	1.00–2.59	0.0502
Running and jogging (risk level 2)	2.09	1.31–3.32	0.0019

(*Continued*)

Table 5 (Continued).

Power lifting (risk level 3)	7.58	3.95–14.55	<0.0001
Other activities	1.02	1.00–1.04	0.0658
Time since last infusion to activity start[§]:			
12–24 vs ≤12 h	1.17	0.52–2.62	0.7055
>24 vs ≤12 h	1.01	0.36–2.86	0.9811
Last infusion dose, IU/kg	1.00	0.97–1.02	0.8205
Age	0.95	0.88–1.02	0.1243
B. Activity tracker data (N = 46)[†]			
Parameter	**Odds ratio**	**95% CI**	**p-value**
Model 1: Activity calories			
Activity calories: 1–1000 vs 0	2.75	0.70–10.88	0.1491
Activity calories: >1000 vs 0	3.00	0.77–11.68	0.1124
Time since last infusion to activity start[††]:	0.92	0.45–1.88	0.8148
>24 vs ≤24 h			
Last infusion dose, IU/kg	0.99	0.96–1.01	0.3102
Age	0.96	0.91–1.02	0.1848
Model 2: Steps performed			
Steps: 1–6000 vs 0	2.49	0.64–9.69	0.1891
Steps: >6000 vs 0	3.15	0.81–12.2	0.0975
Time since last infusion to activity start[††]:	0.92	0.45–1.88	0.8308
>24 vs ≤24 h			
Last infusion dose, IU/kg	0.99	0.96–1.01	0.2959
Age	0.96	0.91–1.02	0.1617
Model 3: Time spent on activities			
1–120 min of activity vs no activity	2.57	0.50–13.24	0.2584
121–240 min of activity vs no activity	2.25	0.67–7.62	0.1920
>240 min of activity vs no activity	3.48	0.89–13.63	0.0731
Time since last infusion to activity start[††]:	0.92	0.45–1.89	0.8206
>24 vs ≤24 h			
Last infusion dose, IU/kg	0.99	0.97–1.01	0.3022
Age	0.96	0.91–1.02	0.1608
Model 4: Time spent on the activity by risk level			
Every 30 min spent on lightly active activity	1.03	0.95–1.12	0.4279
Every 30 min spent on fairly active activity	1.06	0.90–1.26	0.4786
Every 30 min spent on very active activity	1.04	0.73–1.47	0.8360
Time since last infusion to activity start[††]:	0.91	0.45–1.87	0.8055
>24 vs ≤24 h			
Last infusion dose, IU/kg	0.99	0.97–1.01	0.3322
Age	0.96	0.91–1.01	0.1472

Notes: *P*-values of <0.05 indicate statistically significant associations. [†]Logistic regression models were used to evaluate the dynamic association between physical activity and time since last infusion on bleeding risk with time-varying activity level in the current cycle, time since last rAHF infusion to the beginning of the activity cycle, and age at baseline (fixed covariate). Generalized estimating equations were used to account for intra-person correlations. [‡]Among the 47 patients receiving prophylaxis, 22 experienced a total of 50 activity-related bleeding events that were included in this analysis. [§]eDiary data did not record the exact timing of activities. Time since last rAHF infusion was estimated by dividing each day into four cycles (6 AM to 12 PM [morning], 12 PM to 6 PM [afternoon], 6 PM to sleep [evening], 12 AM to 6 AM [night]), with activities assumed to start at the beginning of a designated cycle. [¶]Risk levels rate from 1 (low) to 3 (high); see Table S1 for more details. [††]The activity tracker did not record the exact timing of activity but reported the number of activity minutes by risk level daily. Therefore, the data were structured by date for each patient.
Abbreviation: rAHF, antihemophilic factor (recombinant).

Table 6 Effect of Physical Activity and Time Since Last rAHF Infusion to Start of Activity on the Risk of Bleeding Events Reported as Spontaneous by Patients with Hemophilia A Receiving Prophylactic rAHF: Logistic Regression Model (Activity Tracker Data; N = 46)[†,‡]

Parameter	Odds Ratio	95% CI	*p*-value
Model 1: Activity calories			
Activity calories: 1–1000 vs 0	2.43	1.01–5.87	0.0484
Activity calories: >1000 vs 0	2.25	0.90–5.62	0.0840
Time since last infusion to activity start[§]: >24 vs ≤24 h	2.65	1.05–6.67	0.0386
Last infusion dose, IU/kg	0.98	0.95–1.00	0.0644
Age	0.99	0.96–1.02	0.4659
Model 2: Steps performed			
Steps: 1–6000 vs 0	2.76	1.15–6.62	0.0225
Steps: >6000 vs 0	1.97	0.82–4.72	0.1299
Time since last infusion to activity start[§]: >24 vs ≤24 h	2.65	1.05–6.62	0.0399
Last infusion dose, IU/kg	0.98	0.95–1.00	0.0607
Age	0.99	0.96–1.02	0.5006
Model 3: Time spent on activities			
1–120 min of activity vs no activity	2.68	1.06–6.75	0.0368
121–240 min of activity vs no activity	2.28	0.92–5.64	0.0760
>240 min of activity vs no activity	2.19	0.88–5.47	0.0921
Time since last infusion to activity start[§]: >24 vs ≤24 h	2.65	1.04–6.72	0.0409
Last infusion dose, IU/kg	0.98	0.95–1.00	0.0595
Age	0.99	0.96–1.02	0.4855
Model 4: Time spent on the activity by risk level			
Every 30 min spent on lightly active activity	1.01	0.94–1.09	0.8197
Every 30 min spent on fairly active activity	1.02	0.80–1.22	0.8308
Every 30 min spent on very active activity	0.92	0.68–1.25	0.5969
Time since last infusion to activity start[§]: >24 vs ≤24 h	2.61	1.02–6.69	0.0452
Last infusion dose, IU/kg	0.98	0.95–1.00	0.0594
Age	0.99	0.96–1.02	0.4492

Notes: *P*-values of <0.05 indicate statistically significant associations. [†]Logistic regression models were used to evaluate the dynamic association between activity and time since last infusion on bleeding risk with time-varying activity level in the current cycle, time since last rAHF infusion to the beginning of the activity cycle, and age at baseline (fixed covariate). Generalized estimating equations were used to account for intra-person correlations. [‡]Among the 46 patients in the analysis, 19 had a total of 57 bleeds reported as spontaneous. [§]Activity tracker data did not record the exact timing of activity, but reported the number of activity minutes by risk level daily. Therefore, the data were structured by date for each individual.
Abbreviation: rAHF, antihemophilic factor (recombinant).

tracker data did not show that a longer time between last rAHF infusion and start of physical activity (≤24 vs > 24 hours) was associated with significantly greater risk of occurrence of bleeding reported by patients as physical activity-related. Although most patients infused before physical activity, nearly 65% of infusions occurred >24 hours (35.3% ≤24 hours) before initiating physical activity. rAHF infusion was timed more closely to physical activity and occurred more frequently among patients engaging in higher-risk activities, although 60% of infusion intervals for level 2.5–3 activities exceeded 24 hours. Together, these findings suggest that less than half of patients adjusted rAHF infusion timing for higher-risk physical activities.

The study cohort presented in this analysis represented a generally healthy population with hemophilia A, with a number of pre-study bleeding episodes comparable with that of prophylactically treated patients with severe hemophilia A in the United States.[15] Time since last infusion was not

significantly associated with an increased risk of activity-related bleeds, and this may be partially explained by patient-reported categorization of bleeding types as "any", "spontaneous", "activity-related", or "other". Some patients may have had difficulty identifying an occurrence of bleeding as physical activity-related vs spontaneous. Therefore, "activity-related" occurrence of bleeding may have been under-reported.

The study design did not mandate for exact timing of physical activities to be recorded; activity time was reported in the eDiary as morning, afternoon, and night (the statistical approach to these data was using 6-hour cycles), and assumed all activities occurred before bleeding onset. While this methodology may accurately reflect the sequence of physical activity and physical activity-related occurrence of joint bleeding episodes, it may not for other bleeding types (ie, spontaneous, any, and other). The 6-hour cycles may therefore have been too imprecise to robustly explore the relationship between timing of rAHF infusion and physical activity and bleed risk. Further, patient entries were retrospective and based on memory, thus subject to recall bias.[16] In addition, activity tracker data may have been skewed towards including physically active patients and those interested in tracking physical activity. The results may therefore not be indicative of causal relationships and the findings should be interpreted with caution. Further, it may suggest that the National Hemophilia Foundation "Playing it safe" activity listing could predict high-risk activities inaccurately.

Analysis by physical activity level as a group meant that data were not skewed by individual patient physical activity levels. Nevertheless, further research on patient physical activity in relation to FVIII treatment patterns is warranted, preferably with inclusion of pharmacokinetic data so that correlations between FVIII levels and physical activity can be investigated. The small sample size of regression models for the physical activity level group would likely reduce their ability to control for confounding risk factors such as bleeding history.

Although joint health data were collected at baseline, they were not included in the regression analysis because of small sample size; therefore the study could not assess whether patients with more joint disease at baseline were at higher risk of injury-related and reported spontaneous bleeding. Lastly, only one-third of patients were ≥60% compliant with both eDiary and activity tracker data entry.

ABRs for all bleeds in this study were similar to bleeding rates from other real-world settings,[17,18]

although somewhat higher than rates reported at year 3 of rAHF treatment in the observational AHEAD study,[19] and after ≥5 years of rAHF treatment in the AHEAD study.[20] This may be explained by suboptimal prophylaxis adherence compared with other studies.[18] Hemophilia A treatment adherence rates vary across studies due in part to lack of standardized estimation methods[21] and barriers to prophylaxis adherence in a real-world setting.[22] In the current study, adolescents exhibited worse adherence than adults, which is consistent with findings from elsewhere.[23] We observed a small number of reported AEs or SAEs, none of which were considered related to rAHF.

Conclusions

The findings of this study did not demonstrate that a longer time between last rAHF infusion and physical activity start was associated with risk of occurrence of bleeding reported by patients as physical activity-related. Further studies designed to address the limitations herein described are needed to confirm the association between physical activity level, timing of infusions, and the occurrence of bleeding in patients with hemophilia A.

Data Accessibility

The datasets, including the redacted study protocol, redacted statistical analysis plan, and individual participant data supporting the results reported in this paper, will be made available within 3 months from initial request to researchers who provide a methodologically sound proposal. The data will be provided after its de-identification, in compliance with applicable privacy laws, data protection, and requirements for consent and anonymization. Data requests should follow the process described in the Data Sharing section on https://clinicaltrials.takeda.com/ and https://vivli.org/ourmember/takeda/.

Additionally, Friedrich Maritsch (friedrich.maritsch@takeda.com) can be contacted regarding data-sharing information.

Acknowledgments

(Fairfield, CT, USA), and was funded by Baxalta US Inc., a Takeda company, Cambridge, MA, USA.

Author Contributions

All authors contributed to the study concept and design. BAK, DVQ, LR, MR, VCR, SLC, and ALD were clinical trial investigators, and were involved in the execution of the study and acquisition of data. All authors participated in interpreting the data and critically reviewing the paper. All authors read and approved all versions of the paper before submission, including the final manuscript before submission, and agreed on the journal to which the paper was submitted. All authors take responsibility and are accountable for the contents of the paper.

Disclosure

BAK has received research support from Baxalta, Pfizer, Sanofi, Sigilon, Takeda, and Uniqure; and consulting fees from BioMarin, CSL Behring, Pfizer, Sanofi, Takeda, Sigilon, Spark, and Uniqure. DVQ has received consulting fees/honoraria from Bayer, BioMarin, Genentech, Novo Nordisk, Sanofi, and Octapharma; and has been a speaker for BioMarin, Genentech, Novo Nordisk, Sanofi, and Takeda. LR has participated on advisory boards for Bayer, CSL Behring, Genentech, Roche, HemaBiologics, and XaTek. MR has received research support for Oregon Health & Science University from BioMarin, Bioverativ/ Sanofi, Catalyst Biosciences, Genentech, Hema Biologics, Novo Nordisk, Shire/Takeda, Spark, and uniQure; has been a consultant for the American Thrombosis and Hemostasis Network, Bayer, Bioverativ/Sanofi, CSL Behring, Genentech, Grifols, Kedrion, LFB, Novo Nordisk, Octapharma, Pfizer, Shire/Takeda, and uniQure; has been on the board of directors of the Foundation for Women and Girls with Blood Disorders and Partners in Bleeding Disorders; and is an employee of Oregon Health & Science University. VCR has received research support from Grifols, Pfizer, and Takeda. SLC has received honoraria from CSL Behring, Genentech, Kedrion, and Novo Nordisk; has received research support from the American Thrombosis and Hemostasis Network; and has been on the board of directors for the American Thrombosis and Hemostasis Network and the Hemostasis and Thrombosis Research Society. ALD has received research support from BioMarin and Takeda; and personal fees from CSL Behring, Genentech, Medscape, and uniQure. ML is an employee of Shire US Inc., a Takeda company, and

a Takeda stock owner. MW is an employee of Shire International GmbH, a Takeda company, and a Takeda stock owner. The authors report no other conflicts of interest in this work.

References

1. Aledort L, Mannucci PM, Schramm W, Tarantino M. Factor VIII replacement is still the standard of care in haemophilia A. *Blood Transfus*. 2019;17(6):479–486. doi:10.2450/2019.0211-19
2. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Treatment Guidelines Working Group on Behalf of the World Federation of Hemophilia. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1–e47. doi:10.1111/j.1365-2516.2012.02909.x
3. Srivastava A, Santagostino E, Dougall A, et al. WFH guidelines for the management of hemophilia, 3rd edition. *Haemophilia*. 2020;26 (Suppl 6):1–158. doi:10.1111/hae.14046
4. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535–544. doi:10.1056/NEJMoa067659
5. García-Dasí M, Aznar JA, Jiménez-Yuste V, et al. Adherence to prophy-laxis and quality of life in children and adolescents with severe haemo-philia A. *Haemophilia*. 2015;21(4):458–464. doi:10.1111/hae.12618
6. Krishnan S, Vietri J, Furlan R, Duncan N. Adherence to prophylaxis is associated with better outcomes in moderate and severe haemophi-lia: results of a patient survey. *Haemophilia*. 2015;21(1):64–70. doi:10.1111/hae.12533
7. Manco-Johnson MJ. Collision sports and risk of bleeding in children with hemophilia. *JAMA*. 2012;308(14):1480–1481. doi:10.1001/jama.2012.13303
8. Negrier C, Seuser A, Forsyth A, et al. The benefits of exercise for patients with haemophilia and recommendations for safe and effective physical activity. *Haemophilia*. 2013;19(4):487–498. doi:10.1111/hae.12118
9. Broderick CR, Herbert RD, Latimer J, et al. Association between physical activity and risk of bleeding in children with hemophilia. *JAMA*. 2012;308(14):1452–1459. doi:10.1001/jama.2012.12727
10. Wang M, Álvarez-román MT, Chowdary P, Quon DV, Schafer K. Physical activity in individuals with haemophilia and experience with recombinant factor VIII Fc fusion protein and recombinant factor IX Fc fusion protein for the treatment of active patients: a literature review and case reports. *Blood Coagul Fibrinolysis*. 2016;27 (7):737–744. doi:10.1097/MBC.0000000000000565
11. Trinchero A, Sholzberg M, Matino D. The evolution of hemophilia care: clinical and laboratory advances, opportunities, and challenges. *Hamostaseologie*. 2020;40(3):311–321. doi:10.1055/a-1175-6530
12. Carrasco JJ, Pérez-Alenda S, Casaña J, Soria-Olivas E, Bonanad S, Querol F. Physical activity monitoring and acceptance of a commercial activity tracker in adult patients with haemophilia. *Int J Environ Res Public Health*. 2019;16(20):3851. doi:10.3390/ijerph16203851
13. Qian W, Lam TT, Lam HHW, Li CK, Cheung YT. Telehealth inter-ventions for improving self-management in patients with hemophilia: scoping review of clinical studies. *J Med Internet Res*. 2019;21(7): e12340. doi:10.2196/12340
14. Anderson A, Forsyth A. Playing it safe: bleeding disorders, sports and exercise; 2005. Available from: http://www.hemophilia.ca/files/ PlayingItSafe.pdf. Accessed June 8, 2019.
15. Manco-Johnson MJ, Soucie JM, Gill JC. Joint Outcomes Committee of the Universal Data Collection, US Hemophilia Treatment Center Network. Prophylaxis usage, bleeding rates, and joint outcomes of hemophilia, 1999 to 2010: a surveillance project. *Blood*. 2017;129 (17):2368–2374. doi:10.1182/blood-2016-02-683169

16. Rudrapatna VA, Butte AJ. Opportunities and challenges in using real-world data for health care. *J Clin Invest.* 2020;130(2):565–574. doi:10.1172/JCI129197

17. Lou M, Ullman M, Baker J, et al. Comparison of clinical characteristics and health care utilization among individuals with hemophilia A and B in the hemophilia utilization group studies (HUGS) cohorts. Presented at: World Federation of Hemophilia 2016 World Congress; July 24-28; 2016; Orlando, FL.

18. Kruse-Jarres R, Oldenburg J, Santagostino E, et al. Bleeding and safety outcomes in persons with haemophilia A without inhibitors: results from a prospective non-interventional study in a real-world setting. *Haemophilia.* 2019;25(2):213–220. doi:10.1111/hae.13655

19. Khair K, Mazzucconi MG, Parra R, et al. Pattern of bleeding in a large prospective cohort of haemophilia A patients: a three-year follow-up of the AHEAD (Advate in haemophilia A outcome database) study. *Haemophilia.* 2018;24(1):85–96. doi:10.1111/hae.13361

20. Tsakiris DA, Oldenburg J, Klamroth R, et al. Effectiveness and safety outcomes in patients with hemophilia a receiving antihemophilic factor (recombinant) for at least 5 years in a real-world setting: 6-year interim analysis of the AHEAD international and German studies.Presented at: 62nd American Society of Hematology Annual Meeting; December 5-8, 2020.

21. Young G. New challenges in hemophilia: long-term outcomes and complications. *Hematology Am Soc Hematol Educ Program.* 2012;2012:362–368. doi:10.1182/asheducation.V2012.1.362.3798344

22. Pipe SW. New therapies for hemophilia. *Hematology Am Soc Hematol Educ Program.* 2016;2016(1):650–656. doi:10.1182/asheducation-2016.1.650

23. Thornburg CD, Duncan NA. Treatment adherence in hemophilia. *Patient Prefer Adherence.* 2017;11:1677–1686. doi:10.2147/PPA.S139851

Integrated Hemophilia Patient Care via a National Network of Care Centers in the United States: A Model for Rare Coagulation Disorders

Leonard A Valentino, Judith R Baker, Regina Butler, Miguel Escobar, Neil Frick, Susan Karp, Kollet Koulianos, Susan Lattimore, Diane Nugent, Joseph N Pugliese, Michael Recht, Mark T Reding, Michelle Rice, Constance B Thibodeaux & Mark Skinner

PERSPECTIVES

Integrated Hemophilia Patient Care via a National Network of Care Centers in the United States: A Model for Rare Coagulation Disorders

Leonard A Valentino, [ID] [1,2]
Judith R Baker, [ID] [3] Regina Butler, [4]
Miguel Escobar, [ID] [5] Neil Frick, [1]
Susan Karp, [6] Kollet Koulianos, [1]
Susan Lattimore, [7]
Diane Nugent, [ID] [3]
Joseph N Pugliese, [8]
Michael Recht, [7,9] Mark T Reding, [10]
Michelle Rice, [1]
Constance B Thibodeaux, [11]
Mark Skinner [12,13]

[1]National Hemophilia Foundation, New York, NY, USA; [2]Departments of Internal Medicine and Pediatrics, Rush University, Chicago, IL, USA; [3]Center for Inherited Blood Disorders, Orange, CA, USA; [4]Children's Hospital of Philadelphia, Philadelphia, PA, USA; [5]Department of Internal Medicine, University of Texas Health Science Center at Houston, Houston, TX, USA; [6]University of California, San Francisco, San Francisco, CA, USA; [7]Department of Pediatrics, Oregon Health & Science University, Portland, OR, USA; [8]Hemophilia Alliance, Lansdale, PA, USA; [9]American Thrombosis and Hemostasis Network, Rochester, NY, USA; [10]Department of Internal Medicine, University of Minnesota Medical Center, Minneapolis, MN, USA; [11]Departments of Internal Medicine and Pediatrics, Hemophilia Center, Tulane University School of Medicine, New Orleans, LA, USA; [12]Institute for Policy Advancement Ltd, Washington, DC, USA; [13]Department of Health Research Methods, Evidence and Impact, McMaster University, Hamilton, ON, Canada

Correspondence: Leonard A Valentino
National Hemophilia Foundation, 7 Penn Plaza Suite 1204, New York, NY, 10001, USA
Tel +1 212 328 3700
Fax +1 212 328 3777
Email lvalentino@hemophilia.org

Abstract: Rare, chronic diseases such as hemophilia and other congenital coagulation disorders require coordinated delivery of services for optimal outcomes. Hemophilia Treatment Centers (HTCs) are specialized, multidisciplinary health-care centers providing team-based care to meet the physical, psychosocial, and emotional needs of people with hemophilia (PWH) and may serve as a model for other rare coagulation disorders. Health-care purchasers, as well as the general medical community, may not appreciate the breadth and quality of services provided by HTCs. They exemplify the acculturalization and actualization of integrated care by providing comprehensive diagnostic and treatment services that reduce morbidity, mortality, avoidable emergency room visits, hospitalizations, and overall costs, while promoting a longer lifespan and improved patient functioning and outcomes. This is accomplished by a team-based approach relying upon a shared decision-making model to effectively prevent complications and manage symptoms in PWH, who are dependent on high-cost treatments. This article provides a concise yet comprehensive description of the core components of an HTC and the regional and national networks in the United States, which together achieve their incomparable value for all stakeholders.

Keywords: hemophilia, coagulation disorders, integrated care, patient-centered care, health-care delivery network, multidisciplinary

Plain Language Summary

Hemophilia Treatment Centers (HTCs) and the regional and national network to which they each belong deliver integrated, patient-centered care that reduces morbidity, mortality, and overall costs, while promoting a longer lifespan and improved outcomes. However, a comprehensive presentation of the HTC Network of care in the United States has not been published since 2005.

During the intervening years, there have been dramatic changes in the diagnosis and treatment of hemophilia and other rare coagulation disorders and rapid evolution in the importance of patient-centered, integrated, value-oriented care. For example, there is more widespread acceptance of prophylaxis as the standard of care for severe hemophilia and there are many more factor and non-factor therapies available for patients to consider. In addition, there has been a dramatic shift in the patient populations served by HTCs, including many more middle-aged and older adults, women, and people with other rare coagulation disorders. Moreover, there are new organizations, such as the American Thrombosis and Hemostasis Network (ATHN), that did not exist in 2005 and which now play a central role, and advances in information technology and telemedicine that have revolutionized the ways in which care is provided.

This article was written to help inform the medical and public health communities about the inner workings of a complex but highly coordinated approach to delivery of health-care services to this unique population, and to advance widespread recognition of the benefits of this model of care to ensure that sufficient funding and support continues into the future.

Introduction

Hemophilia is a rare inherited, bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII) or factor IX (FIX), known as hemophilia A or B, respectively. The prevalence (per 100,000 males) is 17.1 cases for all severities of hemophilia A, and 3.8 for hemophilia B.[1] Bleeding occurs most commonly in joints, soft tissues, and muscles; it can be serious, causing debilitating pain and musculoskeletal complications, resulting in morbidity or even death.[1,2] Acute and chronic complications result in a major impact on health-related quality of life (HRQoL) for people with hemophilia (PWH).[2] The mainstay of treatment in PWH is intravenous factor replacement therapy, for either prevention of bleeding (prophylaxis) or its treatment on demand.[1] Prophylaxis has been proven to maintain joint status and function but requires maintenance therapy over a lifetime.[3] Moreover, PWH who develop hemophilic arthropathy require a multimodal approach, including medical treatment, surgery, rehabilitation, and exercise.[4] Women with hemophilia and bleeding require the same care as males during hemostatic challenges such as surgery, pregnancy, childbirth, and menstruation.[5]

Over the past 4 decades, Hemophilia Treatment Centers (HTCs) have emerged as specialized, multidisciplinary health-care centers with unique expertise to meet the physical, psychosocial, and emotional needs of PWH and other congenital coagulation disorders. Optimal outcomes are achieved with a team-based, multidisciplinary, shared decision-making model. Specifically, HTCs offer integrated and comprehensive diagnostic and treatment services, including prevention education, counseling, case management, care coordination, outreach, research, surveillance, and outpatient pharmacy services. These services ensure that PWH and other congenital coagulation disorders have access to highly specialized care to reduce morbidity and enhance wellness, promote a longer lifespan, and improve patient/family functioning; while at the same time reducing avoidable emergency room visits, hospitalizations, and overall costs.[6,7]

In 2020, the last full year for which we have complete data, HTCs in the United States (US) provided care for 37,541 people with bleeding disorders, compared with 25,450 in 2012.[8] In the US, HTCs are at a crossroads: hemophilia treatment is rapidly evolving toward greater complexity, utilization of HTC services by those with other congenital coagulation disorders is on the rise,[9] and the generation of sufficient revenue to fund integrated care services has moved to the forefront.[10–13] The evolution and increased complexity of hemophilia treatment, coupled with the introduction of precision medicine principles, molecular medicines, and requisite surveillance to monitor these developments, along with the ability to provide sufficient education to engage in shared decision-making, requires ever more expertise on the part of clinicians to ensure that PWH receive state-of-the-art care as HTC teams incorporate these advances.[14]

Furthermore, several populations are growing overall HTC utilization. First is the increasing number of PWH who reach older ages over the last 20 years.[15] Previously, viral contamination of hemophilia medications with both hepatitis C and human immunodeficiency virus (HIV) decimated the community.[16] Viral comorbidities, plus progressive painful musculoskeletal deterioration, combine with aging to add complexity to the population's disease management.[17] The lifespan of PWH has extended from 13 years in the early 1900s to approximately 70 years today.[18] Finally, HTCs are increasingly serving diverse populations: between 1990 and 2010, the number of women treated at HTCs has increased by 346%, and the proportion of other congenital coagulation disorders, such as von Willebrand disease (VWD), has also substantially increased.[9]

Purchasers of health care, as well as the general medical community, may not have a complete appreciation of the breadth and quality of services provided by US HTCs. This article is intended to provide a concise yet comprehensive description of the core components of all US HTCs and illustrate how they function as a highly effective national network within a regional structure. Furthermore, it articulates for all stakeholders the value chain of services resulting in the lowest total cost of care while achieving optimal health outcomes for affected individuals and families.

The HTC Model
Integrated Care

HTCs provide value to PWH and other congenital coagulation disorders, their caregivers, health-care providers (HCPs), insurers, and policymakers in the form of integrated disease management. This integration is possible

through a core care team (Table 1) including a hematologist/medical director, nurse coordinator, physical therapist, and social worker, and access to a specialized coagulation laboratory.[7,19,20] This is complemented by an extended team, comprising professionals in related disciplines such as orthopedic surgery, as well as nutritionists, genetic counselors, psychologists, and others available upon referral (Table 1, Figure 1).[7,21] Other extended team members include those who facilitate research activities at the HTC, such as data managers and clinical research nurses.[22]

Expertise

The sustainability of provider expertise is ensured at HTCs, where clinicians focus on congenital coagulation disorders, and where training is regularly provided. Private practice hematologists and health maintenance organizations do not have the clinical experience, teamwork-based model,[23] or capacity to make the investment in quality and

expertise that HTCs have, nor do they have ready access to clinical trials that most HTCs have.[24]

Adherence to Clear Guidelines

Guidelines inform and set a foundation for standardization and assurance of baseline levels of care. To this end, the National Hemophilia Foundation (NHF) partnered with McMaster University to develop an evidence-based clinical practice guideline on "Care Models for Hemophilia Management," which have been implemented at HTCs across the country.[19] The current HTC standard of care is in line with these clinical practice guidelines, ensuring consistent care for PWH within the HTC network.[22,25] Moreover, HTCs also adhere to treatment guidelines, standards, and recommendations established by the Medical and Scientific Advisory Council (MASAC) of the NHF[26] as well as the World Federation of Hemophilia (WFH) Guidelines for the Management of Hemophilia, which

Table 1 Requirements for HTC Funding Through the Hemophilia Care Act of 1975

Core multidisciplinary team comprising: ● Hematologist ● Registered nurse ● Physical therapist ● Social worker
Extended multidisciplinary team: ● Internist ● Pediatrician ● Orthopedic surgeon ● Oral surgeon/dentist ● Psychologist/psychiatrist ● Genetic counselor ● Educational/vocational rehabilitation counselor ● Nutritionist
Facilities: ● Specialized coagulation laboratory that satisfies defined standards ● Blood bank that satisfies defined capability and has appropriate blood product resources
Additional:[a] ● Instruct patient/family members in administering CFC in a home setting (home visits conducted by the HTC nurse) ● Provide a written comprehensive care plan to the patient and their primary physician ● Establish an outreach program and encourage all PWH and health-care providers in the geographic area served by the HTC to participate in the program ● Train professional and other personnel about hemophilia and its management ● Commit to collecting reimbursement from third-party payers (ensuring that PWH receive product for home treatment) ● Establish a database for reporting outcomes ● Participate in the community through the creation of an advisory council ● Create a patient information source

Notes: Adapted with permission from Aledort LM. The evolution of comprehensive haemophilia care in the United States: perspectives from the frontline. *Haemophilia*. 2016;22(5):676–683. © 2016 John Wiley & Sons Ltd.[21] a As HTCs continued to evolve, additional services were provided, including an infusion certification program, vocational guidance, and family planning.
Abbreviations: CFC, clotting factor concentrate; HTC, hemophilia treatment center; PWH, person/people with hemophilia.
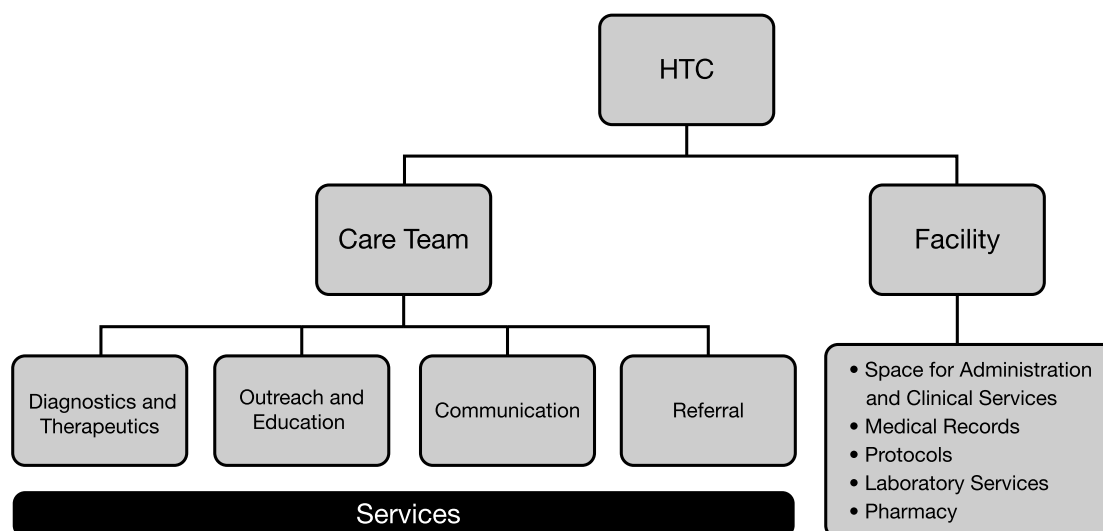
Figure 1 MASAC Standards and Criteria for the Care of Persons with Congenital Bleeding Disorders. Data from Reding and Kenney.[21]

include recommendations for 24-hour patient care and access to safe and effective hemostasis products.[20]

Specialty Coagulation Laboratory Services

Laboratory services and interpretation of test results are a large part of diagnosis, management, and monitoring for PWH and represent a core service in HTCs.[27] Large HTCs have their own specialty laboratories, while smaller HTCs have access to the laboratory services at larger HTCs, as well as those of the Centers for Disease Control and Prevention (CDC). Samples can be shipped from centers within the region, or when necessary, to HTCs outside the region. The quality of care of PWH and others with congenital coagulation disorders depends on the highest quality and timeliness of laboratory services. Speed of results and relationship with hospital-based coagulation laboratories as opposed to commercial laboratories is vital to ensuring optimal outcomes for PWH.[28]

Coordinated Pharmacy Services

Coordination of care reaches beyond communication between HCPs and PWH. Embedding pharmacy services into HTCs enhances care coordination and real-time response, facilitates shared decision-making, and reduces fragmentation that occurs when pharmacy services are handled by a separate entity. Notably, the pharmacies within the HTCs are consistent with the MASAC's[26] minimum standards for specialty pharmacies serving PWH, including

knowledgeable staff, comprehensive access to ancillary services and medications that treat hemophilia, timeliness, accuracy, accessibility, safety, and thoroughness.[29] Most HTCs participate in the federal 340B program, a discounted prescription drug purchasing program, and are registered with Health Resources Services Administration (HRSA) as comprehensive HTC 340B programs (https://www.hrsa.gov/opa/index.html). In accordance with the principal aim of the program to enable covered entities to reach more patients and provide more comprehensive services, any income generated through 340B drug discounts is to be used for the direct benefit of PWH, thereby providing a critical revenue stream to support HTC services including patient care and education, staff salaries, and care coordination.[30]

Although PWH may use any pharmacy allowed by their insurer, using the HTC pharmacy facilitates case management and the opportunity for collaboration between the patient and the medical and pharmacy teams, enabling more agile care coordination in real time, ensuring accurate and complete reporting while achieving the lowest total cost of care and optimal health outcomes.[31,32]

HTCs Function Within a National Network

In addition to the expertise within each HTC, their value is enhanced by the existence of the US Hemophilia Treatment Center Network (USHTCN), which supports fully integrated and consistent disease management and education across all

HTCs. The network not only provides operational organization and administration of HTCs, but also facilitates education and training of HCPs and PWH.[25]

Operational Organization

Eight regional HRSA grantees administer and support 149 HTCs, comprising the USHTCN.[33] Core centers serve many functions including disbursement of funds and monitoring to ensure that HTCs conform to the HRSA and CDC requirements.[29] Regional HTC networks are administered via a Regional Director and Regional Administrator, roles with standardized responsibilities in accordance with the HRSA and the CDC. Regional core centers organize Executive Committees, promulgate policies and procedures, convene ad hoc working groups, engage with partners, and liaise with community-based organizations. These functions provide a consistent foundation for organized communication and education among and between HTCs.[34]

Provider Education and Training

Within the United States, there are a limited number of expert HCPs who are concentrated in the 149 US HTCs, necessitating a highly organized and efficient network model to maximize these scarce resources and support cost-effective educational opportunities. The WFH Guidelines also acknowledge this need and recommend networking strategies to provide educational opportunities benefiting HCPs including regional conferences, discipline-specific work groups, information distribution lists, shadow-experiences in HTC clinics, and mentorship of new colleagues.[20] Beyond educating HCPs who are focused on treating people with congenital coagulation disorders, the network model also supports the education of community physicians and physical therapists, homecare nurses, emergency room staff, school personnel, and others.[11,34,35]

In summary, the USHTCN provides operational organization and excellence to enhance communication and education, thereby creating a living and learning network that constantly evaluates and revises treatment standards and recommendations. For example, only through this network approach could over 35,000 publications in the past decade on various types of coagulation disorders (Figure 2), many of which were generated by HTCs, be assimilated, vetted, and utilized to advance patient care and improve patient
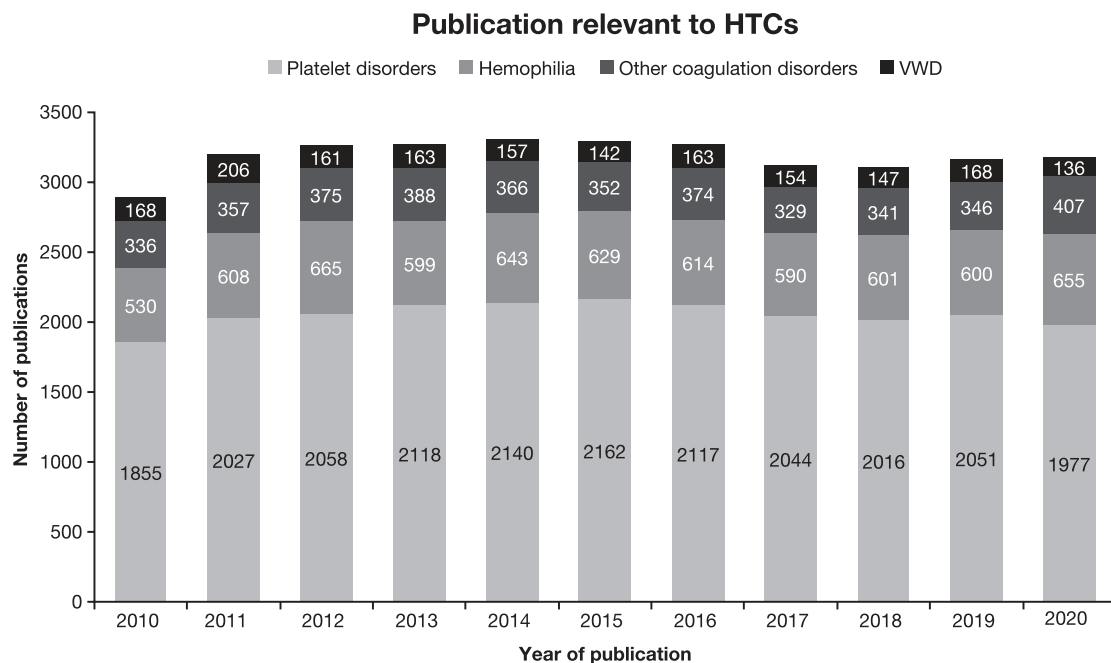
Figure 2 Proliferation of Knowledge Generation in Hemophilia and Other Coagulation Disorders.
Notes: EndNote™ version 20.1 (Clarivate, London, UK) was used to search the literature available in PubMed® National Library of Medicine database and retrieve a list of references by combining the individual year of publication (2010–2020) with each of the following Medical Subject Heading (MeSH) search terms: "hemophilia", "von Willebrand Disease", platelet disorders", or "other coagulation disorders". The number of citations for each MeSH term and year were recorded in an Excel spreadsheet (Microsoft 365, Redmond, Washington).

outcomes. Importantly, much of this quality improvement is neither reimbursed nor paid for by federal grants and exists as unfunded mandates to ensure the highest quality of care for PWH.

Unique Benefits of Integrated Care Provided by HTCs
Comprehensive Care and Educational Support Across the Lifespan

The operational organization of US HTCs enables responsiveness to patient needs, identifying gaps in care and planning educational opportunities around these gaps. The benefits extend to safety and surveillance efforts to enhance outcomes and HRQoL for PWH by reducing the likelihood of serious adverse events due to the bleeding disorder or its treatment while enhancing shared decision-making and self-sufficiency focusing on the PWH as a partner rather than only as a consumer of health care.[34]

Treatment plan development beginning in early childhood and extending through adulthood includes disease and treatment education; case management; rehabilitation; genetic counseling and testing; dental and orthopedic care; pain management; and nutritional, psychosocial, and vocational counseling. Education is provided to parents of newborns and continues as children develop the capacity to participate in decisions regarding their own treatment (eg, self-infusion education). HTCs facilitate the transition from adolescent to adult health services and preparedness for health-care independence.[34]

As adults, PWH are directly engaged in their medical care using the shared decision-making model and are fully supported in their aspiration to live as normal a life as possible with respect to education, employment, physical activity, and all other aspects of living.[36,37] Comprehensive case management by the HTC's core care team enables the provision of genetic testing and counseling, surgical coordination, rehabilitation and physiotherapy expertise, and advanced training.[7,38] Across the lifespan, women with bleeding disorders require specialized care that includes attention to heavy menstrual bleeding pre-menopause,[39] as well as pre- and post-surgery planning to control for heavy blood loss associated with surgery, pregnancy, and postpartum and during times of spontaneous and traumatic joint bleeding.[40–42]

The lifespan of PWH is increasing, necessitating development of care expertise for elderly PWH. Three issues associated with aging for PWH are general age-associated disease, motor coordination/falls, and specific symptomatology associated with hemophilia such as renal insufficiency and joint disease increasing the incidence of falls. Elderly PWH express comorbidities associated with general aging, such as obesity and hypertension. However, the presentation of these diseases can look different in PWH than the general population.[43,44]

Improving Patient Outcomes by Evidence Generation

Comprehensive and strategic collection, curation, analysis, and dissemination of data concerning the care and outcomes of PWH and other congenital coagulation disorders is achieved through a collaboration between the PWH, HTCs, CDC, American Thrombosis and Hemostasis Network (ATHN; https://athn.org/), and the USHTCN. Initially, the focus of this effort was on infectious disease (such as viral hepatitis and HIV) and health complications associated with joint disease, but has expanded to examine specific bleeding complications for women and babies, as well as HRQoL.[45–47] For example, the Community Counts Project, administered by ATHN in partnership with the USHTCN, collects data for public health tracking of the complications of congenital coagulation disorders (Table 2).[45,48] Near real-time reporting of data in an electronic data visualization tool allows for faster detection of trends and potentially faster response times.[45]

Beyond patient care data, the HTC infrastructure also provides PWH with an opportunity to participate in clinical research trials, including novel therapeutics; this is the standard of care at many centers. For example, from 2012 to 2018, the "My Life, Our Future" (MLOF) Research Repository processed information from over 11,000 male and female participants who contributed their genetic data and biologic samples to create the world's largest hemophilia scientific resource. This rich dataset was realized through extensive partnership between PWH, ATHN, Bloodworks Northwest, the NHF, and Bioverativ.[49] ATHN is currently housing the MLOF Research Repository on behalf of the community. Examples of ongoing projects include understanding the genetic, epigenetic, and antibody signatures of inhibitor responses in PWH, and studying factors associated with bleeding in women with hemophilia.[49] Another example of using data to demonstrate better outcomes comes from the 3-year retrospective data abstraction study conducted by the Hemophilia Surveillance System, including data on

Table 2 Comparison of the Universal Data Collection and the Community Counts Surveillance Systems

System Details	Universal Data Collection System	Community Counts System
Time frame	1998 through September 2011	October 2011 to present (however, the full data collection system was not implemented until 2013)
Primary focus	• Track blood safety through HIV and viral hepatitis monitoring • Track hemophilia complications with a focus on infections and joint disease	• Continued tracking of bloodborne infections and joint disease • In-depth tracking of other hemophilia complications and additional indices relevant to the aging hemophilia population
Data source	Federally funded HTCs	Federally funded HTCs
Funding	Cooperative agreement awarded to 12 HTC regional centers	Cooperative agreement awarded to ATHN to serve as Community Counts coordinating center; ATHN administers subcontracts with HTC regional centers, which then administer contracts to individual HTCs
Partnerships	• USHTCN: includes regional medical directors, regional administrators, and health-care providers at individual HTCs • Multidisciplinary committees with representation from CDC and USHTCN established to advise on all aspects of UDC implementation	• USHTCN • ATHN • Multidisciplinary committees with representation from CDC, USHTCN, and ATHN established to advise on all aspects of Community Counts implementation
System components	• Patient registry: clinical data from medical records and direct patient inquiry • Laboratory specimens • Mortality reporting	• Patient registry: clinical data from medical records and direct patient inquiry • Laboratory specimens • Mortality reporting • HTC population profile: minimal data collection about the entire hemophilia population served by HTCs
Consent for registry participation	• UDC designated by CDC as research • Participants (or parents of minor children) asked to provide informed consent	• Community Counts designated by CDC as non-research public health surveillance not requiring consent • Participants still asked to provide written authorization for participation in the registry
Patient registry clinical data forms	• Initial visit form: historic and current clinical data • Subsequent annual visit forms: clinical information since the last registry submission	• Initial visit form: historic and current clinical data • Subsequent annual visit forms: clinical information since the last registry submission
Patient registry: types of data reported on clinical forms	• Demographics • Weight and height • Family history • History of HIV, hepatitis C, and liver disease • Bleeding disorder diagnoses • Treatment regimen and products • Bleeding episodes • Mobility restrictions and joint procedures • HTC laboratory results, including levels of circulating clotting factor and inhibitor titers • Joint range of motion • HIV risk-reduction measures • Optional supplemental quality of life questionnaire (since 2005)	• Demographics • Weight and height • Family history • History of HIV, hepatitis C, and liver disease • Bleeding disorder diagnoses • Treatment regimen and products • Bleeding episodes: more extensive information than UDC • Mobility restrictions and joint procedures • HTC laboratory results, including levels of circulating clotting factor and inhibitor titers • Additional information on patient inhibitors to treatment products • Chronic pain • Opioid use for chronic pain • Health-care use • Comorbid medical conditions (eg, cancer, cardiovascular disease)

(*Continued*)

Table 2 (Continued).

System Details	Universal Data Collection System	Community Counts System
Patient registry data submission	• Primary submission source throughout UDC: paper forms submitted through US postal system, although some HTCs developed and submitted electronic forms via secure FTP • Forms entered and transferred to electronic database and reviewed; HTCs asked to resolve data discrepancies and provide missing data • HTCs encouraged to submit data continuously rather than submitting in batches	• Before 2015: paper forms submitted through US postal system and entered into an electronic database • Since 2015: data submitted electronically via an online data capture system developed and maintained by ATHN • Review of forms submitted on paper and electronically; HTCs asked to resolve data discrepancies and provide missing data • Some data checks integrated into ATHN system (ie, to occur in real time) • HTCs encouraged to submit data continuously rather than in batches

Laboratory specimens and tests	• Serum specimens
	  ◦Hepatitis A, B, and C
	  ◦HIV
	
	• Plasma specimens (for select years)
	  ◦Hepatitis C RNA
		• Serum specimens
		  ◦Hepatitis C
		  ◦HIV
		
		• Plasma specimens
		  ◦Inhibitors to treatment products

Laboratory specimen shipping timeline	• Centrifuged and shipped to CDC on cold packs within 30 hours of blood draw	• Centrifuged and shipped to CDC on cold packs within 72 hours of blood draw or frozen and shipped on dry ice within 30 days of blood draw
Laboratory accreditation	• CLIA-certified laboratory • Test use FDA-approved kits or CLIA-approved in-house developed tests	• CLIA-certified laboratory • Test use FDA-approved kits or CLIA-approved in-house developed tests
Biobank	Both serum and plasma specimens stored long term (with participant permission)	Both serum and plasma specimens stored long term (with participant permission)
Publication of surveillance data	• Publication of key findings in MMWR or peer-reviewed journals • Periodic comprehensive surveillance reports published on CDC website	• Publication of key findings in MMWR or peer-reviewed journals • Periodic comprehensive surveillance reports published on CDC website • Data visualization tool

Notes: Reproduced from Schieve L, Byams V, Dupervil B, et al. Evaluation of CDC's Hemophilia Surveillance Program — universal data collection (1998–2011) and community counts (2011–2019), United States. *MMWR Surveill Summ*. 2020;69(5):1–18.[45]
Abbreviations: ATHN, American Thrombosis and Hemostasis Network; CDC, Centers for Disease Control and Prevention; CLIA, Clinical Laboratory Improvement Amendments; FDA, [US] Food and Drug Administration; FTP, file transfer protocol; HIV, human immunodeficiency virus; HTC, Hemophilia Treatment Center; MMWR, *Morbidity and Mortality Weekly Report*; UDC, Universal Data Collection; USHTCN, US HTC Network.

PWH from 6 states. This study indicated a 40% reduction in mortality in PWH who were treated at HTCs, compared with those treated in other health-care settings.[50] Additionally, a 20% reduction in bleed-related hospitalizations among PWH cared for at an HTC who were on a medically supervised self-infusion program was also reported.[51] More recently, a small study of PWH in Indiana showed a reduction in the use of emergency department services from 33.3% of those treated outside of an HTC to 17.6% of those who were being treated through an HTC.[52]

Quality improvement (QI) is also a key function of the network, with a focus on 3 areas: patient engagement, medical home, and adolescent transition. The data and quality metrics are essential; the network continues to strive to improve the principles and practices used to diagnose and treat PWH so that the manifestations of their disease and its complications are minimized to achieve better outcomes and reduce disease burden on the individual and his/her family, while achieving optimal health outcomes at the lowest total cost of care—all steps toward health equity.[53]

Assessing Patient Satisfaction

Improvements in HRQoL such as reduced unemployment and a reduction in mortality are important outcomes, but patient satisfaction with treatment can have a profound impact on the patient's experience and subsequent treatment adherence. HTCs conducted national patient satisfaction surveys in 2015 and 2018, with another underway in 2021.[54] The 2015 and 2018 surveys engaged 5006 and 4767 PWH, respectively. Nationally, over 90% of

respondents, regardless of age, sex, race, ethnicity, diagnosis, disease severity, or where they lived, reported being usually or always satisfied with overall HTC care; components of HTC care (each >90% satisfaction nationally) were core team members, HTC care processes and services, and adolescent transition to adult care guidance.[55,56] Addressing patient satisfaction also involves finding areas for improvement. In 2013, the National HTC Patient Needs Assessment Survey was mailed to PWH being actively treated at 129 HTCs. Question topics included management and information, access and barriers to care, coping, resources, and transition. Over 90% of respondents agreed or strongly agreed that care at HTCs was patient centered and reported HTC care as important or very important; <3% reported not receiving the services they need. The survey also identified gaps in care including dietary advice, genetic testing, information on aging with hemophilia, sexual health, and basic needs resources; additionally, minority respondents reported more barriers.[57]

Focusing on Value of Care and Collaboration with Payers

Integrated care brings value from a public health perspective, in the form of potential cost savings to the system and to PWH, which can dramatically impact HRQoL. Figure 3 illustrates how many of the features of HTC care and its

clinical benefits contribute to lowering the total cost of care for PWH treated at an HTC. Payers have acknowledged that they did not fully recognize the comprehensive medical and support services that HTCs provide and the positive impact those services have on patient HRQoL and cost of care.[58]

Strengthening relationships and fostering mutual understanding between health plans and HTCs could further improve outcomes for PWH while providing a more favorable cost-to-benefit ratio. The Comprehensive Care Sustainability Collaborative (CCSC), initiated by NHF in 2014, has served as a springboard for bringing payers and HTCs together to explore and develop opportunities to work collaboratively and enhance communication and understanding between payers and providers to assure optimal patient outcomes at the lowest total cost of care.[11] As of 2020, the CCSC advisory board comprises a wide-ranging group of stakeholders, including medical and operational directors representing 8 HTCs, national and regional health plans, and the voice of the self-funded employer. One key learning from the CCSC inaugural consensus meeting was that HTCs are providing a level and range of multidisciplinary care that is far more comprehensive than what has generally been recognized by purchasers of health-care services. After multiple iterations in the CCSC, participants agreed
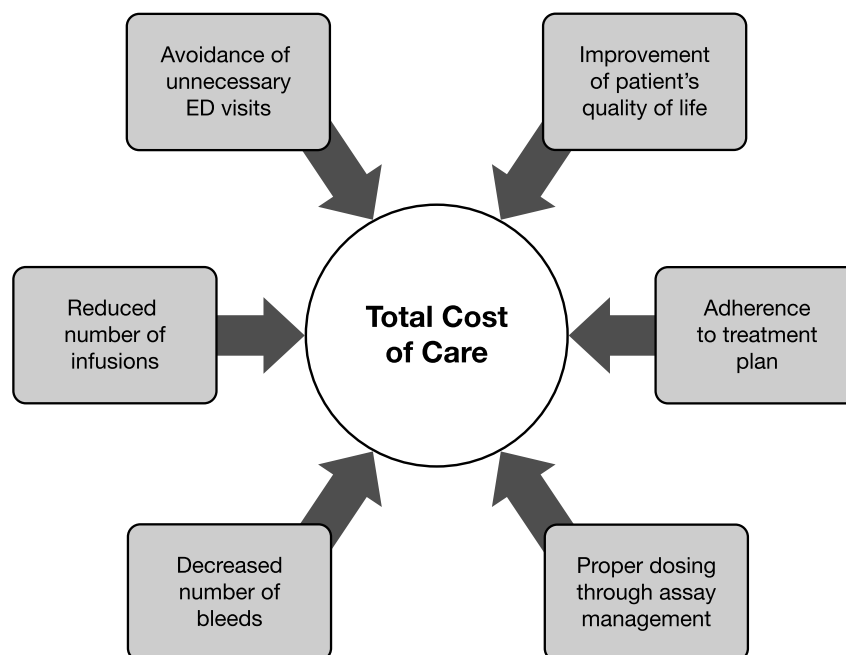

Figure 3 Benefits of Care at an HTC are Associated with Lower Total Cost of Care. Data from National Hemophilia Foundation.[6]
Abbreviation: ED, emergency department.

Table 3 Final HTC- and Payer-Reported Metrics for Use in Pilot Programs

Payer-Reported Metrics	HTC-Reported Metrics

Cost of factor
• Total factor cost
• Total factor cost per patient
• Site of care
  ∘Facility (hospital/ED)
  ∘Ambulatory (infusion center, physician's office, HTC)
  ∘Home/self
	Patient classification
	• Diagnosis (A or B)
	  ∘Severity (mild, moderate, or severe)
	  ∘Inhibitor status (yes or no)

Prescribed dose/dispensed dose/weight (± range) when dispensed via a specialty pharmacy
• Product
• Total units
• U/kg
• Units dispensed
• Prescribed dose/dispensed dose
  ∘±10% according to MASAC guidelines; payers desire ±5%
	Prescribed dose/dispensed dose/weight (± range) when dispensed at an integrated HTC pharmacy
	• Product
	• Total units
	• U/kg
	• Units dispensed
	• Prescribed dose/dispensed dose
	  ∘±10% according to MASAC guidelines; payers desire ±5%

ED visits/hospitalizations • With hemophilia listed as first/second diagnosis code (ie, in first 2 lines of claim)	ED visits/hospitalizations • With hemophilia listed as first/second diagnosis code (ie, in first 2 lines of claim)
Total cost per patient • Total cost of pharmacy claims • All other medical claims costs • Total cost per patient	Home infusion • PWH/families independently infusing at home (%) • PWH/families infusing at home with nursing assistance (%)
	Patient contacts (eg, in person, telephone, e-mail) • Comprehensive care visits • Other visits • Case management contacts • Collaboration with other providers

Notes: Reproduced from Tarantino MD, Pindolia VK. Hemophilia management via data collection and reporting: initial findings from the Comprehensive Care Sustainability Collaborative. *J Manag Care Spec Pharm*. 2017;23(1):51–56. Available from: https://www.jmcp.org/doi/10.18553/jmcp.2017.23.1.51.[59]
Abbreviations: ED, emergency department; HTC, Hemophilia Treatment Center; MASAC, Medical and Scientific Advisory Council; PWH, person/people with hemophilia.

on 2 sets of reporting metrics (payer and HTC). These metrics are being assessed in pilot programs (Table 3).[59]

Advocating for PWH Locally, Regionally, and Nationally

HTCs are actively involved in collaborating with the NHF and other patient advocacy organizations to advance the interests of PWH and their families.[60] Families and PWH rely on the NHF for its infrastructure, which includes 50 chapters that provide community support. Through the NHF and its chapters, families can connect with support networks, essential education, and advocacy efforts. In addition to national advocacy efforts, HTCs build partnerships with local foundations and support advocacy at state and national levels;[61] HTC staff serve in many advisory and other leadership capacities locally and at NHF.

Funding Mechanisms for HTCs

HRSA has identified specific criteria to qualify for a limited grant; these criteria ensure consistency within the network and support a specific standard of care at each center. HTCs that receive HRSA grant funding are required to provide evidence of optimal care using a multidisciplinary team-based approach.[62]

With the passage of the Veterans Health Care Act of 1992, regional HRSA grantees and subcontracting HTCs were included as entities eligible to participate in the 340B drug discount program. Though HTCs receive some federal support, they depend on revenue from their pharmacy programs to help finance clinical, educational, counseling, surveillance, and many laboratory services. Most of the federally designated comprehensive HTCs with established 340B programs who participated in a survey indicated that

they rely on their 340B-associated income to help fund the staff hours of their on-site social workers, physical therapists, and nurses.[30] At issue is the fact that many essential facets of integrated care that are typically delivered via the HTC staff are not billable or are minimally billable. For example, 29 centers in the survey reported that in 2013 alone, 62,640 medical coordination encounters and 57,072 urgent/emergent telephone triage encounters occurred, most of which were non-billable services. In the same survey, 28,880 encounters for psychosocial and vocational services were provided by 30 centers in 2013, again mostly non-billable.[30]

Charting a Course for the Future of HTC-Based Care

The HTC model can be adapted to the changing healthcare needs of PWH as it is primed to take advantage of the evolving technology of the twenty-first century. For example, telehealth and telemedicine at HTCs help to overcome access barriers, allowing HTC care to be offered to anyone regardless of their proximity to a center. These interventions are supported by preliminary evidence of their feasibility and effectiveness, especially for those who face transportation, mobility, or economic barriers, either in the United States or in developing countries;[63] however, the benefits remain to be conclusively demonstrated. Telemedicine increases opportunities for collaboration, promoting communication between HTC staff and local HCPs to ensure seamless care.[64]

One example of successful telehealth implementation includes telerehabilitation for people who need musculoskeletal rehabilitation. Telerehabilitation can greatly improve a patient's experience and attitude toward essential physical movement needed for recovery. Videoconferencing can also be useful for remotely evaluating bleeding or providing integrated care with multiple providers on the line at the same time.[65] The third national HTC Patient Satisfaction Survey, underway in Q1 2021, asks respondents about satisfaction with telehealth/telemedicine, and the extent to which COVID-19 was a barrier to obtaining needed HTC services. Adding these questions illustrates the capacity of the USHTCN to rapidly assess its ability to respond to emerging events (eg, the pandemic) from the patient perspective in a uniform way across centers nationwide.[54]

Monitoring and self-auditing procedures for PWH in HTCs are also evolving with technology. Cellphone tools and apps have been developed to record information,

including details on bleeding episodes, pain, and the use of coagulation factors, potentially allowing for virtual treatment supervision.[65] Enhanced real-world evidence collection would stand to improve patient outcomes through increased health surveillance, and potentially offer payers more evidence in support of the advantages of integrated care offered by HTCs.[6] HTCs serve PWH and their families who express high satisfaction, a high level of needs met, and improved outcomes spanning HRQoL to cost savings.[21,56]

The value of the HTC model and the USHTCN are clear; but HTCs are in a state of financial jeopardy, currently heavily reliant on 340B pharmacy-generated income. To continue to provide essential services for PWH and other rare coagulation disorders, it is imperative not only that HTCs receive optimal and fair reimbursement but also that PWH have access to HTCs. Revisions to federal regulations and funding schemes are essential to maintain the quality and level of services currently being offered to PWH. Increased reimbursement for existing billing codes, as well as new codes that more accurately reflect the services delivered, would be an important step toward securing the future of HTCs. Another critical step would be reducing or eliminating exclusionary practices that limit access within health-care networks.

Looking to the future, HTCs are poised to partner on the critical health priorities of the country. With the current structure, HTCs could serve a larger number of people with VWD and other rare coagulation disorders, who currently make use of the integrated care model, along with PWH. Refinement over 40 years has created the foundation for sustainability and the possibility of growth, with the potential for expanded services to PWH and individuals with other rare coagulation disorders that require a similar level of integrated, multidisciplinary care.

Conclusion

People with congenital coagulation disorders have been well served by the HTCs and USHTCN operating in the United States for over 40 years. Herein, we have described what HTCs do and how they do it through a team-based, multidisciplinary approach that incorporates the affected person in shared decision making. We have shown that PWH/caregivers are highly satisfied and have presented evidence for why this model of care has been and will remain the gold standard in the United States. This article can serve as a reference document for all stakeholders in the care of PWH and other rare coagulation disorders. The

unique benefits of the HTC network system of care have been clearly demonstrated. Taking all the benefits into account, we believe that purchasers of health care will conclude, as we do, that HTCs provide the highest-quality care for their beneficiaries, delivering optimal health outcomes at the lowest total cost of care.

Acknowledgments

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

LAV, JRB, NF, KK, JNP, and MRi declare no conflicts of interest. All grant support declared in the following statements was paid to the author's institution. RB received grant support from Health Resources & Services Administration (HRSA) and American Thrombosis and Hemostasis Network (ATHN), and honoraria from Genentech and Pfizer for advisory panel participation and from Medscape for a webinar series. ME received institutional support for clinical research from ATHN, Novo Nordisk, Sanofi, Takeda, and uniQure; payments for educational lectures from Bayer, BioMarin, Genentech, Novo Nordisk, and Pfizer; and has participated on Advisory Boards of Bayer, CSL Behring, Genentech, Kedrion, NHF, Novo Nordisk, Pfizer, and Sanofi; all outside the submitted work. SK has been reimbursed for travel to and attendance at meetings of the Hemophilia Alliance, for which she was the unpaid board chair (2018–2020) and is a retiree from the University of California, San Francisco. SL has received a grant from HRSA and from ATHN and

honoraria from the National Hemophilia Foundation (NHF) for speakers' bureau/educational events and from uniQure for participation in an advisory board. DN is co-chair of the Steering Committee and member of the Executive Committee of CONNECTS (National Heart, Lund, and Blood Institute), president and founder of the Center for Inherited Blood Disorders, and a member of the Board of Directors of the National Association of Sickle Cell Centers and Pediatric Subspecialty Faculty at CHOC Children's Hospital. MRe reports grants from Spark Therapeutics during the preparation of this manuscript. Outside the submitted work, he has received grants from Bayer, Grifols, LFB, and Octapharma; personal fees from Catalyst Biosciences and Kedrion; and grants and personal fees from BioMarin, CSL Behring, Genentech, Hema Biologics, Novo Nordisk, Pfizer, Sanofi, Takeda, and uniQure. In addition, he is on the board of directors for Foundation for Women and Girls with Blood Disorders and Partners in Bleeding Disorders. MTR has received grant support from Bayer and BioMarin; honoraria for being on the Speakers' Bureau of Bayer, CSL Behring, Sanofi, and Takeda; and honoraria for participation on Advisory Boards of Bayer, CSL Behring, Novo Nordisk, Sanofi, and Takeda, all outside the submitted work; and is a member of NHF's Medical and Scientific Advisory Council. CBT has received honoraria for virtual educational presentations from InFuCare RX and vWD Connect Foundation and sits on the NHF Pain Committee and Social Work Working Group. MS has received consulting fees to his institution from NHF. He is on the Board of Directors of the World Federation of Hemophilia USA and on the governing board of the Institute for Clinical and Economic Review. The authors report no other conflicts of interest in this work.

References

1. Iorio A, Stonebraker JS, Chambost H, et al. Establishing the prevalence and prevalence at birth of hemophilia in males: a meta-analytic approach using national registries. *Ann Intern Med*. 2019;171 (8):540–546. doi:10.7326/M19-1208

2. O'Hara J, Walsh S, Camp C, et al. The impact of severe haemophilia and the presence of target joints on health-related quality-of-life. *Health Qual Life Outcomes*. 2018;16(1):84. doi:10.1186/s12955-018-0908-9

3. Pipe SW. New therapies for hemophilia. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):650–656. doi:10.1182/asheducation-2016.1.650

4. Nacca CR, Harris AP, Tuttle JR. Hemophilic arthropathy. *Orthopedics*. 2017;40(6):e940–e946. doi:10.3928/01477447-20170619-05

5. McLintock C. Women with bleeding disorders: clinical and psychological issues. *Haemophilia*. 2018;24(Suppl 6):22–28. doi:10.1111/hae.13501

6. National Hemophilia Foundation. Comprehensive hemophilia management: a payer's guide to the hemophilia comprehensive care model. Available from: https://www.managedcarehemo.com/wp-content/uploads/2017/09/Payeru2019s-Guide-to-the-Hemophilia-Comprehensive-Care-Model.pdf. Accessed February 9, 2021.

7. Aledort LM. The evolution of comprehensive haemophilia care in the United States: perspectives from the frontline. *Haemophilia*. 2016;22 (5):676–683. doi:10.1111/hae.12970

8. Centers for Disease Control and Prevention. Community counts: HTC population profile. Available from: https://www.cdc.gov/ncbddd/hemophilia/communitycounts/data-reports/2021-03/table-1-patient-characteristics-by-calendar.html. Accessed July 28, 2021.

9. Baker JR, Riske B, Drake JH, et al. US Hemophilia Treatment Center population trends 1990–2010: patient diagnoses, demographics, health services utilization. *Haemophilia*. 2013;19(1):21–26. doi:10.1111/j.1365-2516.2012.02915.x

10. Dalton DR. Hemophilia in the managed care setting. *Am J Manag Care*. 2015;21(6 Suppl):S123–S130.

11. Comprehensive Care Sustainability Collaborative. HTC success in a value-based health care environment: findings from the Comprehensive Care Sustainability Collaborative (CCSC). Available from: https://www.ccschemo.com/webcast20/. Accessed September 17, 2021.

12. Croteau SE, Cheng D, Cohen AJ, et al. Regional variation and cost implications of prescribed extended half-life factor concentrates among U.S. haemophilia treatment centres for patients with moderate and severe haemophilia. *Haemophilia*. 2019;25(4):668–675.

13. Goodman C, Rangarao S, Rubin RJ; The Lewin Group. National hemophilia foundation: strategic summit report. Available from: https://www.hemophilia.org/sites/default/files/document/files/HemophiliaSummitFinalReportOct2012.pdf. Accessed February 4, 2021.

14. Di Minno G, Tremoli E. Tailoring of medical treatment: hemostasis and thrombosis towards precision medicine. *Haematologica*. 2017;102(3):411–418. doi:10.3324/haematol.2016.156000

15. Mazepa MA, Monahan PE, Baker JR, et al. Men with severe hemophilia in the United States: birth cohort analysis of a large national database. *Blood*. 2016;127(24):3073–3081. doi:10.1182/blood-2015-10-675140

16. White GC. Hemophilia: an amazing 35-year journey from the depths of HIV to the threshold of cure. *Trans Am Clin Climatol Assoc*. 2010;121:61–73.

17. Mejia-Carvajal C, Czapek EE, Valentino LA. Life expectancy in hemophilia outcome. *J Thromb Haemost*. 2006;4(3):507–509. doi:10.1111/j.1538-7836.2006.01776.x

18. National Hemophilia Foundation. History from 2AD to the present. Available from: https://www.hemophilia.org/bleeding-disorders-a-z/overview/history. Accessed July 29, 2021.

19. Pai M, Key NS, Skinner M, et al. NHF-McMaster guideline on care models for haemophilia management. *Haemophilia*. 2016;22 (Suppl):36.

20. Srivastava A, Santagostino E, Dougall A, et al. WFH guidelines for the management of hemophilia, 3rd edition. *Haemophilia*. 2020;26 (Suppl 6):1–158. doi:10.1111/hae.14046

21. Reding MT, Kenney J. Hemophilia care coordination and current treatment options: the latest insights for managed care and specialty pharmacy. Available from: https://www.managedcarehemo.com/cmece/. Accessed February 4, 2021.

22. National Hemophilia Foundation. Comprehensive medical care: HTCs. Available from: https://www.hemophilia.org/healthcare-professionals/guidelines-on-care/comprehensive-medical-care. Accessed October 24, 2020.

23. Mitchell P, Wynia M, Golden R, et al. Core principles & values of effective team-based health care. Presented at: National Academy of Medicine (Discussion Paper); October 2, 2012; Washington, DC.

24. Weill Cornell Medicine. Comprehensive hemophilia treatment center: research. Available from: https://hemophilia.weill.cornell.edu/research. Accessed April 17, 2021.

25. Center for Disease Control. Hemphilia treatment centers. Available from: https://www.cdc.gov/ncbddd/hemophilia/documents/a-simmons_htc-brochure-508c.pdf. Accessed July 30, 2021.

26. Medical and Scientific Advisory Council. MASAC documents. Available from: https://www.hemophilia.org/healthcare-professionals/guidelines-on-care/masac-documents. Accessed July 30, 2021.

27. University of Vermont Larner Colllege of Medicine. The vermont regional hemophilia treatment center: coagulation lab. Available from: https://www.med.uvm.edu/medicine/hemonc/patient_care_-vermont_regional_hemophilia_treatment_center. Accessed April 17, 2021.

28. National Hemophilia Foundation. MASAC Document 262 - MASAC resolution on off-site hemostasis testing. Available from: https://www.hemophilia.org/healthcare-professionals/guidelines-on-care/masac-documents/masac-document-262-masac-resolution-on-off-site-hemostasis-testing. Accessed May 5, 2021.

29. Skinner MW, Soucie JM, McLaughlin K. The National Haemophilia Program standards, evaluation and oversight systems in the United States of America. *Blood Transfus*. 2014;12(Suppl 3):e542–e548.

30. Malouin RA, McKernan L, Forsberg A, et al. Impact of the 340B pharmacy program on services and supports for persons served by hemophilia treatment centers in the United States. *Matern Child Health J*. 2018;22(9):1240–1246. doi:10.1007/s10995-018-2545-7

31. New York Blood Center. About New York Blood Center pharmacy: pharmacy services. Available from: https://www.nybloodcenter.org/products-and-services/medical-services/hemophilia-services/pharmacy-services/. Accessed April 17, 2021.

32. National Hemophilia Foundation. MASAC Document 188 - recommendations regarding standards of service for pharmacy providers of clotting factor concentrates for home use to patients with bleeding disorders. Available from: https://www.hemophilia.org/healthcare-professionals/guidelines-on-care/masac-documents/masac-document-188-recommendations-regarding-standards-of-service-for-pharmacy-providers-of-clotting-factor-concentrates-for-home-use-to-patients-with-bleeding-disorders. Accessed November 21, 2020.

33. Centers for Disease Control and Prevention. Hemophilia treatment center directory. Available from: https://dbdgateway.cdc.gov/HTCDirSearch.aspx. Accessed July 30, 2021.

34. Baker JR, Crudder SO, Riske B, Bias V, Forsberg A. A model for a regional system of care to promote the health and well-being of people with rare chronic genetic disorders. *Am J Public Health*. 2005;95(11):1910–1916. doi:10.2105/AJPH.2004.051318

35. Lin TF, Carhill P, Huang JN, Baker JR. Capacity building for rare bleeding disorders in the remote commonwealth of the Northern Mariana Islands. *Am J Public Health*. 2016;106(4):658–661. doi:10.2105/AJPH.2016.303093

36. Recht M, Konkle BA, Jackson S, et al. Recognizing the need for personalization of haemophilia patient-reported outcomes in the prophylaxis era. *Haemophilia*. 2016;22(6):825–832. doi:10.1111/hae.13066

37. Roberts JC, Lattimore S, Recht M, et al. Goal Attainment Scaling for haemophilia (GAS-Hem): testing the feasibility of a new patient-centric outcome measure in people with haemophilia. *Haemophilia*. 2018;24(4):e199–e206. doi:10.1111/hae.13454

38. Escobar MA, Brewer A, Caviglia H, et al. Recommendations on multi-disciplinary management of elective surgery in people with haemophilia. *Haemophilia*. 2018;24(5):693–702. doi:10.1111/hae.13549

39. Foundation for Women & Girls With Blood Disorders. Blood disorders resources. Available from: https://www.fwgbd.org/resources/blood-disorders. Accessed August 3, 2021.

40. Chaudhury A, Sidonio R Jr., Jain N, et al. Women and girls with haemophilia and bleeding tendencies: outcomes related to menstruation, pregnancy, surgery and other bleeding episodes from a retrospective chart review. *Haemophilia*. 2021;27(2):293–304. doi:10.1111/hae.14232

41. American College of Obestricians and Gynecologists. Management of acute abnormal uterine bleeding in nonpregnant reproductive-aged women. *Obstet Gynecol*. 2013;121:891–896. doi:10.1097/01. AOG.0000428646.67925.9a

42. Kirtava A, Crudder S, Dilley A, Lally C, Evatt B. Trends in clinical management of women with von Willebrand disease: a survey of 75 women enrolled in haemophilia treatment centres in the United States. *Haemophilia*. 2004;10(2):158–161. doi:10.1046/j.1351-8216.2003.00832.x

43. Angelini D, Konkle BA, Sood SL. Aging among persons with hemophilia: contemporary concerns. *Semin Hematol*. 2016;53(1):35–39. doi:10.1053/j.seminhematol.2015.10.004

44. Mannucci PM. Aging with hemophilia: the challenge of appropriate drug prescription. *Mediterr J Hematol Infect Dis*. 2019;11(1): e2019056. doi:10.4084/mjhid.2019.056

45. Schieve L, Byams V, Dupervil B, et al. Evaluation of CDC's Hemophilia Surveillance Program — universal data collection (1998–2011) and community counts (2011–2019), United States. *MMWR Surveill Summ*. 2020;69(5):1–18. doi:10.15585/mmwr.ss6905a1

46. Byams VR, Kouides PA, Kulkarni R, et al. Surveillance of female patients with inherited bleeding disorders in United States haemophilia treatment centres. *Haemophilia*. 2011;17(Suppl 1):6–13. doi:10.1111/j.1365-2516.2011.02558.x

47. Kulkarni R, Presley RJ, Lusher JM, et al. Complications of haemophilia in babies (first two years of life): a report from the Centers for Disease Control and Prevention Universal Data Collection System. *Haemophilia*. 2017;23(2):207–214. doi:10.1111/hae.13081

48. Pepper L. National databases improve bleeding disorders care. Available from: https://hemaware.org/bleeding-disorders-z/national-databases-improve-bleeding-disorders-care. Accessed October 25, 2020.

49. American Thrombosis and Hemostasis Network. My life, our future research repository. Available from: https://athn.org/what-we-do/national-projects/mlof-research-repository.html. Accessed August 3, 2021.

50. Soucie JM, Nuss R, Evatt B, et al. Mortality among males with hemophilia: relations with source of medical care. The Hemophilia Surveillance System Project Investigators. *Blood*. 2000;96 (2):437–442.

51. Soucie JM, Symons J 4th, Evatt B, et al. Home-based factor infusion therapy and hospitalization for bleeding complications among males with haemophilia. *Haemophilia*. 2001;7(2):198–206. doi:10.1046/j.1365-2516.2001.00484.x

52. Okolo AI, Soucie JM, Grosse SD, et al. Population-based surveillance of haemophilia and patient outcomes in Indiana using multiple data sources. *Haemophilia*. 2019;25(3):456–462. doi:10.1111/hae.13734

53. American Thrombosis and Hemostasis Network. American thrombosis and hemostasis network: overview. Available from: https://athn.org/who-we-are/about/overview.html. Accessed November 21, 2020.

54. National HTC Patient Satisfaction Survey. Third patient satisfaction survey of the U.S. Hemophilia Treatment Centers. Available from: http://www.htcsurvey.com/examplesurvey. Accessed April 17, 2021.

55. Riske B, Shearer R, Baker JR. Patient satisfaction with US hemophilia treatment center care, teams and services: the first national survey. *Haemophilia*. 2020;26(6):991–998. doi:10.1111/hae.14176

56. Lattimore S, Shearer R, Ashton M, Riske B, Baker J. Patient satisfaction with US hemophilia treatment centers: national trends 2014 and 2017. Presented at: Hempstasis and Thrombosis Research Society; May 2019; New Orleans, LA.

57. Butler RB, Cheadle A, Aschman DJ, et al. National needs assessment of patients treated at the United States federally-funded hemophilia treatment centers. *Haemophilia*. 2016;22(1):e11–e17. doi:10.1111/hae.12810

58. Shapiro A, Pipe SW, Geraghty S, et al. Hemophilia and managed care: partnering to achieve cost-effective care. *Pharm Times*. 2011;3 (5):248–255.

59. Tarantino MD, Pindolia VK. Hemophilia management via data collection and reporting: initial findings from the Comprehensive Care Sustainability Collaborative. *J Manag Care Spec Pharm*. 2017;23 (1):51–56.

60. United States Congress. The Public Health Service Act establishing the hemophilia diagnostic and treatment center program. In: Congress US, editor. *Number 1131 of Public Law 9463*. Washington, D.C.: Government Printing Office; 1975.

61. National Hemophilia Foundation. What we do. Available from: https://www.hemophilia.org/who-we-are/our-story/what-we-do. Accessed August 5, 2021.

62. Health Resources and Services Administration. Comprehensive hemophilia treatment centers. Available from: https://www.hrsa.gov/opa/eligibility-and-registration/specialty-clinics/hemophilia/index.html. Accessed August 5, 2021.

63. Qian W, Lam TT, Lam HHW, Li CK, Cheung YT. Telehealth interventions for improving self-management in patients with hemophilia: scoping review of clinical studies. *J Med Internet Res*. 2019;21(7): e12340. doi:10.2196/12340

64. Valentino LA, Skinner MW, Pipe SW. The role of telemedicine in the delivery of health care in the COVID-19 pandemic. *Haemophilia*. 2020;26(5):e230–e231. doi:10.1111/hae.14044

65. Boccalandro EA, Dallari G, Mannucci PM. Telemedicine and telerehabilitation: current and forthcoming applications in haemophilia. *Blood Transfus*. 2019;17(5):385–390.

Video abstract

Point your SmartPhone at the code above. If you have a
QR code reader the video abstract will appear. Or use:
https://youtu.be/_Nx_76pDKLw

Journal of Blood Medicine

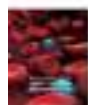**Dove**press

Publish your work in this journal

A Delphi Consensus Approach for Difficult-to-Treat Patients with Severe Hemophilia A without Inhibitors

Sreenivas P Veeranki, Priti Pednekar, Marlon Graf, Rifat Tuly, Michael Recht & Katharine Batt

ORIGINAL RESEARCH

A Delphi Consensus Approach for Difficult-to-Treat Patients with Severe Hemophilia A without Inhibitors

Sreenivas P Veeranki[1]
Priti Pednekar[1]
Marlon Graf[1]
Rifat Tuly[1]
Michael Recht [2,3]
Katharine Batt[1]

[1]PRECISIONheor, Los Angeles, CA, USA;
[2]American Thrombosis and Hemostasis Network, Rochester, NY, USA; [3]The Hemophilia Center, Oregon Health & Science University, Portland, OR, USA

Introduction: Over the past decade, there has been an increase in novel therapeutic options to treat hemophilia A. It is still unclear how these novel treatments are used in the management of patients with hemophilia A, particularly those with challenging clinical scenarios who are typically excluded in clinical trials.

Purpose: This study aimed to understand the areas of consensus and disagreement among hematologists regarding the preferences toward therapeutic approaches for difficult-to-treat patients with severe hemophilia A without inhibitors.

Patients and Methods: During February–June 2020, a three-round modified Delphi study was conducted to generate consensus among 13 US experts in the field of hemophilia. Experts were asked about their preferences toward therapeutic options for patients with challenging clinical situations, including age-related morbidities (eg, myocardial infarction, joint arthropathy), increasing demand for high-impact physical activities, early onset osteoporosis, and newborns with hemophilia A. Consensus was defined as ≥75% agreement between the panelists.

Results: Consensus was reached on many, but not all cases, leaving uncertainty about appropriateness of therapeutic approaches for some patients where clinical evidence is not available or driven by physicians' or patients' preferences toward therapeutic options. A majority of panelists preferred FVIII replacement therapy rather than emicizumab prophylaxis for the challenging cases presented due to established evidence on safety, efficacy, and level of bleed protection for FVIII treatment.

Conclusion: Recommendations emerging from this study may help guide practicing hematologists in the management of challenging hemophilia A cases. Future studies are needed to address treatment options in the clinical cases where no consensus was reached.

Keywords: emicizumab, expert elicitation, FVIII, treatment, management

Introduction

Patients with hemophilia A (PwHA) experience spontaneous bleeds resulting in a significant clinical burden and poor quality of life (QoL).[1,2] While Factor VIII (FVIII) replacement treatment, the standard of care for the management of hemophilia A[3] is safe and effective, it carries a known risk of inhibitor development (approximately 30% of PwHA develop inhibitors), profoundly impacting the patient's QoL, morbidity and mortality with increased need for venous access and infusions, despite less effective bleed control.[4,5] These challenges have led to the emergence of novel, non-factor replacement therapies that can impact bleeding at

Correspondence: Priti Pednekar
PRECISIONheor, 11100 Santa Monica
Blvd, Ste 500, Los Angeles, CA, 90025,
USA
Tel +1 267 438 3588
Email Priti.Pednekar@precisionvh.com

different stages of the coagulation cascade, often times with an easier method of delivery or decreased frequency of administrations.[3,4]

The increased availability of treatment options for PwHA presents a unique opportunity to create individualized, patient-centered regimens. However, the accelerated pace of innovation also demonstrates physician awareness of novel treatment options along with combination with existing treatments may be lacking, particularly when considering the management of severe PwHA (SPwHA) without inhibitors. In routine clinical practice, therapeutic decisions are often challenged by real-world clinical needs, particularly in PwHA without inhibitors with more severe bleeding phenotype, who are involved in high-impact physical activities, and who report arthropathy symptoms or have age-related comorbidities such as cardiovascular diseases, osteoporosis, renal failure, etc. Uncertainty in management of such cases in the absence of robust guidelines, leads to a high degree of variability in therapeutic approaches and poor outcomes.

The current study employed a Delphi method to address uncertainties in the treatment and management of high-risk cases of SPwHA without inhibitors. The Delphi method is recommended for use in obtaining consensus in the healthcare setting when "gold-standard" evidence is not available or available evidence lacks sufficient details to apply to the subset of patients observed in clinical practice.[6,7] The study also identified patients' and clinical characteristics that influence physicians' treatment decision-making in the management of PwHA.

Study Participants and Methods
Delphi Panel Participants Selection

The Delphi panel participants were recruited through recommendations from two hemophilia experts and based on the existing literature. This study included expert panel members who a) were board-certified physicians in either adult or pediatric hematology b) had current or previous clinical practice for at least 5 years, c) had at least 50% of time or effort dedicated to treating patients, d) treated at least 15 patients with hemophilia during the past 12 months, e) had at least 10 relevant peer-reviewed publications in the evaluation and treatment management of patients with hemophilia, and f) practiced in the United States only. Experts who were unable to speak or read English were not included in the panel. A total of 51 potential panelists were identified and contacted with an

introductory email outlining the study and participation requirements.

Delphi Panel Instrument

A targeted literature review was conducted to identify currently available treatments and their advantages and limitations, guidelines for the management of hemophilia A, factors influencing decision-making of hematologists while selecting or switching treatments, and challenging clinical scenarios in SPwHA without inhibitors. Subsequently, a survey instrument was developed based on findings from the targeted literature review and inputs from two hemophilia experts with emphasis on challenging clinical scenarios or situations where treatment-decision making has not been well established.

Delphi Panel Implementation

A modified Delphi panel consisting of three rounds of a web-based questionnaire was conducted between February and June 2020 (Figure 1). The Delphi survey was implemented using the web-based Delphi platform-Welphi.[8] All responses of panelists were kept anonymous and consent from panelists was obtained during each round. Experts who did not complete the Delphi survey round within the allotted time were not eligible to participate in the subsequent rounds. Panel members who agreed to participate were provided honoraria for participation in each round. The study was conducted in a double-blind manner, where the panel members had no knowledge about the study sponsor, and the study sponsor, as well, was not aware of the experts' names and their work- place organizations. Additionally, the study panelists were also blinded from their fellow panelists, thus all responses were anonymous.

Round 1 of the Delphi exercise was intended to establish the experts' baseline – a) preferences for different treatment options in challenging clinical scenarios involving SPwHA without inhibitors, b) assessment of outcomes associated with switching from factor replacement therapy to a non-factor replacement option, and c) identification of key factors considered in the treatment decision-making process. Experts were asked to review each clinical scenario and provide their likelihood estimates for each therapeutic option, as per their clinical knowledge. Likelihood estimates ranged from 1–100 with lower values indicating an expert's lower preference for a particular treatment option in his/her patients. For a few survey questions, experts were asked to rank factors affecting treatment decision-making in
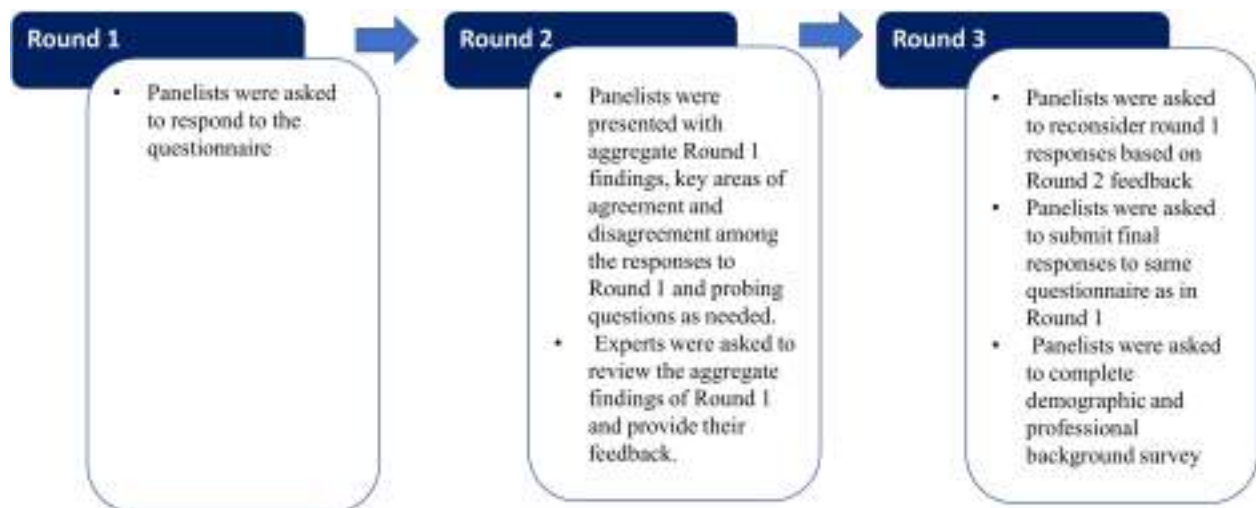
hemophilia A. In round 2, an online forum discussion was conducted in which experts were presented with aggregate group responses from round 1 as well as additional probing questions where needed. Experts were asked to review the aggregate findings from round 1 and provide feedback on any response(s) they chose, using the "comments" feature in Welphi. This round facilitated discussion among the panelists, allowing them to argue for different treatment approaches in any of the clinical scenarios, while also sharing their individual clinical experiences and expertise while avoiding oversaturation and participation fatigue.[9,10] In round 3, the experts completed the same questionnaire as that in round 1, but now responding with the additional knowledge and discussion gleaned from round 2. Final responses were analyzed as described for round 1. Panel members were also asked to complete a brief demographic and professional background survey. Using the Department of Health and Human Services regulations, 45 CFR 46.104 (d)(2), Advarra Institutional Review Board (IRB) exempted this study from IRB oversight.

Descriptive analyses were conducted to summarize panel members' characteristics, including demographics and expertise in hemophilia management. For clinical scenarios, panelists were asked to indicate appropriateness of treatment described. A scale from 1 to 100 was provided, where 1–50 ratings were considered "not appropriate or not recommended" and 51–100 ratings were considered as "appropriate/recommended". In analyzing the responses, consensus was considered to be present if at least 75% of panelists reported "not appropriate" or "appropriate" ratings during round 3.[11–14] For likelihood questions, the median and interquartile range

(IQR) were also calculated.[15] For ranking questions, variations in proportion of panelists choosing different treatment options or responses as high rank compared to lower ranks were analyzed to gauge the presence or absence of consensus among panel members. All analyses were conducted in the programs built into Welphi and Microsoft Office Excel.

Results
Participant Characteristics

Out of 51 hemophilia experts invited, 14 experts agreed to participate; 13 experts completed all three rounds of the Delphi study and therefore, formed a panel. Approximately 25 invited experts expressed interest in the study but could not participate due to clinical responsibilities encountered during the COVID-19 pandemic. All 13 panelists provided consent to participate in the study. A majority of the panelists were male (69.2%), white (76.9%) and aged 31–50 years (46.2%) (Table 1). Most panelists (53.8%) had been treating PwHA for 10–19 years. Most panelists (76.9%) spent their time providing direct patient care in a hemophilia treatment center. Most panelists (61.5%) treated both adult and pediatric PwHA and 46.1% panelists treated more than 50% SPwHA without inhibitors during the past three months.

Panelists' Preferences for Hemophilia Management in Challenging Scenarios
PwHA with Cardiovascular Comorbidities

The panel was asked to indicate their preferred therapeutic option for a 69-year-old SPwHA without inhibitors

Table I Characteristics of Delphi Participants, Their Professional Background, and Hemophilia Patients They Treat

Characteristic	n	%
Total	13	
Demographic characteristics		
Age group		
31–50 years	6	46.2%
51–70 years	6	46.2%
71–90 years	1	7.7%
Sex		
Female	3	23.1%
Male	9	69.2%
Other	1	7.7%
Race		
White	10	76.9%
Asian	2	15.4%
Other	1	7.7%
Professional background		
Year when medical license was obtained		
1950–1990	4	30.8%
1991–2000	4	30.8%
Later than 2000	5	38.4%
Number of years working in the field of hemophilia A		
10–19 years	7	53.8%
20–29 years	5	38.5%
≥ 30 years	1	7.7%
Principal practice location[a]		
Hemophilia treatment center	10	76.9%
University hospital or university affiliated clinic	3	23.1%
Number of hemophilia A patients treated at the principal practice location		
≤125	5	38.5%
126–150	2	15.4%
151–175	1	7.7%
>175	5	38.5%
Characteristics of hemophilia patients treated at clinical practice location		
Hemophilia A patient population typically treated		
Pediatric	5	38.5%
Both pediatric and adult	8	61.5%
SPwHA managed during past three months		
≤40%	4	30.8%
41–50%	3	23.1%
>50%	6	46.1%

(*Continued*)

Table I (Continued).

Characteristic	n	%
SPwHA prescribed emicizumab		
6–10%	3	23.1%
11–20%	5	38.4%
21–30%	1	7.7%
More than 30%	4	30.8%

Note: [a]Principal practice location is the practice location where hematologist spends the most hours per week.
Abbreviation: SPwHA, severe patient with hemophilia A.

diagnosed with a non-ST-elevation myocardial infarction (current therapy: dual antiplatelet therapy (DAPT) with FVIII prophylaxis) (Table 2). The panel reached consensus around "FVIII prophylaxis to achieve desired FVIII levels and then episodic FVIII therapy along with DAPT" as not an appropriate therapeutic option for this patient (84.6% panelists reported likelihood ≤50%). During round 2 discussion, panelists argued that due to high risk of bleeding while on DAPT, continuous FVIII prophylaxis would be an appropriate therapy.

For a patient with recently documented AF with CHA_2DS_2VASc score=2, on episodic FVIII replacement therapy prior to the diagnosis of AF, panelists reached consensus that "continuing episodic FVIII replacement therapy only with DOACs" was not a recommended treatment (100.0% panelists reported likelihood ≤50%) (Table 2). The panel did not reach consensus on whether "emicizumab prophylaxis along with episodic FVIII and DOAC" was an appropriate treatment and reported in round 2 that emicizumab might provide more favorable coverage than FVIII prophylaxis in SPwHA with cardiovascular comorbidities; however, the risk of emicizumab-associated thrombotic complications in these patients is still unknown. During round 2, panelists expressed needing more information on pharmacodynamics and efficacy profile of emicizumab to reach consensus.

PwHA with Increasing Demand for Physical Activity and Musculoskeletal-Related Disorders

The panelists were asked which therapy they would prescribe to a 19-year old SPwHA without inhibitors with a history of joint bleeds yet interested in participating in high-intensity sport activities (current therapy: extended half-life FVIII prophylaxis) (Table 2). All panelists agreed that "FVIII prophylaxis with on-demand FVIII treatment post-bleeds" was not an appropriate treatment for this

916

Journal of Blood Medicine 2021:12

Table 2 Results of Round 3 of the Delphi Panel Survey of 13 Hemophilia Experts About Their Preferences Toward Therapeutic Options for Challenging Clinical Scenarios

Clinical Scenario	Therapeutic Options	Likelihood Estimate		Proportion of Panelists		Consensus[c]
		Median[a]	IQR[b]	Rated Treatment as Not Appropriate (Likelihood Estimate ≤50%)	Rated Treatment as Appropriate (Likelihood Estimate >50%)	
SPwHA without inhibitors with cardiovascular comorbidities						
Scenario 1: PwHA diagnosed with MI and on DAP	Continue with FVIII prophylaxis with dual antiplatelet therapy	75.0	59.0	38.5%	61.5%	No
	FVIII prophylaxis to achieve desired FVIII levels and then episodic FVIII therapy along with dual antiplatelet therapy	20.0	24.0	84.6%	15.4%	Yes
	Emicizumab prophylaxis along with dual antiplatelet therapy	30.0	66.0	61.5%	38.5%	No
Scenario 2: PwHA diagnosed with stroke and on DOACs	Regular FVIII prophylaxis along with episodic FVIII and DOAC	74.0	47.5	46.2%	53.8%	No
	Episodic FVIII replacement therapy only with DOAC	3.0	17.5	100.0%	0.0%	Yes
	Emicizumab prophylaxis along with episodic FVIII and DOAC	50.0	72.5	53.8%	46.2%	No
SPwHA without inhibitors with physical activity and related musculoskeletal disorders						
Scenario 1: PwHA with increasing demand for physical activity (soccer/high activity sports)	Continue current FVIII prophylaxis	3.0	14.5	92.3%	7.7%	Yes
	Episodic regimen prior to activity along with FVIII prophylaxis	80.0	59.5	46.2%	53.8%	No
	FVIII prophylaxis with on-demand FVIII treatment post-bleeds	7.0	8.5	100.0%	0.0%	Yes
	Prophylaxis with emicizumab once a week	30.0	50.5	69.2%	30.8%	No
	Prophylaxis with emicizumab once a week along with episodic regimen prior to activity	55	70.5	46.2%	53.8%	No
Scenario 2: PwHA with arthroscopic surgery	As needed FVIII replacement factor during and immediately post-surgery in addition to patient's stable emicizumab prophylaxis	100.0	8.5	0.0%	100.0%	Yes
	Continue prophylaxis with emicizumab before and after surgery without breakthrough FVIII treatment	2.0	4.0	92.3%	7.7%	Yes

(Continued)

Table 2 (Continued).

Clinical Scenario	Therapeutic Options	Likelihood Estimate		Proportion of Panelists		Consensus[c]
		Median[a]	IQR[b]	Rated Treatment as Not Appropriate (Likelihood Estimate ≤50%)	Rated Treatment as Appropriate (Likelihood Estimate >50%)	
Scenario 3: PwHA with osteoporosis	Continue on emicizumab prophylaxis	87.0	88.5	30.8%	69.2%	No
	Switch to FVIII prophylaxis	5.0	48.5	76.9%	23.1%	Yes
	Switch to FVIII prophylaxis and episodic FVIII regimen	9.0	43.5	76.9%	23.1%	Yes
	Continue emicizumab prophylaxis along with episodic FVIII regimen	82.0	82.5	46.2%	53.8%	No
Newborn PwHA						
Scenario 1: Newborn child with congenital hemophilia with family history of intracranial hemorrhage	Start FVIII prophylaxis immediately	5.0	9.0	92.3%	7.7%	Yes
	Start emicizumab prophylaxis immediately	52.0	76.5	46.2%	53.8%	No
	Wait until 6 months and start FVIII prophylaxis	26.0	44.0	84.6%	15.4%	Yes
	Wait until 6 months and start emicizumab prophylaxis	30.0	60.5	76.9%	23.1%	Yes
Scenario 2: An infant with post-circumcision bleeding	FVIII prophylaxis	5.0	16.5	92.3%	7.7%	Yes
	Emicizumab prophylaxis	15.0	43.0	84.6%	15.4%	Yes
	Episodic FVIII followed by FVIII prophylaxis	30.0	49.0	69.2%	30.8%	No
	Episodic FVIII followed by emicizumab prophylaxis	77.0	27.0	23.1%	76.9%	Yes

Notes: [a]Median likelihood estimate: smaller value indicates less preferred while larger value indicates more preferred therapeutic option by panelists; [b]IQR indicates strength of agreement, where 0=perfect agreement, <20% = strongest agreement, 21–40% = strong agreement, 41–60% = better agreement and >60% = weak agreement; [c]consensus reached if ≥75% panelists reported therapeutic option as "not appropriate" or "appropriate".
Abbreviations: DAP, dual antiplatelet therapy; DOAC, direct oral anticoagulant; IQR, interquartile range; SPwHA, severe patient with hemophilia A.

patient and during round 2, highlighted the need for pre-activity FVIII bolus for these patients. During round 2, panelists discussed that emicizumab prophylaxis may protect patient from joint bleeds; however, in round 3, they did not reach consensus given implicit concerns with the cost of combined treatment as well as broad access/reimbursement issues.

The panel was asked about how they managed SPwHA without inhibitors scheduled for a joint surgery (current therapy: emicizumab prophylaxis). Based on available evidence and extensive clinical experience using FVIII peri-operatively, the panel reached consensus on the most appropriate treatment as "FVIII replacement therapy as needed during and immediately post-surgery in addition to patient's current, stable emicizumab prophylaxis" (100.0% panelists reported likelihood >50%) (Table 2).

For SPwHA suffering early onset osteoporosis and treated historically with on-demand FVIII therapy but recently started on emicizumab prophylaxis, panelists reached consensus on "switching to FVIII prophylaxis" (76.9% panelists reported likelihood ≤50%) and "switching to FVIII prophylaxis along with episodic FVIII regimen" (76.9% panelists reported likelihood ≤50%) as inappropriate treatment (Table 2). The majority of panelists (69.2%) preferred continuing emicizumab prophylaxis with the hope that the patient's physical activity would increase and result in some bone remodeling, however consensus was not reached.

Newborn PwHA

For a newborn PwHA with a brother diagnosed with severe hemophilia, on FVIII prophylaxis with a prior history of intracranial hemorrhage, panelists reached consensus on a least recommended treatment as "starting FVIII prophylaxis immediately" (92.3% panelists reported likelihood <50%) and "waiting until 6 months and starting FVIII prophylaxis" (84.6% panelists reported likelihood ≤50%) (Table 2). Panelists did not reach consensus on starting emicizumab prophylaxis immediately, however, the panel reached consensus on "wait until 6 months and start emicizumab prophylaxis" as not an appropriate therapeutic option (76.9% panelists reported likelihood ≤50%).

The panel, when asked how to manage a newborn PwHA with post-circumcision bleeding, reached consensus on "FVIII prophylaxis" (92.3% panelists reported likelihood ≤50%) and "emicizumab prophylaxis" (84.6% panelists reported likelihood ≤50) as not recommended treatments (Table 2). A majority of panelists agreed that

they would wait for the infant to be 6–9 months before starting FVIII prophylaxis. The panel reached consensus on "episodic FVIII prophylaxis followed by emicizumab prophylaxis" as a recommended treatment (76.9% panelists reported likelihood >50%) with episodic FVIII bolus and provide prophylaxis through easy subcutaneous administration of emicizumab.

In round 2, panelists agreed that FVIII prophylaxis is predictable, has long-standing safety data and can be adjusted, however, panelists expressed their low preference toward FVIII prophylaxis due to the difficulty in accessing veins in infants and the risk of inhibitor development. During round 2 discussion, panelists reported that emicizumab prophylaxis is easy to administer subcutaneously in infants; however, there is a lack of clinical evidence around emicizumab's ability to protect infants from bleeding events and to prevent intracranial hemorrhage. At present, panelists noted that treatment decision-making in the management of newborn PwHA is largely based on the preferences of physicians and patients.

Panelists' Opinions on Outcomes Related to Treatment Switching

Based on currently available efficacy and safety data for emicizumab, the panel agreed to switch treatment from a FVIII therapy to emicizumab prophylaxis if a SPwHA without inhibitors had 4–5 bleeds during the previous year or had more than 5 bleeds during the previous year (Table 3). Panelists, however, did not reach consensus that SPwHA without inhibitors should be switched from FVIII therapy to emicizumab prophylaxis if a patient had 0–1 bleeds or 2–3 bleeds during the previous year. There is no standard cut-off for the number of bleeds during the previous year to determine when to switch from the FVIII treatment to emicizumab prophylaxis. Yet, panelists seemed to prefer emicizumab prophylaxis to provide protection against bleeds if patient had at least 4 bleeds during the previous year while on FVIII therapy. More data on efficacy and safety of emicizumab prophylaxis compared to FVIII treatment may further help clinicians to make treatment decisions. Panelists also reached consensus that switching from prophylaxis with factor replacement therapy to emicizumab is only likely to reduce bleeding events by <10% or 10–40%. Panelists did not reach consensus on whether switching prophylaxis from FVIII replacement therapy to emicizumab would impact medication adherence among SPwHA without inhibitors.

Table 3 Results of Round 3 of the Delphi Panel Survey of 13 Hemophilia Experts About Their Opinions About Switching Treatment from Factor to Non-Factor Replacement Therapy

Question	Options	Likelihood Estimate		Proportion of Panelists		Consensus[c]
		Median[a]	IQR[b]	With Likelihood Estimate ≤50%	With Likelihood Estimate >50%	
Given the current evidence on efficacy and safety for emicizumab, the likelihood that hematologist will switch treatment from a FVIII therapy to emicizumab prophylaxis for different number of bleeds during previous year for SPwHA without inhibitors	0–1 bleeds during previous year	25.0	48.5	69.2%	30.8%	No
	2–3 bleeds during previous year	66.0	61.0	46.2%	53.8%	No
	4–5 bleeds during previous year	88.0	23.0	7.7%	92.3%	Yes
	>5 bleeds during previous year	95.0	18.5	0.0%	100.0%	Yes
Among SPwHA without inhibitors who had ~5 bleeding events while on FVIII prophylaxis during the past 6 months, the likelihood of the reduction in bleeding events if treatment is switched to emicizumab prophylaxis	<10% reduction in bleeding events	5.0	9.0	92.3%	7.7%	Yes
	10–40% reduction in bleeding events	30.0	38.5	76.9%	23.1%	Yes
	41–80% reduction in bleeding events	80.0	13.5	0.0%	100.0%	Yes
	> 80% reduction in bleeding events	69.0	37.5	30.8%	69.2%	No
Among SPwHA without inhibitors who have switched from FVIII prophylaxis to emicizumab prophylaxis, the likelihood of the change in medication adherence (given that other factors associated with adherence such as age, frequency of bleeding, medication beliefs, etc. remain constant)	No change in medication adherence	11.0	67.5	61.5%	38.5%	No
	10–20% increase in doses/medication administered	36.0	62.5	61.5%	38.5%	No
	21–50% increase in doses/medication administered	50.0	54.0	61.5%	38.5%	No
	51–75% increase in doses/medication administered	50.0	50.0	53.8%	46.2%	No
	>75–80% increase in doses/medication administered	30.0	51.5	69.2%	30.8%	No

Notes: [a]Median likelihood estimate: smaller value indicates less preferred while larger value indicates more preferred therapeutic option by panelists; [b]IQR indicates strength of agreement, where 0=perfect agreement, <20% = strongest agreement, 21–40% = strong agreement, 41–60% = better agreement and >60% = weak agreement; [c]consensus reached if ≥75% panelists reported therapeutic option as "not appropriate" or "appropriate".
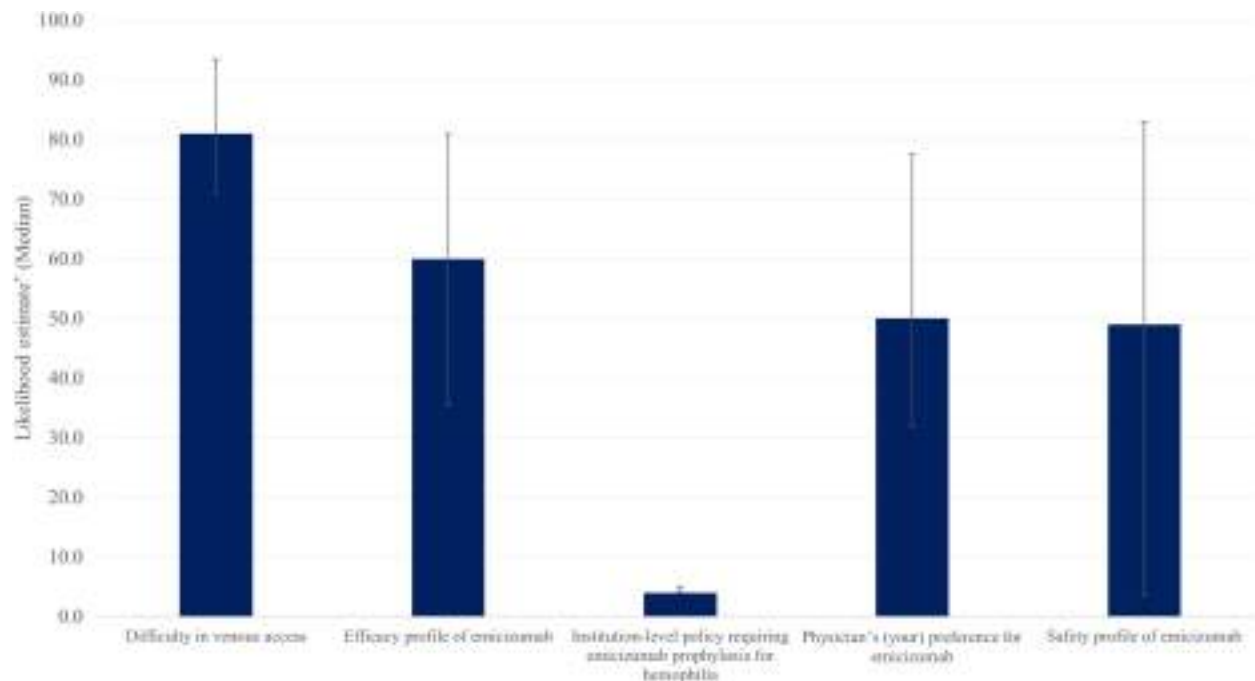**Abbreviations:** IQR, interquartile range; SPwHA, severe patient with hemophilia A.

Figure 2 Factors considered by hematologists while switching SPwHA without inhibitors from FVIII prophylaxis to emicizumab prophylaxis.
Note: *Panelists provided their likelihood estimates ranging from 1–100, where 1 means highly unlikely and 100 means extremely likely.

Factors Affecting Treatment Decision-Making in SPwHA

Panelists reached consensus that the primary factors considered when switching treatment from factor replacement therapy to non-factor treatment in SPwHA were difficulty in venous access (Figure 2), long-term efficacy and/or effectiveness, and physician or patient preferences toward treatment options (Figure 3). Regarding health system related factors, the majority of panelists (38%) reported patients' knowledge of and preferences toward treatment options is most important to consider while switching treatment for SPwHA without inhibitors (Figure 4). While only 8% of panelists ranked institution-level policies (such as those related to prescribing procedures, dose distribution systems, outpatient prescription availability, etc.) as the most important health system related factors considered in treatment decision-making in management of SPwHA, approximately 23% panelists considered insurance coverage policies (such as those related to type of plan, access to and reimbursement of hemophilia treatments, out-of-pocket payment, management tactics employed in pharmacy or medical benefits by health plan) as major factors in treatment decision-making for SPwHA.

Results of round 1 are presented in Tables 4 and 5.

Discussion

Existing guidelines for the management of hemophilia A[3,16] and clinical trials evaluating efficacy and safety of therapeutic options often do not provide recommendations on how to manage specific clinical scenarios. In particular, age-related comorbidities such as cardiovascular disease, early onset osteoporosis, increased bleeding risks associated with physical activity or a major surgery, or newborn infants with severe bleeding are often exclusion criteria for enrollment in randomized clinical trials.[17] This further limits the applicability of evidence-based guidelines, possibly leading to misconceptions about available treatments. These gaps can only be bridged by achieving consensus among clinicians, typically drawn from their practice-based experience(s). Delphi techniques represent the most reliable consensus methods in healthcare.[14,18–20] In the present Delphi study, cases of SPwHA without inhibitors of uncertain management were presented to a panel of 13 specialized clinicians in hemophilia, who answered a questionnaire on the appropriateness of treatment strategies. This is the first study of its kind, aimed at obtaining a consensus on complex and real-life cases of SPwHA without inhibitors, where definitive guidelines are not applicable.
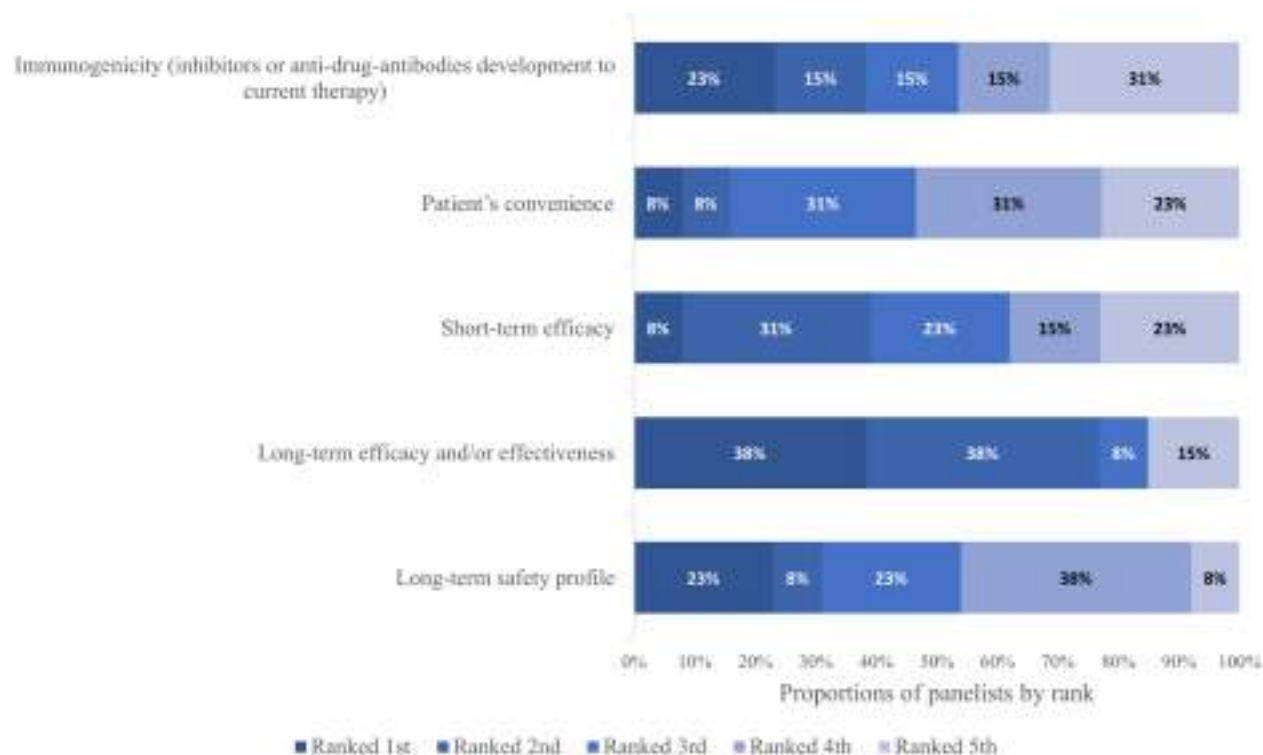
Figure 3 Hemophilia treatment characteristics considered by hematologists while switching treatment for SPwHA without inhibitors.
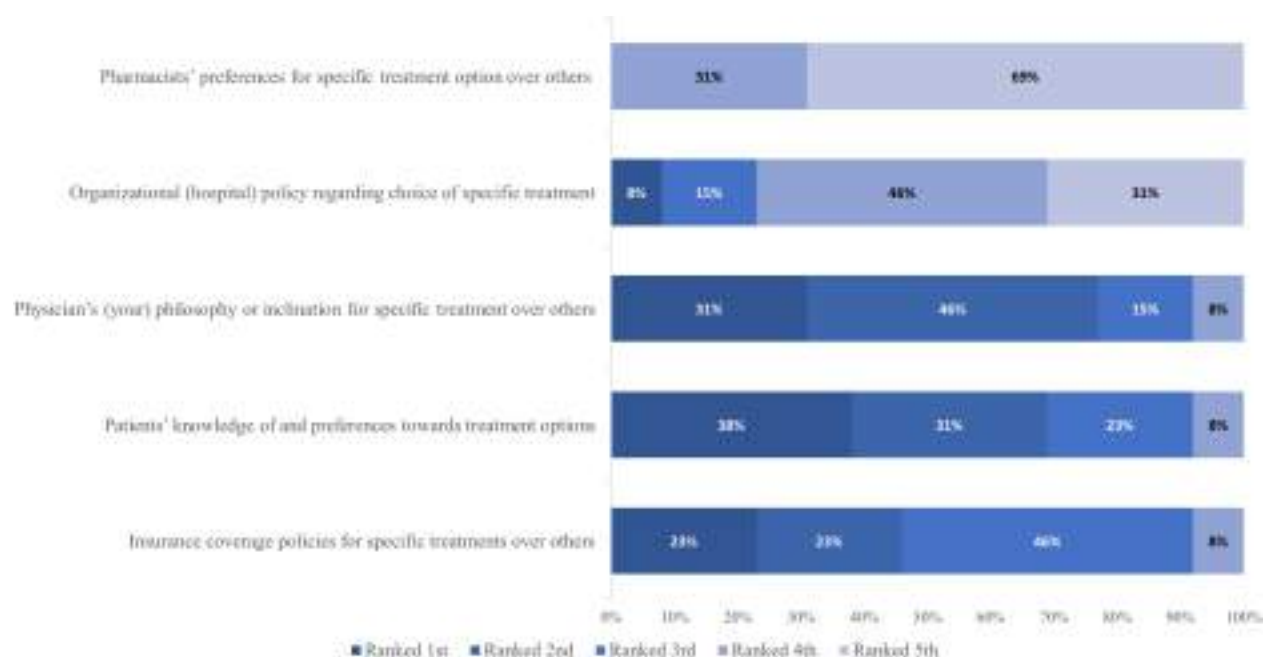

Figure 4 Health system related factors considered by hematologists while switching treatment for SPwHA without inhibitors.

For SPwHA with age-related morbidity such as cardiovascular disease (CVD), panelists reached consensus that episodic FVIII prophylaxis would not be adequate to reach targeted trough levels of FVIII required to avoid risk of bleeding associated with antithrombotic treatments. While the majority of panelists preferred FVIII prophylaxis, as

Table 4 Results of Round 1 of the Delphi Panel Survey of 13 Hemophilia Experts About Their Preferences Toward Therapeutic Options for Challenging Clinical Scenarios

Clinical Scenario	Therapeutic Options	Likelihood Estimate		Proportion of Panelists	
		Median[a]	IQR[b]	Rated Treatment as Not Appropriate (Likelihood Estimate ≤50%)	Rated Treatment as Appropriate (Likelihood Estimate >50%)
SPwHA without inhibitors with cardiovascular comorbidities					
Scenario 1: PwHA diagnosed with MI and on dual antiplatelet therapy (DAP)	Continue with FVIII prophylaxis with dual antiplatelet therapy	75.0	47.5	23.1%	76.9%
	FVIII prophylaxis to achieve desired FVIII levels and then episodic FVIII therapy along with dual antiplatelet therapy	16.0	40.0	76.9%	23.1%
Scenario 2: PwHA diagnosed with stroke and on direct oral anticoagulants (DOACs)	Emicizumab prophylaxis along with dual antiplatelet therapy	20.0	57.5	69.2%	30.8%
	Regular FVIII prophylaxis along with episodic FVIII and DOAC	75.0	37.0	30.8%	69.2%
	Episodic FVIII replacement therapy only with DOAC	5.0	40.0	84.6%	15.4%
	Emicizumab prophylaxis along with episodic FVIII and DOAC	50.0	59.0	53.8%	46.2%
SPwHA without inhibitors with physical activity and related musculoskeletal disorders					
Scenario 1: PwHA with increasing demand for physical activity (soccer/high activity sports)	Continue current FVIII prophylaxis	3.0	24.0	92.3%	7.7%
	Episodic regimen prior to activity along with FVIII prophylaxis	75.0	69.5	38.5%	61.5%
	FVIII prophylaxis with on-demand FVIII treatment post-bleeds	7.0	20.5	92.3%	7.7%
	Prophylaxis with emicizumab once a week	37.0	63.5	61.5%	38.5%
	Prophylaxis with emicizumab once a week along with episodic regimen prior to activity	24.0	67.5	61.5%	38.5%
Scenario 2: PwHA with arthroscopic surgery	As needed FVIII replacement factor during and immediately post-surgery in addition to patient's stable emicizumab prophylaxis	97.0	10.0	0.0%	100.0%
	Continue prophylaxis with emicizumab before and after surgery without breakthrough FVIII treatment	5.0	10.0	84.6%	15.4%

(*Continued*)

Table 4 (Continued).

Clinical Scenario	Therapeutic Options	Likelihood Estimate		Proportion of Panelists	
		Median[a]	IQR[b]	Rated Treatment as Not Appropriate (Likelihood Estimate ≤50%)	Rated Treatment as Appropriate (Likelihood Estimate >50%)
Scenario 3: PwHA with osteoporosis	Continue on emicizumab prophylaxis	90.0	59.5	23.1%	76.9%
	Switch to FVIII prophylaxis	10.0	53.5	76.9%	23.1%
	Switch to FVIII prophylaxis and episodic FVIII regimen	7.0	32.0	84.6%	15.4%
	Continue emicizumab prophylaxis along with episodic FVIII regimen	15.0	76.5	53.8%	46.2%
Newborn PwHA					
Scenario 1: Newborn child with congenital hemophilia with family history of intracranial hemorrhage	Start FVIII prophylaxis immediately	5.0	7.0	92.3%	7.7%
	Start emicizumab prophylaxis immediately	53.0	83.5	46.2%	53.8%
	Wait until 6 months and start FVIII prophylaxis	32.0	62.0	69.2%	30.8%
	Wait until 6 months and start emicizumab prophylaxis	52.0	70.5	46.2%	53.8%
Scenario 2: An infant with post-circumcision bleeding	FVIII prophylaxis	5.0	14.0	92.3%	7.7%
	Emicizumab prophylaxis	10.0	50.0	76.9%	23.1%
	Episodic FVIII followed by FVIII prophylaxis	30.0	77.0	61.5%	38.5%
	Episodic FVIII followed by emicizumab prophylaxis	66.0	57.0	46.2%	53.8%

Notes: [a]Median likelihood estimate: smaller value indicates less preferred while larger value indicates more preferred therapeutic option by panelists; [b]IQR indicates strength of agreement, where 0=perfect agreement, <20% = strongest agreement, 21–40% = strong agreement, 41–60% = better agreement and >60% = weak agreement.
Abbreviations: DAP, dual antiplatelet therapy; DOAC, direct oral anticoagulant; IQR, interquartile range; SPwHA, severe patient with hemophilia A.

Table 5 Results of Round 1 of the Delphi Panel Survey of 13 Hemophilia Experts About Their Opinions About Switching Treatment from Factor to Non-Factor Replacement Therapy

Question	Options	Likelihood Estimate		Proportion of Panelists	
		Median[a]	IQR[b]	With Likelihood Estimate ≤50%	With Likelihood Estimate >50%
Given the current evidence on efficacy and safety for emicizumab, the likelihood that hematologist will switch treatment from a FVIII therapy to emicizumab prophylaxis for different number of bleeds during previous year for SPwHA without inhibitors	0–1 bleeds during previous year	15.0	66.0	61.5%	38.5%
	2–3 bleeds during previous year	66.0	78.0	46.2%	53.8%
	4–5 bleeds during previous year	84.0	36.5	23.1%	76.9%
	>5 bleeds during previous year	93.0	24.0	15.4%	84.6%
Among SPwHA without inhibitors who had ~5 bleeding events while on FVIII prophylaxis during the past 6 months, the likelihood of the reduction in bleeding events if treatment is switched to emicizumab prophylaxis	<10% reduction in bleeding events	7.0	35.5	76.9%	23.1%
	10–40% reduction in bleeding events	30.0	61.0	61.5%	38.5%
	41–80% reduction in bleeding events	81.0	18.5	7.7%	92.3%
	> 80% reduction in bleeding events	69.0	33.5	30.8%	69.2%
Among SPwHA without inhibitors who have switched from FVIII prophylaxis to emicizumab prophylaxis, the likelihood of the change in medication adherence (given that other factors associated with adherence such as age, frequency of bleeding, medication beliefs, etc. remain constant)	No change in medication adherence	10.0	74.0	61.5%	38.5%
	10–20% increase in doses/medication administered	38.0	72.5	61.5%	38.5%
	21–50% increase in doses/medication administered	50.0	52.5	53.8%	46.2%
	51–75% increase in doses/medication administered	48.0	51.0	61.5%	38.5%
	>75–80% increase in doses/medication administered	20.0	52.0	76.9%	23.1%

Notes: [a]Median likelihood estimate: smaller value indicates less preferred while larger value indicates more preferred therapeutic option by panelists; [b]IQR indicates strength of agreement, where 0=perfect agreement, <20% = strongest agreement, 21–40% = strong agreement, 41–60% = better agreement and >60% = weak agreement.
Abbreviations: IQR, interquartile range; SPwHA, severe patient with hemophilia A.

recommended by the World Federation of Hemophilia (WFH) guidelines[21] and available limited evidence in the literature,[22,23] a few panelists differed in their opinions around prophylaxis and consensus was not achieved. The uncertainty expressed by panelists toward emicizumab prophylaxis along with antithrombotic therapies was based on limited experience and lack of evidence-based guidelines on the use of emicizumab in elderly patients with CVD. Data on real-world adverse effects of emicizumab are still being collected; to this end, the thrombotic risk of emicizumab in elderly patients receiving antithrombotic treatments, remains unknown.[24,25]

In children and young adolescents with severe hemophilia A, physical activity, particularly high contact sports or prolonged activity, significantly increases the risk of bleeding.[26] The management of hemophilia A becomes particularly challenging if a patient is not highly adherent to prophylaxis.[27] For such patients, the panelists highlighted that this patient requires pre-activity episodic FVIII replacement therapy to mitigate the risk of bleeding; an on-demand, episodic regimen would not be adequate. Panelists needed more efficacy and/or effectiveness data to use emicizumab prophylaxis in these patients. HAVEN-3 trial results showed that emicizumab prophylaxis reduced joint bleeds as compared to no prophylaxis; however, no results were reported for young adults undergoing extensive physical activity.[28]

The surgical setting represents a challenge in the management of SPwHA due to the risk of peri-operative bleeding. The panel agreed that for a SPwHA without inhibitors undergoing a major surgical procedure such as arthroscopic surgery, FVIII replacement therapy is required during and post-surgery to secure hemostasis and wound healing; in such a scenario, emicizumab prophylaxis is not adequate as the hemostatic efficacy is not yet well established. Although HAVEN-3's intraindividual comparison of PwHA demonstrated emicizumab prophylaxis had significantly lower bleeds than previous FVIII prophylaxis, this cannot be extrapolated to a surgical setting, particularly given that the trial excluded patients experiencing bleeds due to surgery/procedures.[28,29]

SPwHA are also at increased risk of developing reduced bone mineral density, which poses greater risk of fractures and osteoporosis.[30,31] For these patients, panelists reached consensus that switching to FVIII prophylaxis with or without episodic FVIII regimen would not be appropriate. While the role of FVIII in overall bone health is promising, there is very limited evidence supporting the role of FVIII prophylaxis in maintaining bone health.[32] Although panelists preferred emicizumab prophylaxis with or without episodic FVIII more than FVIII prophylaxis with or without episodic FVIII, consensus was not reached. While a recent analysis of data from HAVEN-3 trial found that emicizumab promoted joint health in people with hemophilia A,[28] the long-term effect of emicizumab prophylaxis on bone density compared to routine FVIII prophylaxis is still unknown.

Strengths and Limitations

Our study has several strengths. Our study panel consisted of experts in the field of hemophilia who have wide experience treating PwHA in real-world settings. Use of the Delphi method as a structured expert elicitation technique enabled capturing of perspectives and opinions across a broad spectrum of experts. This methodology facilitated an in-depth exploration of attitudes and opinions that is not possible in quantitative surveys. Anonymity among panelists during online discussion forum in round 2 coupled with careful moderation of the forum by the moderators helped avoidance of an individual dominance that may result from strong verbalization or professional dominance, which may have led to biased results. This is often a concern in group-based approaches in qualitative studies, however anonymity and thoughtful direction helped prevent it in the study. In addition, anonymity allowed panelists to change their opinion on the basis of arguments presented by the other panel members during online discussion forum and avoided group pressure for conformity. These advantages of Delphi method are likely to increase reliability of consensus, as elucidated in previous studies.[33,34]

The study was limited by its small sample size and questions' generalizability. However, there is no gold standard of sample size for Delphi panels. Moreover, the sample size of this study aligns with the commonly observed number of experts involved in the previously published Delphi studies in hematology.[35–38] In addition, the clinical scenarios were designed using typical practice patterns in the US and may not adequately reflect clinical variations encountered worldwide. Given that panelists volunteered and were compensated to participate in the study, self-selection bias may be present. However, study participants did not differ meaningfully in clinical expertise or practice setting from those who declined to participate. While we undertook a comprehensive literature search covering a full spectrum of challenging clinical

scenarios, it is possible that relevant clinical scenarios were missed. Nevertheless, we anticipate that these study results provide unique views and valuable insights from the perspective of hematologists into current issues surrounding treatment decision-making for SPwHA.

Conclusion

The panelists achieved consensus for appropriate and inappropriate use of FVIII and emicizumab prophylaxis including, appropriate use of FVIII replacement therapy during and immediately post-surgery in addition to emicizumab prophylaxis for patients with elective surgery, and episodic FVIII followed by emicizumab prophylaxis for a newborn with post-circumcision bleeding. A majority of panelists preferred FVIII replacement therapy rather than emicizumab prophylaxis due to established evidence on safety, efficacy, and level of bleed protection for FVIII treatment. The recommendations emerging from this study may support or extend guidelines for practicing physicians when treating SPwHA without inhibitors. Further studies are needed to identify appropriate therapeutic approaches in those clinical cases for which consensus was not reached.

Ethics Approval

Using the Department of Health and Human Services regulations, 45 CFR 46.104(d)(2), Advarra Institutional Review Board (IRB) exempted this study from IRB oversight.

Funding

Disclosure

Sreenivas P. Veeranki was a former employee and Priti Pednekar, Marlon Graf, and Rifat Tuly are current employees of PRECISIONheor, a research consultancy to the health and life sciences industries. Sreenivas P. Veeranki is now affiliated with Optum LifeSciences, Eden Prairie, MN. Katharine Batt served as a consultant on this project through PRECISIONheor. Michael Recht's institutions have received research funding from Bayer, BioMarin, CSL Behring, Genentech, Grifols, Hema Biologics, LFB, Novo Nordisk, Octapharma, Pfizer, Sanofi, Spark, Takeda, and uniQure. He has served as a consultant to Catalyst Biosciences, CSL Behring, Genentech, Hema Biologics, Kedrion, Novo Nordisk, Pfizer, Sanofi, Takeda, and uniQure. He serves on the Board of Directors for the Foundation for Women and Girls with Blood Disorders and Partners in Bleeding Disorders. The authors report no other conflicts of interest in this work.

References

1. Mahlangu J, Oldenburg J, Callaghan MU, et al. Health-related quality of life and health status in persons with haemophilia A with inhibitors: a prospective, multicentre, non-interventional study (NIS). *Haemophilia*. 2019;25(3):382–391. doi:10.1111/hae.13731
2. Chen SL. Economic costs of hemophilia and the impact of prophylactic treatment on patient management. *Am J Manag Care*. 2016;22 (5 Suppl):s126–s133.
3. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1–e47.
4. Meeks SL, Batsuli G. Hemophilia and inhibitors: current treatment options and potential new therapeutic approaches. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):657–662. doi:10.1182/asheducation-2016.1.657
5. Centers of Disease Control & Prevention (CDC). Inhibitors and Hemophilia. Available from: https://www.cdc.gov/ncbddd/hemophilia/inhibitors.html#ref1. Accessed September 29, 2021.
6. Ayanian JZ, Landrum MB, Normand S-LT, Guadagnoli E, McNeil BJ. Rating the appropriateness of coronary angiography — Do practicing physicians agree with an expert panel and with each other? *N Engl J Med*. 1998;338(26):1896–1904. doi:10.1056/NEJM199806253382608
7. Campbell SM, Cantrill JA, Roberts D. Prescribing indicators for UK general practice: Delphi consultation study. *BMJ*. 2000;321 (7258):425. doi:10.1136/bmj.321.7258.425
8. Roberts M, Russell LB, Paltiel AD, et al. Conceptualizing a model: a report of the ISPOR-SMDM modeling good research practices task force–2. *Value Health*. 2012;15(6):804–811. doi:10.1016/j.jval.2012.06.016
9. Eubank BH, Mohtadi NG, Lafave MR, et al. Using the modified Delphi method to establish clinical consensus for the diagnosis and treatment of patients with rotator cuff pathology. *BMC Med Res Methodol*. 2016;16(1):56. doi:10.1186/s12874-016-0165-8
10. Armstrong C, Grant S, Kinnett K, et al. Participant experiences with a new online modified-Delphi approach for engaging patients and caregivers in developing clinical guidelines. *Eur J Pers Cent Healthc*. 2019;7(3):476–489.
11. Santaguida P, Dolovich L, Oliver D, et al. Protocol for a Delphi consensus exercise to identify a core set of criteria for selecting health related outcome measures (HROM) to be used in primary health care. *BMC Fam Pract*. 2018;19(1):152. doi:10.1186/s12875-018-0831-5
12. Santaguida PL, Oliver D, Gilsing A, et al. Delphi consensus on core criteria set selecting among health-related outcome measures (HROM) in primary health care. *J Clin Epidemiol*. 2020;127:105–116. doi:10.1016/j.jclinepi.2020.04.028
13. Diamond IR, Grant RC, Feldman BM, et al. Defining consensus: a systematic review recommends methodologic criteria for reporting of Delphi studies. *J Clin Epidemiol*. 2014;67(4):401–409. doi:10.1016/j.jclinepi.2013.12.002
14. Boulkedid R, Abdoul H, Loustau M, Sibony O, Alberti C. Using and reporting the Delphi method for selecting healthcare quality indicators: a systematic review. *PLoS One*. 2011;6(6):e20476. doi:10.1371/journal.pone.0020476
15. Birko S, Dove ES, Ozdemir V. Evaluation of nine consensus indices in Delphi foresight research and their dependency on Delphi survey characteristics: a simulation study and debate on Delphi design and interpretation. *PLoS One*. 2015;10(8):e0135162. doi:10.1371/journal.pone.0135162

16. National Hemophilia Foundation. MASAC Recommendations. Available from: https://www.hemophilia.org/Researchers-Healthcare-Providers/Medical-and-Scientific-Advisory-Council-MASAC/MASAC-Recommendations. Accessed July 2, 2020.

17. US Food and Drug Administration. Evaluating inclusion and exclusion criteria in clinical trials. FDA report; 2019.

18. Wood L, Bjarnason GA, Black PC, et al. Using the Delphi technique to improve clinical outcomes through the development of quality indicators in renal cell carcinoma. *J Oncol Pract.* 2013;9(5):e262–e267. doi:10.1200/JOP.2012.000870

19. Meshkat B, Cowman S, Gethin G, et al. Using an e-Delphi technique in achieving consensus across disciplines for developing best practice in day surgery in Ireland. *J Hosp Adm.* 2014;3(4):1–8. doi:10.5430/jha.v3n4p1

20. Colonna P, Andreotti F, Ageno W, Pengo V, Marchionni N. Clinical conundrums in antithrombotic therapy management: a Delphi Consensus panel. *Int J Cardiol.* 2017;249:249–256. doi:10.1016/j.ijcard.2017.09.159

21. World Federation of Hemophilia. *Guidelines for the Management of Hemophilia.* 2020. doi:10.1111/hae.14046

22. Mannucci PM, Schutgens RE, Santagostino E, Mauser-Bunschoten EP. How I treat age-related morbidities in elderly persons with hemophilia. *Blood.* 2009;114(26):5256–5263. doi:10.1182/blood-2009-07-215665

23. Coppola A, Tagliaferri A, Franchini M. The management of cardiovascular diseases in patients with hemophilia. *Semin Thromb Hemost.* 2010;36(1):91–102. doi:10.1055/s-0030-1248728

24. Dane KE, Lindsley JP, Streiff MB, Moliterno AR, Khalid MK, Shanbhag S. Successful use of emicizumab in a patient with refractory acquired hemophilia A and acute coronary syndrome requiring percutaneous coronary intervention. *Res Pract Thromb Haemost.* 2019;3(3):420–423. doi:10.1002/rth2.12201

25. Knoebl P, Sperr W, Schellongowski P, et al. Emicizumab for the treatment of acquired Hemophilia A: lessons learned from 4 very different cases. *Hamostaseologie.* 2019;39(S 01):SY13-SY15-AB.

26. Broderick CR, Herbert RD, Latimer J, et al. Association between physical activity and risk of bleeding in children with hemophilia. *JAMA.* 2012;308(14):1452–1459. doi:10.1001/jama.2012.12727

27. Zanon E, Tagliaferri A, Pasca S, et al. Physical activity improved by adherence to prophylaxis in an Italian population of children, adolescents and adults with severe haemophilia A: the SHAPE Study. *Blood Transfus.* 2020;18(2):152–158.

28. Mahlangu J, Oldenburg J, Paz-Priel I, et al. Emicizumab prophylaxis in patients who have Hemophilia A without inhibitors. *N Engl J Med.* 2018;379(9):811–822. doi:10.1056/NEJMoa1803550

29. National Institute of Health. A clinical trial to evaluate prophylactic emicizumab versus no prophylaxis in Hemophilia A participants without inhibitors (HAVEN 3); 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT02847637. Accessed September 29, 2021.

30. Roushan N, Meysamie A, Managhchi M, Esmaili J, Dormohammadi T. Bone mineral density in hemophilia patients. *Indian J Hematol Blood Transfus.* 2014;30(4):351–355. doi:10.1007/s12288-013-0318-4

31. Sahin S, Sadri S, Baslar Z, Ar MC. Osteoporosis in patients with hemophilia: single-center results from a middle-income country. *Clin Appl Thromb Hemost.* 2019;25:1076029619861689. doi:10.1177/1076029619861689

32. Gamal Andrawes N, Hashem Fayek M, Salah El-Din N, Atef Mostafa R. Effect of low-dose factor VIII prophylaxis therapy on bone mineral density and 25(OH) vitamin D level in children with severe haemophilia A. *Haemophilia.* 2020;26(2):325–332. doi:10.1111/hae.13917

33. Gupta UG, Clarke RE. Theory and applications of the Delphi technique: a bibliography (1975–1994). *Technol Forecast Soc Change.* 1996;53(2):185–211.

34. Fletcher AJ, Marchildon GP. Using the Delphi method for qualitative, participatory action research in health leadership. *Int J Qual Methods.* 2014;13(1):1–18. doi:10.1177/160940691401300101

35. Bonnet P, Gringeri A, Gomperts E, et al. Modeling costs and outcomes associated with a treatment algorithm for problem bleeding episodes in patients with severe hemophilia A and high-titer inhibitors. *Am Health Drug Benefits.* 2011;4(4):219.

36. Martinoli C, Alberighi OD, Di Minno G, et al. Development and definition of a simplified scanning procedure and scoring method for Haemophilia Early Arthropathy Detection with Ultrasound (HEAD-US). *Thromb Haemost.* 2013;109(06):1170–1179. doi:10.1160/TH12-11-0874

37. Neufeld EJ, Solimeno L, Quon D, et al. Perioperative management of haemophilia B: a critical appraisal of the evidence and current practices. *Haemophilia.* 2017;23(6):821–831. doi:10.1111/hae.13279

38. Seuser A, Khayat CD, Negrier C, Sabbour A, Heijnen L. Evaluation of early musculoskeletal disease in patients with haemophilia: results from an expert consensus. *Blood Coagul Fibrinolysis.* 2018;29(6):509. doi:10.1097/MBC.0000000000000767

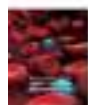# A Case Report of COVID-Associated Catastrophic Antiphospholipid Syndrome Successfully Treated with Eculizumab

Anusha Chidharla, Salman B Syed, Tulika Chatterjee & Michael D Tarantino

CASE REPORT

A Case Report of COVID-Associated Catastrophic Antiphospholipid Syndrome Successfully Treated with Eculizumab

Anusha Chidharla [ID][1]
Salman B Syed [ID][2]
Tulika Chatterjee[2]
Michael D Tarantino[3]

[1]Department of Medical Oncology, Kansas University Cancer Center, Kansas, KS, USA; [2]Department of Internal Medicine, University of Illinois College of Medicine, Peoria, IL, USA; [3]Department of Hematology, Bleeding and Clotting Disorders Institute, Peoria, IL, USA

Abstract: Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by multiple episodes of venous and arterial thromboses or recurrent fetal losses in the presence of antiphospholipid antibodies against $\beta_2 GP1$, frequently accompanied by moderate thrombocytopenia. Catastrophic APS (CAPS) is a severe manifestation of APS. COVID-19 may have an intense hypercoagulable state in critically ill patients. SARS-CoV2 may potentiate pathogenic APS effects, including the activation of endothelial cells, monocytes, platelets, and complement, resulting in a proinflammatory state and prothrombotic events. The endothelial tropism of SARS-CoV2 may also modify the clinical presentation of COVID-19 in susceptible individuals and trigger flares of underlying vascular diseases. We report a case of a 64-year-old woman with a history of triple-positive APS who had multiple thrombotic and bleeding episodes after being found to have a COVID-19 infection temporally associated with CAPS development that was successfully treated with eculizumab, preventing further macro- and microvascular thrombotic events at 1 month follow-up. Our case highlights the need for more research regarding the mechanism by which COVID-19 may potentiate APS and lead to the development of CAPS.

Keywords: antiphospholipid syndrome, phospholipid autoantibodies, catastrophic APS, COVID-19, severe acute respiratory syndrome coronavirus 2, eculizumab

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by multiple episodes of venous and arterial thromboses or recurrent fetal losses in the presence of antiphospholipid antibodies (aPL Abs) against $\beta_2 GP1$, and is frequently accompanied by moderate thrombocytopenia.[1] Rarely, APS may be associated with fulminant multiorgan failure or catastrophic APS (CAPS). CAPS is characterized by widespread small-vessel occlusions that lead to multiorgan failure. In an international study of CAPS-registry patients' lupus anticoagulant, IgG anticardiolipin and IgG anti $\beta_2 GP$ Abs were the most often implicated aPL Abs in CAPS. Although affecting only 1% of patients with APS, CAPS is frequently fatal if not recognized and treated promptly.[2] In spite of its clinical significance, with an approximate mortality rate of 40%, the pathophysiology remains somewhat enigmatic. Activation of the complement is required for the full clinical manifestation of APS. APS serum activates the complement in vitro, and patients with CAPS have been found to have a high rate of complement-gene mutations.[3] In

Correspondence: Anusha Chidharla
9219 West 157th Terrace, Overland Park, KS, 66221, USA
Tel +1 913-568-5708
Email anushaaedma@gmail.com

a descriptive analysis of 500 CAPS patients, the most common precipitating factor of CAPS was infection.[4] Infections, including COVID-19, are considered trigger factors of CAPS. It could be possible that SARS-CoV2 can induce CAPS; however, no evidence has been found for this. A study of COVID-19 patients showed aPL Abs were common in critically ill patients.[5] El Hasbani et al wrote a review article that discussed APL in the setting of COVID-19.[6] Eculizumab inhibits the activation of complement C5, and is approved for use in paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. We present a patient with a history of APS who had multiple thrombotic and bleeding episodes consistent with CAPS, likely triggered by SARS-CoV2 infection, that was successfully treated with eculizumab.

Case Presentation

A 64-year-old woman with a history of type 2 diabetes mellitus and triple-positive APS presented with acute encephalopathy. Her vital signs were normal on admission, with no significant physical examination findings except for altered mental status. Laboratory studies revealed hemoglobin (Hb) concentration of 131g/L, white blood cell (WBC) count of $11.8×10^9$/L, absolute lymphocyte count (ALC) of $1.5×10^9$/L, platelet count of $173×10^9$/L, and D-dimer of 0.54 μg/mL. MRI of the brain revealed chronic microvascular ischemia. Therapeutic unfractionated heparin was initiated, given her history of APS and multiple venous thrombotic events. On day 10, she developed a fever and hypoxia and tested positive for SARS-CoV2. Dexamethasone and remdesivir were initiated. Remdesivir was discontinued after 3 days, due to worsening kidney function. Subsequent laboratory testing revealed Hb concentration 86 g/L, WBC count $11×10^9$/L, ALC $0.77×10^9$/L, and platelet count $93×10^9$/L. APL Ab testing revealed a positive lupus anticoagulant, cardiolipin IgG >112 U/mL, IgA >65 U/mL, $β_2$GP1 IgG >112 U/mL, and IgA >65 U/mL. Her platelet count had decreased to $67×10^9$/L. A coagulation profile showed a prothrombin time of 23.3 seconds, international normalized ratio of 2.1, activated partial thromboplastin time of 132 seconds, fibrinogen activity of 832 mg/dL, and D-dimer of 1.56 μg/mL. On the following day, the patient developed acute respiratory distress requiring noninvasive ventilation. CT of the chest demonstrated diffuse bilateral ground-glass opacity a 4.3 cm left adrenal mass, concerning in terms of adrenal hemorrhage (Figure 1). Intravenous (IV)
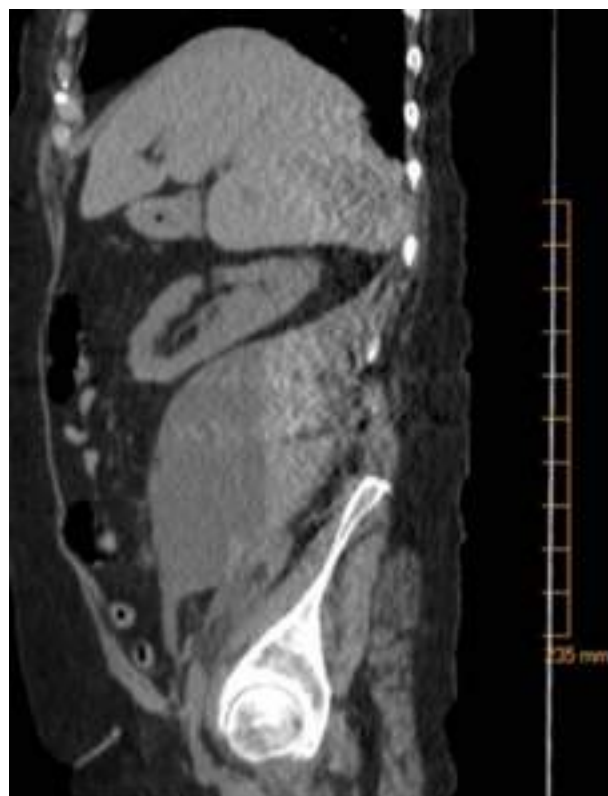

Figure 1 CT of abdomen and pelvis without contrast, showing large right-sided retroperitoneal mass (10×11×16 cm) concerning for hematoma of varying age.

methylprednisolone 500 mg was initiated. On day 19, the patient showed a significant decrease in Hb concentration and had retroperitoneal hemorrhage on imaging. Her anticoagulation was stopped. She later had acute venous thrombosis of the right superficial femoral vein. Because of intolerance to anticoagulation, an inferior vena cava filter was placed. She developed acute right-sided weakness on day 21 of admission, and the brain MRI showed new areas of subacute watershed infarctions with areas of concern in terms of hemorrhagic conversion (Figure 2). A direct antiglobulin test was positive, serum haptoglobin concentration was low, and serum lactate dehydrogenase concentration was elevated on further evaluation of anemia and thrombocytopenia, which raised concern regarding autoimmune hemolytic anemia. With new-onset ischemic stroke, acute venous thrombosis, and adrenal hemorrhage, the patient met the criteria for CAPS. She underwent single-volume plasma exchange daily for 5 consecutive days. She also received two doses of IV rituximab 1,000 g and two doses of intravenous immunoglobulin 1 mg/kg. APL Abs and coagulation parameters were monitored every other day, and initially decreased,
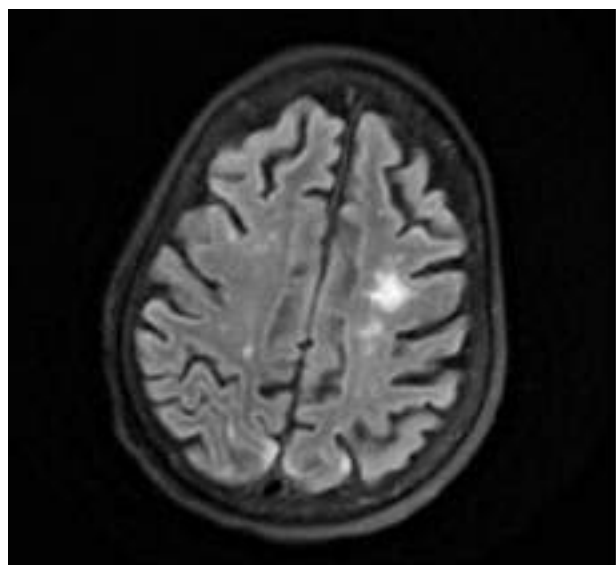
Figure 2 Brain MRI showing new areas representing subacute watershed infarctions with several punctate areas of acute infarction within the bilateral anterior cerebral artery/middle cerebral artery watershed territories.

but later continued to increase. Because of the rise in Ab titers and limited improvement in her clinical status, she was given IV eculizumab 900 mg weekly for refractory CAPS. She received two doses of eculizumab prior to discharge, and was started on prophylactic enoxaparin. The patient did not have any recurrent thrombotic events, and her mentation and right-sided weakness had continued to improve at 1-month follow-up.

Discussion

CAPS is a severe manifestation of APS. It is characterized by multiple thromboses involving three or more organs, systems, or tissue types, histopathology of small-vessel occlusion in at least one organ, acuity of manifestations, and aPL Ab positivity. When all four criteria are present, a "definite CAPS" diagnosis is confirmed. With our patient, she met the criteria for CAPS. Known risk factors of CAPS include infection, surgery, malignancy, anticoagulation withdrawal, low INR, obstetric complications, drugs, and systemic lupus erythematosus flares.[7]

Critically ill patients with COVID-19 are known to have a profound hypercoagulable state. The exact mechanism of COVID-associated thrombophilia is still being elucidated, but it is likely to have a multifactorial etiopathogenesis. Hypoxia and prolonged immobilization are important predisposing factors, but the diffuse inflammatory state and resulting "cytokine storm" seem to be the primary triggers.[6,8] In addition, endothelial dysfunction,

characterized by increased levels of von Willebrand factor, systemic inflammation, activation of Toll-like receptors, a procoagulatory state, and tissue factor–pathway activation are among the suggested mechanisms.[6] Another possible underrecognized mechanism may be the induction of aPL Ab production in severe COVID.[9] Molecular mimicry leading to anti-β_2GP1 Ab production has also been a proposed cause of secondary APS and CAPS. A small case series reported elevated aPL Abs in COVID-19 patients, and CAPS is associated with consistently elevated aPL Abs.[6,9–11]

Given that mortality rate in CAPS is approximately 50%, early identification and treatment is crucial. Nevertheless, due to the rarity of the disease, there have been no randomized controlled trials to guide therapy. The typical treatment approach for CAPS is multiagent therapy that includes high-dose corticosteroids, intravenous immunoglobulin, and plasma exchange.[12] Recent reports suggest that rituximab treatment may improve clinical signs and immunological profiles in CAPS management, and it is thus used in patients who have failed to respond to the standard therapy described.[13] In our patient's case, despite rituximab therapy, she continued to experience rising aPL Ab levels and worsening coagulation-profile results. As such, our patient received a trial of eculizumab, to which she responded well clinically. Also, the aPL titers and coagulation parameters decreased while our patient was on eculizumab. These parameters were consistently followed on an inpatient/outpatient basis.

Eculizumab is a humanized monoclonal Ab approved for use in paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome, but has been used in the treatment of refractory CAPS.[14–16] To our knowledge, this is the first case describing successful treatment of COVID-associated CAPS successfully treated with eculizumab. As complement activation plays a crucial role in animal models of APS thrombosis, eculizumab is considered a last resort for CAPS treatment. To date, data are restricted to eleven isolated case reports that indicated a dramatic improvement of CAPS after eculizumab. Inconsistent results with its use on clinical outcomes have been reported by Yelnik et al.[17] Ongoing randomized trials are investigating the role of anti-C5 drugs in severe COVID-19; however, several case series have suggested promising effects on clinical outcomes in patients with severe COVID-19.[18] Ours is the first case describing successful treatment of COVID-associated CAPS with eculizumab. The absence of further episodes of thrombosis despite

stagnant aPL Ab levels in our patient treated with eculizumab highlights the potential role of complement-mediated thrombosis in CAPS.

Conclusion

In a subset of patients with severe COVID-19, increased production of aPL Abs may play a role in the development of CAPS. Studies are needed to evaluate the presence of aPL Abs and CAPS in patients with COVID-19. SARS-Co V2 may exaggerate the clinical manifestations of APS, including activation of endothelial cells, monocytes, platelets, and complement, resulting in proinflammatory and prothrombotic states. The endothelial tropism of SARS-CoV2 may also modify the clinical presentation of COVID-19 in susceptible patients. Few studies have showen that patients with CAPS have mutations in complements, which increase complement activation. However, further studies are needed to elucidate the role of complement in CAPS. There have been case series reporting eculizumab use in refractory CAPS. High-quality studies like randomized control trials are needed to evaluate the efficacy of eculizumab in refractory CAPS. Our case highlights the need for more research regarding the mechanism(s) by which COVID-19 may potentiate APS predisposing to CAPS and treatment of refractory CAPS with eculizumab.

Consent

Written informed consent was obtained from the patient's health-care power of attorney/next of kin to have the case details and any accompanying results published. Institutional approval was not required to publish the case details.

Disclosure

Dr Michael D Tarantino is the CMO and CEO of the Bleeding and Clotting Disorders Institute, and reports grants and/or personal fees for consultancy, speakers' bureaux and/or clinical trial PI from Amgen, BioMarin, Dova, Genentech, Grifols, Octapharma, Principia, Sobi, Spark Therapeutics, Takeda, and UCB outside the submitted work. The authors report no other relevant conflicts of interest in this work.
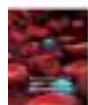
References

1. Gezer S. Antiphospholipid syndrome. *Dis Mon*. 2003;49(12):696–741. doi:10.1016/j.disamonth.2003.10.001

2. Rodriguez-Pintó I, López-Benjume B, Espinosa G, Cervera R. Catastrophic antiphospholipid syndrome. *Rev Colomb De Reumatol*. 2021;28:39–43. doi:10.1016/j.rcreu.2021.02.004

3. Chaturvedi S, Braunstein EM, Yuan X, et al. Rare Germline mutations in complement regulatory genes make the antiphospholipid syndrome catastrophic. *Blood*. 2019;134(Supplement_1):4. doi:10.1182/blood-2019-129115

4. Rodríguez-Pintó I, Moitinho M, Santacreu I, et al. Catastrophic antiphospholipid syndrome (CAPS): descriptive analysis of 500 patients from the International CAPS registry. *Autoimmun Rev*. 2016;15 (12):1120–1124. doi:10.1016/j.autrev.2016.09.010

5. Xiao M, Zhang Y, Zhang S, et al. Antiphospholipid antibodies in critically ill patients with COVID-19. *Arthritis Rheumatol*. 2020;72 (12):1998–2004. doi:10.1002/art.41425

6. El Hasbani G, Taher AT, Jawad A, Uthman I. COVID-19, antiphospholipid antibodies, and catastrophic antiphospholipid syndrome: a possible association? *Clin Med Insights Arthritis Musculoskelet Disord*. 2020;13:1179544120978667. doi:10.1177/1179544120978667

7. Mendoza-Pinto C, García-Carrasco M, Cervera R. Role of infectious diseases in the antiphospholipid syndrome (including its catastrophic variant). *Curr Rheumatol Rep*. 2018;20(10):62. doi:10.1007/s11926-018-0773-x

8. Merrill JT, Erkan D, Winakur J, James JA. Emerging evidence of a COVID-19 thrombotic syndrome has treatment implications. *Nat Rev Rheumatol*. 2020;16(10):581–589. doi:10.1038/s41584-020-0474-5

9. Mendoza-Pinto C, Escárcega RO, García-Carrasco M, Bailey DJO, Gálvez-Romero JL, Cervera R. Viral infections and their relationship with catastrophic antiphospholipid syndrome: a possible pathogenic mechanism of severe COVID-19 thrombotic complications. *J Intern Med*. 2020;288(6):737–739. doi:10.1111/joim.13123

10. Upson S, Murphy M, Ashraf F, Shahzad S. Double trouble: a case of suspected antiphospholipid syndrome in the setting of hypercoagulable COVID-19 pneumonia. *Chest*. 2020;158(4):A862. doi:10.1016/j.chest.2020.08.801

11. Yin S, Huang M, Li D, Tang N. Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS-CoV2. *J Thromb Thrombolysis*. 2020. doi:10.1007/s11239-020-02105-8

12. Gansner JM, Berliner N. The rheumatology/hematology interface: CAPS and MAS diagnosis and management. *Hematology*. 2018;2018(1):313–317. doi:10.1182/asheducation-2018.1.313

13. Rymarz A, Niemczyk S. The complex treatment including rituximab in the management of catastrophic antiphospholid syndrome with renal involvement. *BMC Nephrol*. 2018;19(1):132. doi:10.1186/s12882-018-0928-z

14. Kazzaz NM, McCune WJ, Knight JS. Treatment of catastrophic antiphospholipid syndrome. *Curr Opin Rheumatol*. 2016;28 (3):218–227. doi:10.1097/BOR.0000000000000269

15. Tinti MG, Carnevale V, Inglese M, et al. Eculizumab in refractory catastrophic antiphospholipid syndrome: a case report and systematic review of the literature. *Clin Exp Med*. 2019;19(3):281–288. doi:10.1007/s10238-019-00565-8

16. Nauseef JT, Lim HI, DeSancho MT. Successful outcome with eculizumab treatment in a patient with antiphospholipid syndrome presenting with an unusual thrombotic storm. *J Thromb Thrombolysis*. 2020. doi:10.1007/s11239-020-02343-w

17. Yelnik CM, Miranda S, Mékinian A, et al. Patients with refractory catastrophic antiphospholipid syndrome respond inconsistently to eculizumab. *Blood*. 2020;136(21):2473–2477. doi:10.1182/blood.2020007499

18. Fodil S, Annane D. Complement inhibition and COVID-19: the story so far. *ImmunoTargets Ther*. 2021;10:273–284. doi:10.2147/ITT.S284830

Indirect Treatment Comparison of Damoctocog Alfa Pegol versus Turoctocog Alfa Pegol as Prophylactic Treatment in Patients with Hemophilia A

Parth Vashi, Katharine Batt, Robert Klamroth, Maria Elisa Mancuso, Renata Majewska, Andreas Tiede & Lorenzo Giovanni Mantovani

Published online: 01 Nov 2021.

Submit your article to this journal ⬚

View related articles ⬚

View Crossmark data ⬚

ORIGINAL RESEARCH

Indirect Treatment Comparison of Damoctocog Alfa Pegol versus Turoctocog Alfa Pegol as Prophylactic Treatment in Patients with Hemophilia A

Parth Vashi[1],*
Katharine Batt[2],*
Robert Klamroth [3]
Maria Elisa Mancuso[4]
Renata Majewska [5,6]
Andreas Tiede [7]
Lorenzo Giovanni Mantovani[8,9]

[1]US Data Generation and Observational Studies, Bayer Corporation, Whippany, NJ, USA; [2]Department of Internal Medicine, Section on Hematology/ Medical Oncology, Wake Forest University Baptist Medical Center, Winston-Salem, NC, USA; [3]Department for Internal Medicine - Vascular Medicine and Coagulation Disorders, Vivantes Klinikum im Friedrichshain, Berlin, Germany; [4]Center for Thrombosis and Hemorrhagic Diseases, IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy; [5]HEOR, Creativ-Ceutical, Krakow, Poland; [6]Department of Epidemiology, Epidemiology and Preventive Medicine, Jagiellonian University Medical College, Krakow, Poland; [7]Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany; [8]Center for Public Health Research, University of Milan - Bicocca, Monza, Italy; [9]Value-Based Healthcare Unit, IRCCS Multimedica, Sesto San Giovanni, Italy

*These authors contributed equally to this work

Correspondence: Renata Majewska
Creativ-Ceutical, ul. Przemysłowa 12, Kraków, 30-701, Poland
Tel +33176704790
Email renata.majewska@creativ-ceutical. com

Purpose: To assess the efficacy and FVIII consumption of BAY 94-9027 versus N8-GP in prophylaxis in adolescent and adult patients with severe hemophilia A (HA).

Patients and Methods: A systematic literature review was conducted to identify studies on the efficacy of BAY-94-9027 and N8-GP for prophylaxis in patients with HA aged ≥12 years without a history of inhibitors. Eight studies met systematic literature review inclusion criteria, but only data from PROTECT VIII on BAY 94-9027 and PATHFINDER 2 on N8-GP could be used for an indirect comparison. Matching-adjusted indirect comparison (MAIC) and simulated treatment comparison were performed.

Results: No significant differences (unadjusted and adjusted) were observed in the mean annualized bleeding rate (ABR) for any bleed and proportion of patients with zero bleeds when comparing BAY 94-9027 to N8-GP. The adjusted treatment difference [incidence rate ratio (IRR)] in terms of ABR was 1.11 (95% CI, 0.85–1.44). The odds ratio (OR) of any bleed, measuring the relative effect of BAY 94-9027 versus N8-GP on the proportion of patients with zero bleeds, was 1.03 (95% CI, 0.60–1.77). FVIII consumption was significantly lower in BAY 94-9027 [mean adjusted difference=−1292.57 IU/kg/year (95% CI, −2152.44 to –432.70)]; a 26.7% reduction in consumption of BAY-94-9027. The results of the sensitivity analyses were similar to the main analysis for mean ABRs, percentages of patients with zero bleeds, and significant reduction in rFVIII consumption. For patients on BAY 94-9027 every-5-days and every-7-days, no differences versus every-4-days N8-GP were observed for the mean ABR for any bleed [IRR=0.90 (95% CI, 0.68–1.20)] and proportion of patients with zero bleeds [OR=1.06 (95% CI, 0.56–2.02)].

Conclusion: BAY 94-9027 prophylaxis demonstrated 26.7% lower annual consumption when compared to N8-GP with similar efficacy in terms of ABR and percentage of patients with zero bleeds.

Keywords: BAY 94-9027, N8-GP, prophylaxis, bleed, coagulation factor VIII, adults, factor VIII consumption

Introduction

The current standard of care in patients with hemophilia (HA) is a regular replacement of or prophylactic treatment with coagulation factor VIII using either recombinant (rFVIII) or plasma-derived (pdFVIII) clotting factor concentrates intended to prevent recurrent bleeds and subsequent joint damage.[1,2] Patients on prophylaxis have been shown to require fewer hospital admissions and surgical interventions, less frequent unscheduled visits for breakthrough bleeds, and report better quality of life and less time off school/ work.[1]

Although hemophilia is a rare disease, it is associated with high aggregate costs and imposes a high financial burden on individuals, healthcare systems, and society in general. Hemophilia is a chronic condition that requires lifelong treatment, with individual costs varying based on disease severity, complications, and treatment regimen.[3] Medications to treat hemophilia cost more than $270,000 annually per patient on average in the US.[4] If complications occur, the annualized cost can exceed $1 million.[5]

The latest advances in product development aim to extend the half-life of FVIII using fusion to protein conjugates (Fc part of IgG1 or albumin), chemical modification (PEGylation), and protein sequence modification.[6] Extended half-life rFVIII products, such as damoctocog alfa pegol (BAY 94-9027, Jivi, Bayer AG, Berkeley, USA), have decreased the frequency of infusions from about three to one to two per week.[7,8] BAY 94-9027 effectively prevented and treated bleeds with individually tailored dose regimens at intervals up to every 7 days.[8] Little is known about the comparative efficacy of different extended half-life (EHL) rFVIII products. There are no head-to-head efficacy trials, and the comparison is even more difficult because of lack of randomized trials with a common comparator arm. A comparison of BAY 94-9027 versus two EHL rFVIII agents (efmoroctocog alfa (rFVIIIFc), Elocta from Sobi, Orphan Biovitrum AB, Stockholm, Sweden and rurioctocog alfa pegol (BAX 855), Adynovate/ Adynovi, Takeda, Lexington, MA, USA) for prophylaxis in patients with severe HA was previously performed using the method of matching-adjusted indirect comparison (MAIC).[9] The results showed that a mean FVIII consumption was over 20% lower with BAY 94-9027 than with rFVIIIFc and median consumption tended to be lower with BAY 94-9027 than with BAX 855. Furthermore, mean annualized bleeding rates (ABRs) and percentages of patients with zero bleeds were similar between BAY 94-9027 and comparators. Prophylaxis with BAY-94-9027 and turoctocog alfa pegol (N8-GP, Esperoct, Novo Nordisk, Bagsvaerd, Denmark) has not been compared yet as N8-GP was not yet available (FDA approval received Feb 2019[10]). Therefore, we conducted this analysis to assess the efficacy and FVIII consumption of those agents in adolescent and adult patients with severe HA based on existing clinical trial data using the same methodology as earlier.

Materials and Methods
Data Sources and Feasibility Analysis

A systematic literature review was conducted to identify relevant sources of data. The objective of the systematic literature review was to identify all clinical evidence in patients with HA aged ≥12 years regarding the efficacy of BAY-94-9027 and N8-GP for prophylaxis in patients without a history of inhibitor. Searches in key databases (MEDLINE and MEDLINE In-Process, EMBASE and Cochrane Library) were performed on December 3, 2019 and November 18, 2019. Additionally, a hand search of ClinicalTrials.gov was performed to identify other relevant data. The search was restricted to full-text articles.

After screening titles and abstracts of 76 publications, 7 were included in the full-text review. A total of 5 studies (6 records) met the final inclusion criteria and were included in the systematic literature review. Three studies assessed BAY 94-9027: the PROTECT VIII study[8] was partially randomized, an extension of the PROTECT VIII[11] study was an open-label study, and Phase I trial[12] was nonrandomized. Three studies assessed N8-GP (the PATHFINDER 2 study[13] was partially randomized, but the two follow-ups on some patients who completed the PATHFINDER 2 study[14] were nonrandomized). As the baseline characteristics of patients continuing in the extension phase of PATHFINDER-2 were not available, extension studies were excluded. It was not possible to adjust for differences in characteristics of patients between extension studies. Additionally, outcomes were reported in the PATHFINDER 2 extension publication by regimen, with patients contributing to several regimens if they switched. Therefore, if we used data by regimen, there would be an additional problem of comparability due to lack of data on periods of time for which patients were observed on different regimens. If we used pooled data from different regimens, then some patients would be double counted. The phase I trial was excluded as well.

In summary, the analysis was based on the main phases of two clinical trials: PROTECT VIII on BAY 94-9027 and PATHFINDER 2 on N8-GP. The PROTECT VIII study is an open-label Phase II/III trial of parallel design with one on-demand and three prophylaxis arms (different schedules and doses) and nonrandomized allocation between on-demand and prophylaxis arms. The PATHFINDER 2 trial on N8-GP is an open-label, nonrandomized Phase III trial of parallel design with one on-demand and one prophylaxis arm. Both studies had similar inclusion criteria (indication, age range, length of previous treatment), but there were differences in characteristics of included populations. The reported outcomes were defined similarly in the PROTECT VIII and PATHFINDER 2 studies: annualized medians and means of any treated bleeds and medians of spontaneous bleeds and rates of patients with

no bleeds during the study. Moreover, both studies reported a mean annualized consumption in consistent units (IU/kg). The length of the main phase observation period was 36 weeks in the PROTECT VIII trial and approximately 1 year in the PATHFINDER 2 trial. Individual patient data (IPD) for the PROTECT VIII trial were provided by Bayer. For the PATHFINDER 2 trial, only aggregate study-level information was available.

Study Outcomes

The primary outcomes of interest were ABRs (any bleeds) and annualized consumption of factor VIII (IU/kg/y). Due to different observation periods between compared studies, the proportion of patients with zero bleeds was considered as a secondary outcome only.

Statistical Analyses

Although both PROTECT VIII and PATHFINDER 2 trials had control arms (on-demand arms), methods for unanchored (without a common comparator) trials were applied, ie, the data from on-demand arms were not considered. Patients were not randomly allocated to on-demand arms; therefore, the outcomes observed in prophylaxis and on-demand arms were not comparable. MAIC and regression methods [simulated treatment comparison (STC)] – both recommended by the National Institute for Health and Care Excellence Decision Support Unit for indirect treatment comparison (ITC) of nonrandomized trials – were applied.[15] MAIC was chosen as the primary scenario as it uses fewer assumptions regarding the type of relationship between adjustment variables and outcomes. The purpose of a MAIC is to reduce bias caused by differences in the characteristics of the populations (eg, baseline demographic characteristic, disease severity, or prior treatments) of included trials.

In this analysis, MAIC was implemented in the following steps. First, a weight was assigned to each participant of the PROTECT VIII study such that the weighted study-level baseline characteristics of PROTECT VIII reflected the baseline characteristics of the PATHFINDER 2 study. Second, the parameters measuring the effect of BAY 94-9027 (as mean of ABR) were estimated using the assigned weights such that the greater a weight assigned to a patient, the higher the impact on the estimated treatment effect. In each of these two steps IPD of PROTECT VIII was used. Finally, a relative effect of BAY 94-9027 compared to N8-GP was estimated using the weighted effect of BAY 94-9027, as estimated in the previous steps of the MAIC, and the effect of N8-GP as reported in publications

of the PATHFINDER 2 study. This process is illustrated in Appendix Figure 1.

Outcomes of interest were compared between trials in planned prophylaxis treatment groups [intention-to treat (ITT)].

Primary analyses focused on a comparison of ABR and consumption using pooled prophylaxis arms in the PROTECT VIII study. Prophylaxis arms [twice weekly (n=24), every 5 days (n=43), and every 7 days (n=43)] were assumed comparable and pooled into one prophylaxis group, as there were no significant differences in outcomes of interest between groups. Prophylaxis primary efficacy outcome (ABR) as well as a mean annualized total FVIII consumption (IU/kg/year) were based on data from weeks 11 to 36 (end of run-in/randomization to end of the main phase). The same rule was applied to the percentage of patients with zero bleeds, a secondary efficacy outcome. Patients in the prophylaxis arm were initially treated with 25 IU/kg BAY 94-9027 twice weekly during a 10-week run-in period, which was used to identify patients who experienced more frequent bleed and therefore were not expected to benefit from less frequent infusions and were therefore not randomized.[8]

To assess the mean ABR for any bleed, a Poisson regression model on the number of bleeds per patient, allowing for overdispersion using imputed ABR for missing data was used in the PATHFINDER 2 study.[13] The same methodology was applied to the patient-level ABR data from the PROTECT VIII study. The following covariates available in both trials were considered for population adjustment and weight calculations: age (years); race (white/Asian/other), weight (kg), prior prophylaxis treatment (yes/no), prior number of bleeds >2 in prior prophylaxis treatment, and prior number of bleeds >19 in prior on-demand treatment.

Relative treatment effects, comparing BAY 94-9027 vs N8-GP for assessed outcomes, and associated 95% confidence intervals (CI) were estimated for both weighted (adjusted comparison) and unweighted (naïve comparison) estimates from the IPD analysis of PROTECT VIII compared to previously published estimates from PATHFINDER 2:

- Incidence rate ratio (IRR) with 95% CI was reported for ABR for any bleeds,
- Odds ratio (OR) with 95% CI was reported for the percentage of patients without bleeding events,

- Mean differences with 95% CI were reported for annualized consumption.

All calculations were done using R 4.0.2.

Sensitivity Analysis

Four sensitivity analyses were conducted for primary and secondary outcomes to test the robustness of the results for the comparison between BAY-94-9027 and N8-GP used as prophylactic treatment in patients with severe HA. Sensitivity analysis 1 was an ITC analysis using STC with all pooled prophylaxis arms of the PROTECT VIII trial. Sensitivity analysis 2 was a MAIC analysis using ITC between the prophylaxis arm of the PATHFINDER 2 study and pooled every-5-days and every-7-days prophylaxis arms of the PROTECT VIII study. Sensitivity analysis 3 was a MAIC analysis using ITC between the prophylactic arm of the PATHFINDER 2 study and pooled biweekly and every-5-days prophylaxis arms of the PROTECT VIII study. In the last analysis, we compared all outcomes of interest including the run-in period; therefore, outcomes were summarized from week 0 to 36 for all pooled prophylaxis arms of the PROTECT VIII trial.

Results

Baseline Characteristics and Effective Sample Size

Baseline characteristics before and after matching of all variables simultaneously (main scenario) are presented in Table 1. Before matching, the mean age of patients from the PROTECT VIII study was higher than that of the PATHFINDER 2 study (P < 0.01). There were also higher percentages of patients of Asian race with number of bleeds >19 in the prior on-demand treatment group in the PROTECT VIII study compared to the PATHFINDER 2 study (P > 0.05 and P < 0.05, respectively).

The post-matching characteristics in the PROTECT VIII study were comparable to those in the PATHFINDER 2 study. The effective sample size of the PROTECT VIII study was 61.94 (60.73% of the original sample of 102 patients).

ITC of BAY 94-9027 vs N8-GP

Adjusted estimates for mean ABR for any bleed were close to the crude mean ABR number reported in the PROTECT VIII trial (Figure 1). No statistically significant differences were observed in the mean ABR, primary efficacy outcomes

when comparing BAY 94-9027 to N8-GP, nor in unadjusted or adjusted analyses. The adjusted treatment difference between BAY 94-9027 to N8-GP considering ABRs, ie the IRR of bleed, was 1.11 (95% CI, 0.85–1.44). Statistically significant differences were observed in factor VIII consumption, noted lower in BAY 94-9027 compared to N8-GP with the mean adjusted difference of –1292.57 IU/kg/y (95% CI, –2152.44 to –432.70). This difference reflects a 26.7% reduction in the units consumption of BAY-94-9027 compared to N8-GP. A lack of significant difference was also observed for the proportion of patients without bleeds when comparing BAY 94-9027 to N8-GP. The OR of any bleed, measuring the relative effect of BAY 94-9027 versus N8-GP on the proportion of patients with zero bleeds was 1.03 (95% CI, 0.60–1.77). Moreover, the MAIC-adjusted median ABR for spontaneous bleeds were the same between compared treatments and equaled zero.

Sensitivity Analysis

The results of the sensitivity analyses were similar to the main analysis with no statistical difference in mean ABRs and a statistically significant reduction in rFVIII consumption. The adjusted treatment effect in terms of ABR, ie the IRR of bleed, was 0.88 (95% CI, 0.66–1.16) using the STC method instead of MAIC, 0.90 (95% CI, 0.68–1.20) when pooling only patients treated with every 5 days and every 7 days regimens, 1.09 (95% CI: 0.02–66.36) when pooling those treated with biweekly and every 5 days regimens for BAY 94-9027 and 1.11 (95% CI 0.86–1.45) when pooling all prophylaxis arms and including run-in period (Table 2). The STC-adjusted mean annualized consumption was significantly lower in the PROTECT VIII study compared to the PATHFINDER 2 study with an adjusted difference of –1336.31 IU/kg/year (95% CI, –2064.74 to –607.88). A similar difference in consumption was observed when pooling patients treated with every 5 days and every 7 days regimens for BAY 94-9027; (adjusted difference of –1453.26 (95% CI, –2291.62 to –614.91) IU/kg/year and when pooled biweekly and every 5 days prophylaxis PROTECT VIII arms were used (adjusted difference of –1137.62 IU/kg/year (95% CI, –1855.45 to –419.79), see Table 2.

The results of the sensitivity analyses were also similar when the secondary outcome was considered. No statistically significant difference in the proportion of patients with zero bleeds between BAY 94-9027, and N8-GP was observed for none of the sensitivity analysis scenario (Table 2).

Table 1 Quality of Baseline Characteristics Matching (Main Scenario)

Variables	Prematching Characteristics			Postmatching Characteristics	
	PATHFINDER 2	PROTECT VIII (IPD)	P value	PROTECT VIII	P value
N	175	102*		61.94 (60.73%)	
Age, mean [years] (SD)	30.60 (12.50)	34.54 (12.95)	0.0021	30.60 (12.49)	1.000
Weight, mean [kg] (SD)	75.00 (14.40)	76.74 (17.15)	0.3652	75.00 (14.40)	1.000
Asian race (%)	18	26	0.1152	18	1.000
White race (%)	77	71	0.2682	77	1.000
Prior PPX (%)	85	80	0.2844	85	1.000
Prior no. of bleeds >2 in prior PPX (%)	50	57	0.3090	50	1.000
Prior no. of bleedings >19 in prior OD (%)	50	85	0.0145	50	1.000

Notes: *Eight patients had missing values for at least one variable used for matching.
Abbreviations: IPD, individual patient data; OD, on-demand treatment; PPX, prophylaxis.

Discussion

HA is a rare disease, which may explain why no head-to-head efficacy studies comparing active treatments were identified in the systematic literature review. For ethical reasons, existing studies are nonrandomized, and moreover, patients with a prophylaxis history should not be allocated to on-demand arms. Naive, unanchored, comparisons of results observed in different treatments in different studies are of low credibility due to many confounding factors (eg, population, outcomes, study design). Methods such as MAIC and STC used for this analysis may reduce bias related to known differences between study populations because the population of one study is adjusted to reflect the population of the other one.

According to the previous MAICs,[9] ABR (considered the most clinically meaningful efficacy endpoint[16]) and percentages of patients with zero bleeds were similar were similar comparing BAY 94-9027 with rFVIIIFc, BAX 855, and rAHF-PFM. (Appendix Table 1). A recent MAIC with rFVIIIFc[17] suggested no difference in the proportion of patients with zero bleeds between individualized rFVIIIFc and BAY 94-9027 pooled prophylaxis arms, but a significantly lower mean ABR in the rFVIIIFc individualized prophylaxis group versus the BAY 94-9027 pooled prophylaxis population (mean difference [MD] − 1.9; 95% confidence interval [CI] − 3.5 to − 0.4). In the publication by Batt et al,[9] the mean ABR after weighting for difference in baseline characteristics was similar between BAY 94-9027 and individualized rFVIIIFc: 4.25 vs 2.91 (P > 0.05). The difference in the results might be explained by different set of variables used in weighting of individualized data, population selection, as well as different summary measures compared.

The publication of Hakimi et al[17] has a relatively high effective sample size, which helps reaching statistical significance, but this was associated with the use of fewer adjustment factors. There was no adjustment on prior use of prophylaxis. Additionally, the population was selected to be specific to only one of the treatment arms studied for rFVIIIFc compared to a pooled analysis of all treatment arms from the PROTECT VIII trial. Furthermore, the recent publication[17] was based on a comparison of arithmetic means of ABR, while that of Batt et al[9] compared mean ABRs based on a negative binomial model. ABR was highly skewed in the PROTECT VIII trial, therefore means based on a negative binomial model would be a better summary here than arithmetic means. The study by Hakimi et al also did not consider the difference in the quantity of medication consumed between rFVIIIFc and BAY 94-9027.

The present analysis demonstrates similar findings for BAY 94-9027 and N8-GP in both ABR (IRR of 1.11 (95% CI, 0.85–1.44; p-value 0.4490)) and number of patients with zero bleeds (1.03 (95% CI, 0.60–1.77; p-value 0.9039)). As ABR appears to be similar between products, other criteria need to be considered when choosing between products. The quantity of medications used for treating HA and preventing complications correlate with cost and are a key factor. This study showed that the use of medication is over 20% lower with BAY 94-9027 than with N8-GP, as previously shown vs rFVIIIFc, the reduction of the mean annualized consumption of FVIII was here 26% (3552.43 IU/kg/year vs 4845.00 IU/kg/year, respectively). This might be considered as an important advantage of BAY 94-9027.

Our main finding of reduced factor consumption while maintaining similar bleed rates has relevance to a clinical
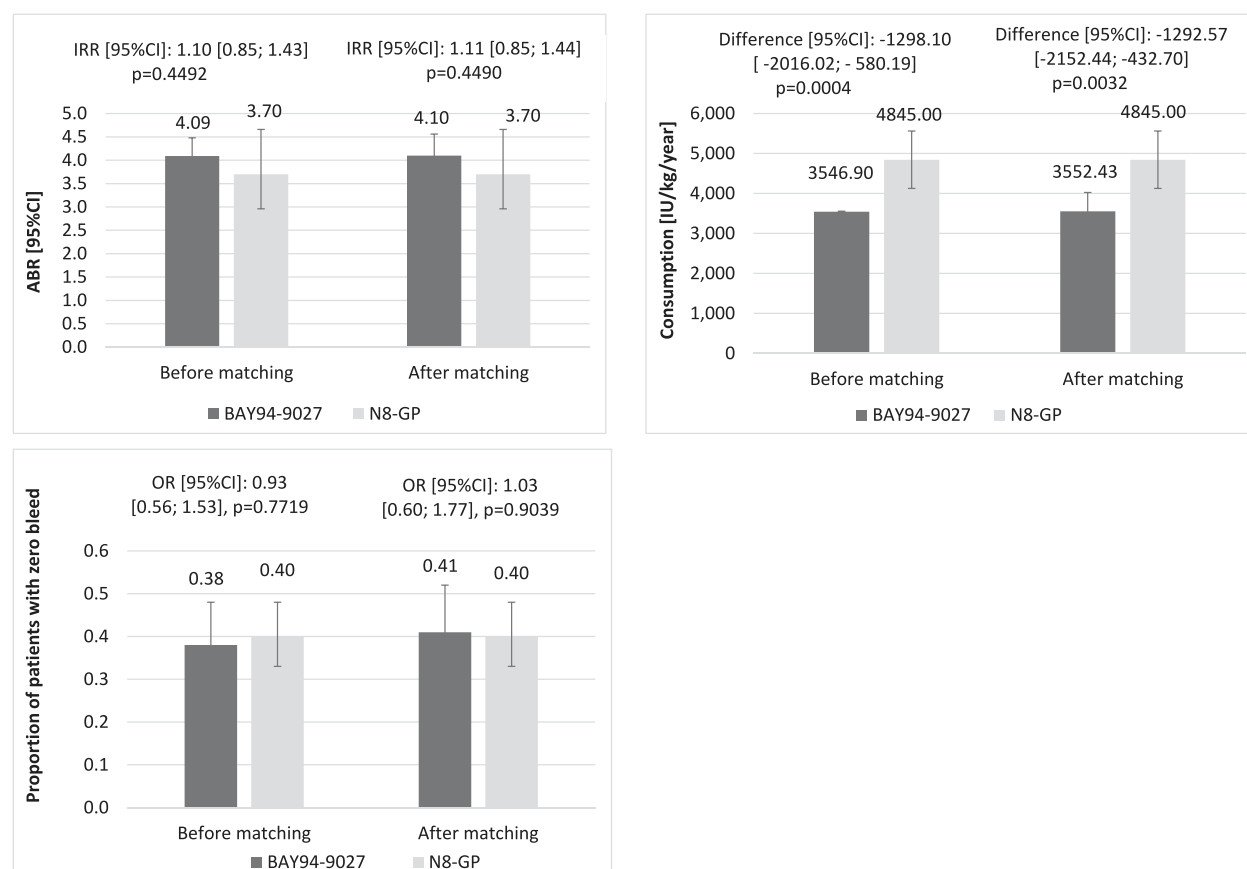
Figure 1 Comparison of ABR, consumption and proportion of patients with zero bleed between BAY94-0927 and N8-GP.
Abbreviations: ABR, annualized bleeding rate; CI, confidence interval; OR, odds ratio; p, p-value; IRR, incidence rate ratio.

audience, given the financial burden HA care has on health care budgets. It is therefore important to consider the underlying reasons for our findings and the implications for translation into clinical practice benefits under real-world conditions. First, differences in pharmacokinetics may account in part for the effects observed in our study. Head-to-head pharmacokinetic studies comparing BAY 94-9027 and N8-GP are not available. Compared to the PEGylated rFVIII concentrate, BAX 855, BAY 94-9027 showed significantly increased exposure (area under the curve (AUC) ratio 1.22 [95% confidence interval 1.11–1.33], $P = 0.0004$) due to slower clearance (ratio 0.82 [0.75–0.90], $P = 0.0004$).[18] Likewise, compared to rFVIIIFc, BAY 94-9027 showed higher exposure (AUC ratio 1.26 [1.14–1.38], $P = 0.0001$) and slower clearance (ratio 0.80 [0.72–0.87], $P = 0.0001$).[19] The differences in FVIII clearance is thought to be linked to the structure of the low-density lipoprotein receptor and size of PEGylation extending the FVIII half-life. Also, the type of conjugation, eg, site-specific conjugation as used in

BAY 94-9027, may contribute to extending the pharmacokinetics and/or pharmacodynamics or change its dynamics.[18,20] Thus, PEGylation is one of the factors likely explaining the similar >20% reduction in factor use when comparing the BAY 90-9027 clinical trials to it's the other factor concentrates. Second, differences in study design may in part explain the reduced factor consumption with BAY 94-9027 as compared with N8-GP as seen in our study. N8-GP was used at the same dosing regimen (50 IU/kg every 4 days) for all patients in the PATHFINDER 2 study. In contrast, the PROTECT VIII study had a run-in phase of 10 weeks (BAY 94-9027 dosed 25 IU/kg twice-weekly) that stratified patients with low bleeding risk (0 or 1 breakthrough bleeds, later-on randomized to prophylaxis every 5 or every 7 days) vs higher bleeding risk (≥2 breakthrough bleeds, later-on prophylaxis twice-weekly). This stratification resulted in spending more factor concentrate on prophylaxis in patients with higher bleeding risk as compared to patients with lower bleeding risk and finally resulted in similar ABR across all

Table 2 Indirect Treatment Comparison Between BAY-94-9027 and N8-GP in Prophylaxis Hemophilia Treatment (Sensitivity Analyses)

Variables	PATHFINDER 2	PROTECT VIII		Comparison of PROTECT-VIII vs PATHFINDER 2	
		Crude Estimates	Adjusted Estimates	Crude Estimates	Adjusted Estimates
ABR for any bleeds*	**Mean (95% CI)**	**Mean (95% CI)**	**Mean (95% CI)**	**IRR (95% CI); p**	**IRR (95% CI); p**
STC – pooled all PPX arms	3.7 (2.96–4.66)	4.09 (3.72–4.48)	3.24 (2.81–3.73)	1.10 (0.85–1.43); 0.4492	0.88 (0.66–1.16); 0.3490
MAIC – pooled Q5D and Q7D PPX arms	3.7 (2.96–4.66)	3.81 (3.40–4.26)	3.34 (2.86–3.90)	1.03 (0.79–1.34); 0.8351	0.9 (0.68–1.20); 0.4818
MAIC – pooled BIW and Q5D PPX arms	3.70 (2.96–4.66)	4.01 (3.54–4.53)	4.02 (0.07–243.84)	1.08 (0.83–1.42); 0.5617	1.09 (0.02–66.36); 0.9686
MAIC – pooled all PPX arms (including run-in period)	3.70 (2.96–4.66)	4.10 (3.75–4.50)	4.12 (3.70–4.58)	1.11 (0.86–1.43); 0.4269	1.11 (0.86–1.45); 0.4226
Consumption (IU/kg/year)	**Mean (95% CI)**	**Mean (95% CI)**	**Mean (95% CI)**	**Difference (95% CI); p**	**Difference (95% CI); p**
STC – pooled all PPX arms	4845.00 (4127.17–5562.83)	3546.90 (3536.15–3557.64)	3508.69 (3247.15–3445.14)	−1298.1 (2016.02 to −580.19); 0.0004	−1336.3 (−2064.74 to −607.88); 0.0001
MAIC – pooled Q5D and Q7D PPX arms	4845 (4127.17–5562.83)	3436.50 (3343.39–3529.61)	3391.74 (2958.67–3824.81)	−1408.5 (−2132.34 to −684.65); 0.0001	−1453.3 (−2291.62 to −614.91); 0.0007
MAIC - pooled BIW and Q5D PPX arms	4845 (4127.17–5562.83)	3681.2 (3531.25–3831.16)	3707.38 (3706.53–3708.23)	−1163.8 (−1897.12 to −430.47); 0.0019	−1137.6 (−1855.45 to −419.79); 0.0019
MAIC – pooled all PPX arms (including run-in period)	4845 (4127.17–5562.83)	3353.06 (3343.47–3362.65)	3341.52 (2893.12–3789.93)	−1491.94 (−2209.84 to −774.04); <0.0001	−1503.48 (−2349.85 to −657.10); 0.0005
Proportion of patients with zero bleed (%)	**Proportion (95% CI)**	**Proportion (95% CI)**	**Proportion (95% CI)**	**OR (95% CI); p**	**OR (95% CI); p**
STC – pooled all PPX arms	40 (33–48)	38 (29–48)	42 (32–51)	0.93 (0.56–1.53); 0.7719	1.08 (0.66–1.77); 0.7655
MAIC – pooled Q5D and Q7D PPX arms	40 (33–48)	41 (30–52)	41 (28–55)	1.04 (0.61–1.80); 0.8779	1.06 (0.56–2.02); 0.8587
MAIC – pooled BIW and Q5D PPX arms	40 (33–48)	38 (26–49)	41 (0–1)	0.90 (0.50–1.62); 0.7261	1.05 (0.00–19×10^6); 0.9951
MAIC – pooled all PPX arms (including run-in period)	40 (33–48)	33 (24–42)	35 (25–46)	0.73 (0.44–1.21); 0.2229	0.83 (0.48–1.42); 0.4907

Notes: *Based on Poisson model. The effective sample size when pooling Q5D and Q7D PPX arms was 40.31 (51.68% of the original sample) and 33.14 (51.78% of the original sample) when pooling BIW and Q5D PPX arms of PROTECT VIII study.
Abbreviations: ABR, annualized bleeding rate; BIW, twice weekly, IRR, incidence rate ratio; MAIC, matching-adjusted indirect comparison; OD, odds ratio; PPX, prophylaxis; Q5D, every 5 days; Q7D, every 7 days; STC, simulated treatment comparison.

dosing tiers. Therefore, not only product characteristics but guidance for dosing and individualized regimens based on response to treatment have an impact on the balance between resource utilization and clinical outcomes.

In the absence of randomized studies with an identical comparator, the ITC methodological guidelines allows for the use of the MAIC as a reasonable method for comparing nonrandomized, unanchored trials with adjustments made for patient characteristics at baseline, thereby minimizing the bias of comparing effects from trials with

different baseline.[15] By comparing studies with similar outcome definitions (excluding those from the observation period), we minimized study design. Finally, by performing sensitivity analyses around different pooled treatment regimens and other ITC approach (STC), we confirmed the results as they were in line with those obtained in the main analysis.

The use of MAIC or STC instead of naive (nonadjusted) comparison carries some limitations as it reduces bias only if all key treatment modifiers and prognostic factors are included for an adjustment. In our case,

a clinically important factor of the prior number of target joints bleeds[21,22] was not considered as this parameter was not reported in the PATHFINDER 2 study. It is also possible that there are other unknown effect modifiers that were not considered in the analysis. It is notable that MAIC adjusts for differences in patient characteristics only, but not for any difference in the study design or outcome definitions. Furthermore, the main phase of the PATHFINDER 2 study was twice as long as in the PROTECT VIII study, which could have additional implications. First, it could affect the comparison of proportions of patients with zero bleeds and result in a bias in favor of BAY 94-9027, because the longer patients are followed, the more likely they are to experience at least one bleed. This efficacy outcome was considered as secondary endpoint of interest. And second, it may also affect the comparison of ABR as the frequency of bleeding tends to decrease over time in patients treated on prophylaxis.[23] The same trend was observed in PROTECT-VIII extension study. Mean ABR (based on negative binomial model) observed at the end of main phase of PROTECT VIII was 4.07,[9] while the one observed in extension study (5 years of follow-up) was 3.49.[11] Therefore, the ABR comparison may be biased against BAY 94-9027. Finally, considered study periods were short, and the study with longer data would provide more robust evidence to changes in ABR.

Conclusions

Prophylactic treatment with BAY 94-9027 shows similar efficacy in terms of ABR and percentage of patients with zero bleeds and is consistent with a 26.7% lower annual factor consumption, suggesting potential cost savings with its use.

Abbreviations

ABR, annualized bleeding rate; CI, confidence interval; EHL, extended half-life; Fc, fragment crystallizable; FVIII, factor VIII; HA, hemophilia A; Ig, immunoglobulin; IRR, incidence rate ratio; ITC, indirect treatment comparison; IU, International units; OR, odds ratio; SD, standard deviation; STC, simulated treatment comparison.

Ethics Approval and Informed Consent

Both the PATHFINDER 2 and PROTECT VIII studies were conducted in accordance with the Declaration of Helsinki and local regulations. The protocols were approved by the authorities and the ethics committees of the respective institutions, and signed informed consent was obtained from all patients. Informed consent for this analysis was not required given the deidentified nature of the PROTECT VIII individualized patient-level data, and the use of aggregated previously published data from the PATHFINDER 2 study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

AT received grants or research support or honoraria for lectures or consultancy in the field of hemophilia from Bayer, Biotest, Chugai, CSL Behring, Novo Nordisk, Octapharma, Pfizer, Roche, SOBI, Takeda. KB has no competing interests; she has consulted with Bayer, HEMA, Takeda, CHEORS, Precisionheor, Kezar and Forma Therapeutics. LGM received personal fees from Bayer in the field of hemophilia and from Roche, Pfizer, Biogen, Takeda outside the field of hemophilia; research grants from Bayer, Roche and Takeda in the field of hemophilia and from Roche, Biogen and Bayer outside the field of hemophilia. PV is an employee of Bayer. RK received honoraria for Advisory boards and presentations from Bayer, Biotest, Biomarin, CSL Behring, Grifols, NovoNordisk, Octapharma, Pfizer, Sanofi, SOBI, Takeda, Unique. RM is an employee of Creative-Ceutical, which received funds to conduct the study. MEM has acted as paid consultant/advisor/speaker for Bayer Healthcare, Biomarin, Catalyst

Bioscience, CSL Behring, Grifols, Kedrion, LFB, Novo Nordisk, Octapharma, Pfizer, Roche, Sobi, Spark Therapeutics, Takeda and UniQure. The authors report no other conflicts of interest in this work.

References

1. Makris M. Prophylaxis in haemophilia should be life-long. *Blood Transfus*. 2012;10(2):165–168.
2. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1–e47.
3. Chen SL. Economic costs of hemophilia and the impact of prophylactic treatment on patient management. *Am J Manag Care*. 2016;22 (5 Suppl):s126–s133.
4. Report ES. Express scripts 2015 drug trend report; 2016. Available from: https://californiahealthline.org/wp-content/uploads/sites/3/ 2018/02/express-scripts-2015-commercial-dtr.pdf. Accessed October 10, 2020.
5. Medicaid Health Plans of America Center for Best Practices. Clinical brief. Addressing the Needs of members with hemophilia in Medicaid managed care: issues and implications for health plans; 2013. Available from: https://www.hca.wa.gov/assets/program/bdc_ MHPA_hemophilia_issue_brief_082113.pdf. Accessed October 10, 2020.
6. Graf L. Extended half-life factor VIII and factor IX preparations. *Transfus Med Hemotherapy*. 2018;45(2):86–91. doi:10.1159/ 000488060
7. Konkle BA, Stasyshyn O, Chowdary P, et al. Pegylated, full-length, recombinant factor VIII for prophylactic and on-demand treatment of severe hemophilia A. *Blood*. 2015;126(9):1078–1085. doi:10.1182/ blood-2015-03-630897
8. Reding MT, Ng HJ, Poulsen LH, et al. Safety and efficacy of BAY 94-9027, a prolonged-half-life factor VIII. *J Thromb Haemost*. 2017;15(3):411–419. doi:10.1111/jth.13597
9. Batt K, Gao W, Ayyagari R, et al. Matching-adjusted indirect comparisons of annualized bleeding rate and utilization of BAY 94-9027 versus three recombinant factor VIII agents for prophylaxis in patients with severe hemophilia A. *J Blood Med*. 2019;10:147–159. doi:10.2147/JBM.S206806
10. U.S. Food and Drug Administration. ESPEROCT (STN: BL 125671); 2019. Available from: https://www.fda.gov/vaccines-blood-biologics/esperoct. Accessed June 30, 2021.
11. Lalezari S, Reding MT, Pabinger I, et al, BAY 94-9027 prophylaxis is efficacious and well tolerated for up to >5 years with extended dosing intervals: PROTECT VIII extension interim results. *Haemophilia*. 2019;25(6):1011–1019. doi:10.1111/hae.13853
12. Coyle TE, Reding MT, Lin JC, Michaels LA, Shah A, Powell J. Phase I study of BAY 94-9027, a PEGylated B-domain-deleted recombinant factor VIII with an extended half-life, in subjects with hemophilia A. *J Thromb Haemost*. 2014;12(4):488–496. doi:10.1111/ jth.12506

13. Giangrande P, Andreeva, T., Chowdary, P., Ehrenforth, S., Hanabusa, H., Leebeek, FW, Lentz, SR, Nemes, L., Poulsen, LH, Santagostino, E., You, CW, Clausen, WH, Jönsson, PG, Oldenburg, J. Clinical evaluation of glycoPEGylated recombinant FVIII: efficacy and safety in severe haemophilia A. *Thromb Haemost*. 2017;117 (2):252–261. doi:10.1160/TH16-06-0444
14. Curry N, Albayrak, C., Escobar, M., Holme, PA, Kearney, S., Klamroth, R., Misgav, M., Négrier, C., Wheeler, A., Santagostino, E., Shima, M., Landorph, A., Tønder, SM, Lentz SR. Once-weekly prophylaxis with glycoPEGylated recombinant factor VIII (N8-GP) in severe haemophilia A: safety and efficacy results from pathfinder 2 (randomized phase III trial). *Haemophilia*. 2019;25:373–381. doi:10.1111/hae.13712
15. Phillippo D, Ades AE, Dias S, Palmer S, Abrams KR, Welton NJ. *Nice DSU Technical Support Document 18: Methods for Population-Adjusted Indirect Comparisons in Submissions to Nice*. NICE DSU; 2016.
16. Keipert C, Müller-Olling M, Gauly F, Arras-Reiter C, Hilger A. Annual bleeding rates: pitfalls of clinical trial outcomes in hemophilia patients. *Clin Transl Sci*. 2020. doi:10.1111/cts.12794
17. Hakimi Z, Santagostino E, Postma MJ, Nazir J. Recombinant FVIIIFc versus BAY 94-9027 for treatment of patients with haemophilia A: comparative efficacy using a matching adjusted indirect comparison. *Adv Ther*. 2021;38(2):1263–1274. doi:10.1007/s12325-020-01599-1
18. Solms A, Shah A, Berntorp E, et al. Direct comparison of two extended half-life PEGylated recombinant FVIII products: a randomized, crossover pharmacokinetic study in patients with severe hemophilia A. *Ann Hematol*. 2020;99(11):2689–2698. doi:10.1007/s00277-020-04280-3
19. Shah A, Solms A, Wiegmann S, et al. Direct comparison of two extended-half-life recombinant FVIII products: a randomized, crossover pharmacokinetic study in patients with severe hemophilia A. *Ann Hematol*. 2019;98(9):2035–2044. doi:10.1007/s00277-019-03747-2
20. Mei B, Pan C, Jiang H, et al. Rational design of a fully active, long-acting PEGylated factor VIII for hemophilia A treatment. *Blood*. 2010;116(2):270–279. doi:10.1182/blood-2009-11-254755
21. Thorat T, Neumann PJ, Chambers JD. Hemophilia burden of disease: a systematic review of the cost-utility literature for hemophilia. *J Manag Care Spec Pharm*. 2018;24(7):632–642. doi:10.18553/ jmcp.2018.24.7.632
22. Arima K, Gupta S, Gadkari A, et al. Burden of atopic dermatitis in Japanese adults: analysis of data from the 2013 national health and wellness survey. *J Dermatol*. 2018;45(4):390–396. doi:10.1111/1346-8138.14218
23. Noone D, O'Mahony B, Prihodova L. A survey of the outcome of prophylaxis, on-demand or combined treatment in 20-35 year old men with severe haemophilia in four European countries. *Haemophilia*. 2011;17(5):e842–e843.

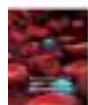# A Rare Association of Thrombotic Thrombocytopenic Purpura with Systemic Lupus Erythematosus in a Sudanese Woman: Case Report

Amel Awad Ibn Idris Rodwan, Osama Khder Ahmed Elmansour, Amar F Eldow Ahmed, Elnour Mohammed Elagib, Noha Ibrahim Ahmed Eltahir, Abubaker Hassan, Sara M El-Sadig, Abdel Gaffar Abdel Allah Mohammed, Huyam H Awadalla, Abubakr Abdalwahab Mohammed & Mohammed Elmujtba Adam Essa

CASE REPORT

A Rare Association of Thrombotic Thrombocytopenic Purpura with Systemic Lupus Erythematosus in a Sudanese Woman: Case Report

Amel Awad Ibn Idris Rodwan[1]
Osama Khder Ahmed Elmansour[1,2]
Amar F Eldow Ahmed[3]
Elnour Mohammed Elagib[1,4]
Noha Ibrahim Ahmed Eltahir[4,5]
Abubaker Hassan[6]
Sara M El-Sadig[7]
Abdel Gaffar Abdel Allah Mohammed[8]
Huyam H Awadalla[9]
Abubakr Abdalwahab Mohammed[5]
Mohammed Elmujtba Adam Essa [ID][6,10]

[1]Department of Rheumatology, Sudan Medical Specialization Council, Khartoum, Sudan; [2]Department of Rheumatology, Faculty of Medicine, Shendi University, Shendi, Sudan; [3]Department of Internal Medicine, Wayne State University, Detroit, MI, USA; [4]Department of Rheumatology, Omdurman Military Hospital, Khartoum, Sudan; [5]Department of Medicine, Faculty of Medicine, Karary University, Khartoum, Sudan; [6]Department of Clinical Medicine, Medical and Cancer Research Institute, Nyala, Sudan; [7]Department of Medicine, Faculty of Medicine, University of Khartoum, Khartoum, Sudan; [8]Rheumatology Department, Security Forces Hospital, Makkah, Saudi Arabia; [9]Department of Internal Medicine, Detroit Medical Centre, Detroit, MI, USA; [10]Faculty of Medicine, Al Fashir University, Al Fashir, Sudan

Correspondence: Mohammed Elmujtba Adam Essa
Department of Clinical Medicine, Medical and Cancer Research Institute, Nyala, Sudan
Tel +249-90-700-938
Email Awadali818@yahoo.com

Abstract: Thrombotic thrombocytopenic purpura (TTP) is an uncommon life-threatening condition characterized by hemolytic disorders. The coexistence of systemic lupus erythematous (SLE) with TTP is extremely rare, although Africans are at increased risk due to inherited risk factors. This report describes a rare clinical manifestation of TTP associated with SLE in a Sudanese patient. A 41-year-old Sudanese woman presented to the emergency department with symptoms and features that were suggestive of malaria, for which she had been treated accordingly. However, a few days later she complained of fever, and was found to have a body temperature of 39.5°C, jaundice, anemia, and thrombocytopenia. Soon after admission, she developed confusion and unrecordable blood pressure. After the patient had stabilized, clinical assessment, immune-system investigation (antinuclear antibody profile, complements, blood panel), and imaging revealed a diagnosis of TTP associated with SLE. The patient received imipenem 500 mg, five sessions of plasmapheresis (60 mL/kg), methylprednisolone 1 g pulse for 3 days, and rituximab 375 mg/week. Three weeks later, the patient was discharged after her condition had improved, and she is now on regular follow-up.

Keywords: TTP, SLE, African female, rituximab and plasmapheresis

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the presence of autoantibodies directed against nuclear antigens.[1] The disease can presents with different features such as fever, skin changes, and hair loss. In severe cases of SLE patients can present with renal, hematological or central nervous system involvements.[2]

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening condition characterized by affecting the capillaries and arterioles of multiple organs, mainly caused by deficiency in ADAMTS13, also known as von Willebrand factor–cleaving protease (VWFCP).[3] VWFCP is significant in inhibiting spontaneous microvascular platelet clumping, an essential pathophysiological finding in TTP. In most TTP patients, ADAMTS13, the main regulator of VWF size, is severely deficient.[4]

TTP can occur through autoimmune inhibitors in acquired cases, such as infections, SLE, and neoplasms, or hereditarily through mutation of the *ADAMTS13* gene in the plasma. TTP in SLE patients is extremely rare — <0.5%.[5] Surprisingly, connective tissue diseases, including SLE, may occur with

low ADAMTS13 levels, suggesting probable pathophysiology for the coexistence of TTP and SLE.[6] The aim of this report was to describe a rare occurrence of TTP as the first presentation of SLE in Sudanese patients.

Case Presentation

A 41-year-old Sudanese woman presented to the emergency department with a 2-day history of high-grade fever (39.5°C) associated with fatigue, nausea, and vomiting. Clinical evaluation and a blood test revealed a diagnosis of malaria, for which treatment was administered. During the next few days, her symptoms worsened, in addition to yellow sclera and low hemoglobin (5.5 g/dL) and platelets (43 cells/mL). The patient was transferred to our rheumatology department at Omdurman Military Hospital for further evaluation. Clinical findings were normal except for jaundice (bilirubin 3.2 mg/dL). She indicated that her sister had Behçet's disease. Upon admission, she developed a severe headache associated with confusion and fever, her blood pressure was unrecordable, and her Glasgow Coma Scale score was 9/15. The rapid-response team was called, and she was admitted to a high-dependency unit, where she received six units of fresh frozen plasma, two pools red blood cells, and kept under close observation. After her condition had stabilized, laboratory investigations were requested (Table 1). A diagnosis of SLE and TTP was made, and she was given imipenem 500 mg, acyclovir 75 mg, electrolyte correction, IV normal saline 0.9%, skin and bladder care (five sessions of plasmapheresis), and methylprednisolone 1 g pulse for 3 days, followed by prednisolone 1 mg/kg in a tapering manner. A few days later, after the infection had been controlled, the patient received four doses of rituximab 375 mg/week with caution. Two weeks later, she was discharged in good general condition, with normal results on complete blood count and renal function testing. She was discharged on hydroxychloroquine 200 mg, azathioprine 50 mg, prednisolone 10 mg, pantoprazole 40 mg, calcium supplement 500 mg, and omega 3. On review at two weeks, she was completely well without any complaints.

Discussion

TTP is an uncommon life-threatening condition, and occurs in <0.5% of SLE patients.[7] It was first reported in

Table I Lab results

Investigations	Results	References
WBC count	18.6 cells/µL	4–11×10^9/L cells/µL
Hb	5.5 g/dL	12–16 g/dL
Platelets	43 cells/µL	150–450 cells/µL
Serum creatinine	1.6 mg/dL	0.5–1.1 mg/dL
Serum K	2.2 mmol/L	3–3.5 mmol/L
Serum albumin	2.5 g/dL	2.4–4 g/DL
Total protein	5.6 g/dL	6–8.3 g/dL
Total bilirubin	3.2 mg/dL	0.2–1.3 mg/dL
Alanine aminotransferase	26 U/L	10–130 U/L
Aspartate aminotransferase	76 U/L	10–34 U/L
Alkaline phosphatase	56 U/L (24–147 U/L)	24–147 U/L
Urine general	Cast granular + no bilirubin	
	7–9 pus cells	
	Fatty cast ++	
Peripheral blood smear	BFFM and ICT negative	
Reticulocyte count	9.1%	0.5%–1.5%
Coagulation profile	INR (1.5) PT (17 seconds) APTT (28.6 seconds)	INR (1:2) PT (10–12 seconds) PTT (30–45 seconds)
Septic screen for blood and urine	No growth	
Viral screen (HIV/ HCV/HBV)	Negative	
Direct Coombs test	Negative	

(*Continued*)

Table 1 (Continued).

Investigations	Results	References
ANA profile	Titer: 1/1,000 Pattern: fine speckled nuclear	
	Positive for anti-Sm, SSA/Ro60, borderline for SSA/Ro52.	
Complements	C3 156 mg/dL (90–180), C4 42 mg/dL (10–40)	
Antiphospholipid antibodies	Negative	
ECG	Sinus tachycardia	
Chest X-ray	Normal	
Abdominal US	Normal	
Echocardiography	Normal	
Brain MRI	Normal	

1924, but the pentad of microangiopathic hemolytic anemia, severe thrombocytopenia, renal insufficiency, neurological abnormalities, and fever was first published in 1966.[8] SLE is a chronic autoimmune disease that affects mainly women of reproductive age,[9] and is characterized by the production of various autoantibodies that target different organs, resulting in many clinical symptoms and signs, such as malaise, fever, headache, myalgia, arthralgia, and loss of weight.[10,11] In this case, the patient presented with clinical features of TTP and exhibited a progressive deteriorating course. The enormous potential for misrecognizing this disorder may be due to the complex picture of SLE presenting as TTP.[12] The differential diagnosis for this case could be Evans syndrome, as it also occurs with fever, fatigue, mucosal bleeding, pallor, cytopenia, and jaundice,[13] which are the same features that presented in this patient. However, Evans syndrome is characterized by autoimmune hemolytic anemia and a positive direct Coombs test result,[13,14] in contrast to anemia in TTP, which is microangiopathic anemia due to fragmented red blood cells and a negative direct Coombs test result, as in this patient. Other main differentials include disseminated intravascular coagulopathy (DIC), which is also characterized by cytopenic anemia and hemolysis, but in contrast to Evans syndrome and TTP, coagulation in DIC is deranged. An absence of

coagulopathy is the key differential, as all coagulation, fibrinolysis, and platelet systems are activated in DIC.[15]

Despite the fact that many patients with TTP in sub-Saharan Africa are at greater risk of mortality due to the absence of prompt diagnostic facilities and access to adequate therapy, an African patient with SLE and TTP may be at even greater risk. The clinical presentation of a TTP patient may not essentially compel a clinician to undertake workup for a primary connective tissue disease, such as SLE, as both conditions can have overlapping features. In our case, TTP was the first presentation of SLE, contrary to previous reports of TTP usually presenting in patients previously managed for SLE for years, with a high SLE Disease Activity Index score and coexisting nephritis.[12,16] Children with hematological conditions, such as venous thromboembolism, are managed by heparin, which may cause by what is known as heparin-associated thrombocytopenia. This may present similarly to TTP.[17]

Arthritis, polyarthralgia, hair loss, fever, osteonecrosis, and myopathy are common presentations reported in previous studies of TTP patients with SLE.[18] However, in addition to hair loss and fever, our patient also presented with confusion, jaundice, anemia, and low platelet count, which led to a high index of suspicion. We thus undertook a complement assay, and immunological findings reinforced the diagnosis of SLE. Furthermore, the occurrence of neurological complications in SLE patients of African descent may cause difficulty in diagnosis and proper management.

The clinical overlap between these two diseases has been described more commonly in young black female patients, and since certain severe manifestations have been found in SLE patients of African descent, TTP may constitute another such manifestation with notable links to genetic attributes.[19]

Our patient exhibited clinical and laboratory features for the diagnosis of SLE, which included skin changes, such as malar rash, positive immunological markers like anti-Smith antibodies and anti-dsDNA, abnormal antinuclear antibody profile, and high antinuclear antibody titers.

Plasma exchange continues to be the mainstay of treatment in patients with TTP, even with concomitant SLE and showing a low response[20,21] (Table 2), emphasizing the significance of looking out for this association, early diagnosis, and aggressive management with plasma exchange and immunosuppression, which is life-saving. Although there was no significant improvement in platelet count, after hepatitis infection had been ruled out, four doses of IV rituximab 375 mg/m^2 once

Table 2 Clinical manifestations and treatment

	SLE	TTP	TTP in SLE
Response to plasma exchange	Average	Average	Low
Sex predominance	Female	Equal	Female higher
Need for immunosuppressants	In some cases	In some cases	Most of cases
Relapse	High	High	Very high
Mortality	10%–15%	10%	30%–60%
Morbidity	Moderate–high	Moderate	Lupus nephritis: most require ICU and patients overall are sicker
ADAMTS13	—	Low	Variable

weekly led to dramatic improvement in the patient's condition and normalization of the platelet count.[22] Critical care specialists need to develop a multidisciplinary approach urgently, emphasizing early recognition and initiation of treatment to ensure better outcomes in this scenario.

Limitation

ADAMTS13-antibody testing, one of the investigations to confirm acquired TTP, was not done on the patient, as it is not available in Sudan.

Conclusion

A middle-aged Sudanese woman presented with fever, jaundice, confusion, unrecordable blood pressure, anemia, and thrombocytopenia. Immunological studies confirmed a diagnosis of TTP on top of SLE, which is a very rare association. The patient received antibiotics, plasmapheresis, methylprednisolone, and rituximab. Her condition improved well, and she was discharged and followed up. Initial diagnosis can overlap with malaria, as happened in this case, as severe malaria can cause thrombocytopenia. Therefore, it is important to consider TTP in patients presenting with hemolytic anemia and thrombocytopenia, as SLE and TTP can occur concomitantly and share similar hematological manifestations.

Data Sharing Statement

All the data used in the study are available from the first and corresponding authors on reasonable request.

Consent

Signed consent was obtained from the patient for publication of the case details.

Ethics Approval and Consent to Publish

Obtained from the Sudan Federal Ministry of Health.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Manson JJ, Rahman A. Systemic lupus erythematosus. *Orphanet J Rare Dis*. 2006;1:6. PubMed PMID: 16722594. Pubmed Central PMCID: PMC1459118. doi:10.1186/1750-1172-1-6
2. Adelowo OO, Oguntona SA. Pattern of systemic lupus erythematosus among Nigerians. *Clin Rheumatol*. 2009;28(6):699–703. PubMed PMID: 19242770. doi:10.1007/s10067-009-1139-6
3. Fujimura Y, Isonishi A. [Pathophysiology of thrombotic thrombocytopenic purpura]. *Nihon Jinzo Gakkai Shi*. 2014;56(7):1043–1051. Japanese. PubMed PMID: 25420404.
4. Kremer Hovinga JA, Lammle B. Role of ADAMTS13 in the pathogenesis, diagnosis, and treatment of thrombotic thrombocytopenic purpura. *Hematology Am Soc Hematol Educ Program*. 2012;2012:610–616. PubMed PMID: 23233642. doi:10.1182/asheducation.V2012.1.610.3798654
5. Ali Z, Zafar MU, Wolfe Z, Akbar F, Lash B. Thrombotic thrombocytopenic purpura induced by immune checkpoint inhibitiors: a case report and review of the literature. *Cureus*. 2020;12(10):e11246. PubMed PMID: 33274128. Pubmed Central PMCID: PMC7707147.
6. Shah AA, Higgins JP, Chakravarty EF. Thrombotic microangiopathic hemolytic anemia in a patient with SLE: diagnostic difficulties. *Nat Clin Pract Rheumatol*. 2007;3(6):357–362. PubMed PMID: 17538567. doi:10.1038/ncprheum0511
7. Crawley JT, Scully MA. Thrombotic thrombocytopenic purpura: basic pathophysiology and therapeutic strategies. *Hematology Am Soc Hematol Educ Program*. 2013;2013:292–299. PubMed PMID: 24319194. doi:10.1182/asheducation-2013.1.292
8. Luken BM, Turenhout EA, Hulstein JJ, Van Mourik JA, Fijnheer R, Voorberg J. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. *Thromb Haemost*. 2005;93(2):267–274. PubMed PMID: 15711742. doi:10.1160/TH04-05-0301
9. Fanouriakis A, Tziolos N, Bertsias G, Boumpas DT. Update on the diagnosis and management of systemic lupus erythematosus. *Ann Rheum Dis*. 2021;80(1):14–25. PubMed PMID: 33051219. doi:10.1136/annrheumdis-2020-218272

10. Catalina MD, Owen KA, Labonte AC, Grammer AC, Lipsky PE. The pathogenesis of systemic lupus erythematosus: harnessing big data to understand the molecular basis of lupus. *J Autoimmun*. 2020;110:102359. PubMed PMID: 31806421. doi:10.1016/j.jaut.2019.102359

11. Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD. Manifestations of systemic lupus erythematosus. *Maedica*. 2011;6(4):330–336. PubMed PMID: 22879850. Pubmed Central PMCID: PMC3391953.

12. Merayo-Chalico J, Demichelis-Gomez R, Rajme-Lopez S, et al. Risk factors and clinical profile of thrombotic thrombocytopenic purpura in systemic lupus erythematosus patients. Is this a distinctive clinical entity in the thrombotic microangiopathy spectrum?: a case control study. *Thromb Res*. 2014;134(5):1020–1027. PubMed PMID: 25257921. doi:10.1016/j.thromres.2014.09.005

13. Jaime-Perez JC, Aguilar-Calderon PE, Salazar-Cavazos L, Gomez-Almaguer D. Evans syndrome: clinical perspectives, biological insights and treatment modalities. *J Blood Med*. 2018;9:171–184. PubMed PMID: 30349415. Pubmed Central PMCID: 6190623. doi:10.2147/JBM.S176144

14. Michel M, Chanet V, Dechartres A, et al. The spectrum of Evans syndrome in adults: new insight into the disease based on the analysis of 68 cases. *Blood*. 2009;114(15):3167–3172. PubMed PMID: 19638626. doi:10.1182/blood-2009-04-215368

15. Wada H, Matsumoto T, Suzuki K, et al. Differences and similarities between disseminated intravascular coagulation and thrombotic microangiopathy. *Thromb J*. 2018;16(1):14. doi:10.1186/s12959-018-0168-2

16. Kwok SK, Ju JH, Cho CS, Kim HY, Park SH. Thrombotic thrombocytopenic purpura in systemic lupus erythematosus: risk factors and clinical outcome: a single centre study. *Lupus*. 2009;18(1):16–21. PubMed PMID: 19074164. doi:10.1177/0961203308094360

17. Molinari AC, Banov L, Bertamino M, Barabino P, Lassandro G, Giordano P. A practical approach to the use of low molecular weight heparins in VTE treatment and prophylaxis in children and newborns. *Pediatr Hematol Oncol*. 2015;32(1):1–10. PubMed PMID: 25325764. Epub 2014/ 10/18.eng. doi:10.3109/08880018.2014.960119

18. Torrente-Segarra V, Monte TCS, Corominas H. Musculoskeletal involvement and ultrasonography update in systemic lupus erythematosus: new insights and review. *Eur J Rheumatol*. 2018;5 (2):127–130. PubMed PMID: 30183613. Pubmed Central PMCID: PMC6072693. doi:10.5152/eurjrheum.2017.17198

19. Terrell DR, Vesely SK, Kremer Hovinga JA, Lammle B, George JN. Different disparities of gender and race among the thrombotic thrombocytopenic purpura and hemolytic-uremic syndromes. *Am J Hematol*. 2010;85(11):844–847. PubMed PMID: 20799358. Pubmed Central PMCID: PMC3420337. doi:10.1002/ajh.21833

20. George P, Das J, Pawar B, Kakkar N. Thrombotic thrombocytopenic purpura and systemic lupus erythematosus: successful management of a rare presentation. *Indian J Crit Care Med*. 2008;12(3):128–131. PubMed PMID: 19742252. Pubmed Central PMCID: PMC2738311. doi:10.4103/0972-5229.43682

21. Adam Z, Sokwala A, Shah J, Ali SK. A delay in diagnosis: thrombotic thrombocytopenia purpura occurring in systemic lupus erythematous. *Pan Afr Med J*. 2019;34:103. PubMed PMID: 31934246. Pubmed Central PMCID: 6945373. doi:10.11604/pamj.2019.34.103.20524

22. Aggarwal V, Singer Z, Ledingham D, Othman I. Refractory acquired thrombotic thrombocytopenic purpura in a patient with sickle cell trait successfully treated with caplacizumab. *Hematology*. 2021;26 (1):590–593. PubMed PMID: 34396933. doi:10.1080/16078454.2021.1959984

Life after Autologous Hematopoietic Stem Cell Transplantation for Systemic Sclerosis

Daniela A Moraes & Maria Carolina Oliveira

Published online: 09 Nov 2021.

Submit your article to this journal 🗗

Article views: 157

View related articles 🗗

View Crossmark data 🗗

Citing articles: 8 View citing articles 🗗

REVIEW

Life after Autologous Hematopoietic Stem Cell Transplantation for Systemic Sclerosis

Daniela A Moraes[1]
Maria Carolina Oliveira [ID][2]

[1]Division of Clinical Immunology, Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil; [2]Center for Cell-Based Therapy, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

Abstract: Stem cell transplantation has been investigated as treatment for severe and progressive systemic sclerosis (SSc) for the past 25 years. To date, more than 1000 SSc patients have been transplanted worldwide. Overall and event-free survival have increased over the years, reflecting stricter patient selection criteria and better clinical management strategies. This review addresses long-term outcomes of transplanted SSc patients, considering phase I/II and randomized clinical trials, as well as observational studies and those assessing specific aspects of the disease. Clinical outcomes are discussed comparatively between studies, highlighting advances, drawbacks and controversies in the field. Areas for future development are also discussed.

Keywords: systemic sclerosis, stem cell transplantation, long-term outcomes, progression-free survival

Introduction

Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by tissue fibrosis, pronounced alterations in the microvasculature and frequent abnormalities in cellular and humoral immunity.[1] Combined pathogenic mechanisms of inflammation, fibrosis and microvascular damage affect the skin and internal organs, including the lungs, heart, gastrointestinal tract and kidneys.[2] Conventional treatment includes systemic immunosuppression, vasodilators and more recently, anti-fibrotic therapy.[3] However, a subset of patients with severe and progressive disease is refractory to these approaches. A meta-analysis from 2012 showed that despite newly available medications, more standardized treatment protocols and strategies to enable early diagnosis, mortality in SSc had not decreased in 40 years.[4] In fact, none of the available conventional treatments reverse the natural course of the disease or demonstrate prolonged benefit.[5] Currently, interstitial lung disease, pulmonary hypertension and cardiac involvement are the major causes of death in patients with SSc.[2,6] Patients with rapidly progressive cutaneous involvement and visceral involvement have poor prognosis, with mortality rates reaching 30% after 5 years of diagnosis, despite conventional treatment.[7,8]

In the mid-1990s, given the lack of effective therapeutic options for refractory autoimmune diseases, and after reports of patients who underwent stem cell transplantation for hematological indications but that presented improvement of coincidental autoimmune conditions, autologous hematopoietic stem cell transplantation (AHSCT) was considered as treatment for patients with severe SSc.[9,10] Since then, several studies with series of patients and phase I/II clinical trials have shown the

Correspondence: Maria Carolina Oliveira
Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo, Avenida dos Bandeirantes, 3900, Ribeirão Preto, SP, 14048-900, Brazil
Email mcarolor@usp.br

efficacy of AHSCT in SSc.[11–15] More recently, randomized controlled trials have demonstrated the superiority of transplantation over conventional treatment.[16–18]

Experience with AHSCT for SSc has increased over time, and protocols have been refined, in special regarding patient selection. Consensus meetings and discussions within medical specialty societies also established recommendations and guidelines to improve patient outcomes.[9,19–21] Today, almost 25 years after the first transplant, SSc is the most frequently transplanted rheumatic disease in the world. Toxicity associated with the procedure has decreased and long-term disease control has improved. As a consequence, transplanted SSc patients have lived longer lives and it is possible to collect data from long-term follow-up. In this study, we revisit the literature, with special emphasis on the last 10 years, discussing short and long-term clinical outcomes of patients with SSc undergoing AHSCT.

We searched the literature in PubMed and Science Direct databases, within a defined period from 1995 to 2021, using words "stem cell transplantation" and "systemic sclerosis". Only studies in English were included. The articles were initially evaluated by title and abstract and, if necessary, in more detail. Among the available articles, only those with clinical data on autologous hematopoietic stem cell transplantation were selected. Research articles were preferred over case reports, review articles, commentaries, and editorials. Studies were excluded if included less than 5 patients, addressed conditions different than systemic sclerosis or included only pediatric patients. We mostly selected articles from the past 10 years, although older important publications have been referenced. Within the selected articles, the following data were extracted: transplant-related mortality, overall survival, progression-free survival, relapse or progression of SSc, disease progression, changes in modified Rodnan Skin Score (mRSS), changes in lung function, quality of life, fertility and long-term complications such as malignancies and secondary autoimmune diseases.

Transplant Procedure

Autologous stem cell transplantation is a form of intensive immunotherapy that aims to eradicate the autoreactive adaptive immune system. Autologous stem cells are harvested and cryopreserved before beginning of the procedure. These cells, thawed and reinfused intravenously to the patient after administration of an immunoablative conditioning regimen, provide accelerated hematopoietic reconstitution and enables reinstatement of a renewed immune system, with long-lasting tolerance to autoantigens (Figure 1). Autologous hematopoietic stem cell transplantation deeply modifies the immune system, promoting an immunological balance that halts inflammation and tissue destruction, enabling disease control, and, to some degree, tissue repair.[22]

Most transplant centers use a non-myeloablative conditioning regimen consisting of high doses of cyclophosphamide plus anti-thymocyte globulin. Higher intensity regimens including total body irradiation or thiotepa have been preferred by a few centers.[14,18,23,24] Different regimens have been compared and discussed for their advantages and drawbacks, mainly addressing aspects related to safety and efficacy, but so far, there are no specific recommendations.[25] There is also considerable debate about the benefits of graft selection, as numerous transplant centers manipulate the harvested autologous hematopoietic stem cells before cryopreservation, positively selecting the graft for CD34+ cells. Graft selection eliminates most mature lymphocytes and may reduce the risks of reinfusing autoreactive cells within the graft. On the

Figure 1 Schematic representation of the transplant procedure. Autologous stem cells are mobilized from the bone marrow to the peripheral blood, from where they are harvested by leukapheresis and cryopreserved (1). The graft can be selected before cryopreservation or remain unmanipulated. The patient then undergoes an immunoablative conditioning regimen (2), followed by intravenous administration of the autologous cells, which are thawed immediately before infusion (3). After a period of aplasia, there is reconstitution of the immune system (4), and the patient is discharged from the hospital. Long-term outcomes are evaluated over time.

other hand, it delays immunological recovery and may increase the incidence of viral infections after AHSCT.[26,27] Attempts to compare clinical outcomes from patients transplanted with or without graft selection have conflicting results, and further prospective investigations are needed.[28–30]

Overall Survival and Toxicity

Since the initial reports from the end of the last century, numerous phase I/II studies have shown feasibility and beneficial outcomes of AHSCT (Table 1).[11–15] In the last decade, three randomized controlled studies – ASSIST (Autologous Systemic Sclerosis Immune Suppression Trial, 2011), ASTIS (Autologous Stem Cell Transplantation versus Immunosuppression trial, 2014) and SCOT (Scleroderma: Cyclophosphamide or Transplantation trial, 2018) – have shown that AHSCT is superior in efficacy and safety than monthly intravenous cyclophosphamide (CY) pulses (Table 2).[16–18] A fourth study retrospectively compared the results of AHSCT with those of a historical cohort of SSc patients who shared similar clinical characteristics.[38] This was a single-center study, and updated knowledge on patient selection and intra-transplant management translated into lower transplant-related mortality and higher overall survival. Table 2 describes the main characteristics and patient outcomes from each trial.

ASSIST was a groundbreaking study in 2011, and the first to show benefits of transplantation over conventional therapy, despite the small number of enrolled patients and short follow-up of only 2 years.[16] According to the authors, study enrollment was stopped earlier than originally planned. An interim analysis showed failure of equipoise, since there was significant difference in outcomes between groups after inclusion of only 19 patients, favoring transplant. ASTIS, published in 2014, was a multicenter European trial that had a higher transplant-related mortality and lower overall survival than the other two studies.[17] The higher transplant-related toxicity of ASTIS was ascribed to the lack of a thorough evaluation of patients for cardiac involvement before transplantation.[39] The importance of cardiac screening only became fully known and incorporated into practice around 2010, at the end of patient recruitment in ASTIS.[32,40] As a result, it is possible that patients with severe cardiac involvement were enrolled for transplantation, increasing the death rate. Nevertheless, after 1 year of follow-up, in accordance with the other comparative trials,

ASTIS showed superiority of transplantation over conventional cyclophosphamide treatment.

The SCOT trial used a myeloablative and thus high-intensity conditioning regimen, including total body irradiation.[18] Although the described transplant-related mortality was acceptable in this trial, and lower than that of ASTIS, the 85% incidence of major (grade 4) transplant-related adverse effects evidences the potential toxicity of the regimen. In the past, total body irradiation, which included irradiation of the lung tissue, was associated with severe adverse events and high mortality in transplants for SSc patients.[11] Total body irradiation regimens may also be associated with scleroderma renal crises, as patients undergoing AHSCT that include TBI are more likely to develop acute kidney insufficiency.[41] As a result of this experience, lung and renal shielding are adopted in TBI-based regimens for SSc patients.[18,42] The available evidence in the literature is not sufficient to define whether the intensity of the transplant regimen associates with better or worse clinical outcomes. Higher intensity regimens may provide more efficient and long-lasting eradication of the autoreactive immune system, but non-myeloablative regimens may be safer. It is most likely that multiple factors are involved and that other aspects, such as patient selection, have a stronger influence on the process.

Transplant-associated cardiac toxicity is a current concern in AHSCT for SSc patients. Most conditioning regimens include high doses of cyclophosphamide, and this alkylating agent is associated with dose-dependent acute myocardial injury through direct endothelial capillary damage.[43] On the other hand, cardiac involvement is a frequent and underdiagnosed manifestation of SSc, as patients may be asymptomatic, and at early stages, echocardiography may overlook diastolic dysfunction.[44,45] To date, pre-transplant cardiac evaluation has been formally recommended by the European Society for Blood and Marrow (EBMT) and partners, and once incorporated by transplant centers, should contribute to a reduction in transplant-related mortality.[20] In parallel, alternative conditioning regimens, including lower doses of cyclophosphamide or different non-cardiotoxic agents, are also under investigation.[24,46]

Over the 20 past years, transplant-related mortality has declined from over 17% to less than 6%, and some centers have reported zero deaths from the procedure.[16,38,47] Systemic sclerosis is a complex disease and internal organ damage increases the toxicity of the procedure.

Table I Main Characteristics of Non-Randomized Phase I/II, Retrospective and Observational Studies

	Henes et al. 2012[31]	Burt et al. 2013[32]	Helbig et al. 2018[33]	Nakamura et al. 2018[34]	Ayano et al. 2019[29]	Guillaume-Jugnot et al. 2019[35]	Van-Bijnen et al. 2020[36]	Henes J et al. 2021[30]	Henrique-Neto et al 2021[37]
Country	Germany	USA/Brazil	Poland	Japan	Japan	France	Netherlands	Europe/Brazil	Brazil
Study design	Prospective Phase I/II 1997–2009	Retrospective 2002–2011	Prospective Phase I/II 2003–2016	Long-term follow-up of phase II 2000–2012	Post-hoc analysis of phase I/II 2002–2009	Retrospective 1997–2013	Retrospective multicenter 1998–2017	Prospective observational multicenter 2012–2016	Retrospective study 2009–2016
Inclusion criteria	Inefficacy of CY pulse therapy or progressive disease with indicators of bad prognosis	mRSS ≥14 plus interstitial lung disease or abnormal EKG or GI tract involvement	≤70 y of age Karnofsky >80 Disease <10 y mRSS ≥15 or mRSS <15 plus progressive pulmonary disease	Diffuse SSc Disease <3y mRSS ≥15 plus refractory digital ulcers or interstitial lung disease	16–65 y of age mRSS ≥15 plus worsening lung or heart or kidney involvement, or mRSS <15 plus pulmonary progression	Not described	Same as in ASTIS trial[17]	18–65 y Progressive disease	18–60 y of age Worsening diffuse skin if mRSS >14 or progressive lung function
Exclusion criteria	Karnofsky <70 PAPsys >50mmHg DLCO<40% predict	PAPsys >40mmHg LVEF <40% FVC<45% predict	PAH Cardiac Insuf. Renal Insuf. DLCO<40% predict	>60 years of age <16 years of age Uncontrolled arrhythmia LVEF <50% DLCO<45% predict	Uncontrolled arrhythmia Severe heart failure PAH Pulmonary or renal failure	Not described	Same as in ASTIS trial[17]		LVEF <40% Diastolic dysfunction PAPsys >40mmHg PAPm >27mmHg FVC <45% predict DLCO<40% predict
N	26	90	18	14	19	56	89	80	70
Median age	39 y	42 y	51.5 y	44.5 y	53.7 y	48 y	46 y	43 y	35.9
Median disease duration	27 y	25 mo	14 mo	22.5 mo	15 mo	25 mo	18 mo	23.8 mo	2y

	CY200 + rATG or CY100 + Thiotepa	CY200 + rATG	CY200 + rATG CY 200 + Alemtuz Mel + Alemtuz CY200 alone	CY200	CY 200	Multiple regimens	Cy200 + rATG	CY200 + ATG CY100 +Thiotepa + ATG	CY200 + rATG Flu + Mel + ATG
Transplant regimen									
CD34+ selection	Yes	No	No	Yes in 5 patients	Yes in 11 patients	Yes in most, but not specified for SSc patients	Yes	Yes in 35 patients	No
Follow-up	4.4 y	5 y	42 mo	11 y	8 y	7 y	4.6 y	2 y	8 y
Overall survival	74% at 3 y	78% at 5 y	61% at 42 m	93% at 5 y 93% at 10 y	79% (15/19)	73.7% at 5 y 55.4% at 10 y	77% at 55 mo	91.2% at 1 y 90% at 2y	91.8% at 3 y 85% at 5 y 81% (8y)
Transplant-related mortality	4% (1/26)	6% (5/90)	22% (4/18)	7.1% (1/14)	Zero	8.9% (5/56)	11% (8/89)	6% (5/80)	4% (3/80)
Progression-free survival or Event-free survival	Progression-free survival 53% at 3 y	Event-free survival 70% at 5 years	Progression-free survival 33% at 42 mo	Event-free survival 50% at 5 y 40% at 10 y	Progression-free survival 68.4% at 5 y 51.3% at 8 y	Progression-free survival 44.2% at 5 y 44.1% at 10 y	Event-free survival 78% at 5 y 76% at 10 y 66% at 15 y	Progression-free survival 87.5% at 1 y 81.8 at 2 y	Progression-free survival 71.8% at 3 y 71.8% at 5 y 70.5% at 8 y
Relapse/progression	30.4% at 3 y	14.4% at 5 y	27.7% at 42 mo	42% at 5 y	Not available	Not available	24% at 5 y	Progression of 6.3% at 1 y and 11.9% at 2 y	24%
mRSS	78.3% improved at 6 mo	Improved up to 5y	No scores available. Improved at 12mo	71% of patients improved at 12 mo	Improved up to 5 y Stable up to 8 y	Not available	Improved up to 5 y	Improved up to 2 y	Improved up to 5 y
FVC and DLCO	FVC improved at 12 mo DLCO unchanged	FVC improved up to 36 mo DLCO stable	No scores available Stable over 12 mo	85% of patients stable at 12 mo	FVC improved until 8 y DLCO stable	Not available	FVC and DLCO improved up to 5 y	FVC improved up to 2 y DLCO stable	FVC and DLCO stable up to 5 y

Abbreviations: CY, cyclophosphamide; CY200, CY at the dose of 200mg/kg; ATG, anti-thymocyte globulin; rATG, rabbit ATG; Flu, fludarabine; Mel, melphalan; Alemtuz, alemtuzumab; SSc, systemic sclerosis; mRSS, modified Rodnan's skin score; LVEF, left ventricle ejection fraction; FVC, forced vital capacity; DLCO, diffusing lung capacity for carbon monoxide; predict, % of predicted value; y, years; mo, months; GI, gastrointestinal; PAH, pulmonary artery hypertension; PAP, pulmonary artery pressure; PAPsys, systolic PAP; PAPm, mean PAP; EKG, electrocardiography; progression-free survival, proportion of patients who were alive and with no worsening of disease when compared to baseline; event-free survival, proportion of patients who were alive and with no worsening of disease from best improvement after transplant.

Table 2 Main Characteristics of Comparative Studies, Including Randomized Controlled Trials

	Burt et al 2011 (ASSIST)[16]	van Laar et al 2014 (ASTIS)[17]	Sullivan et al 2018 (SCOT)[18]	Del Papa et al. 2017[38]
Study design	Phase II, randomized 1:1, open-label cross-over to HSCT allowed at 12 mo North American single-center 2006–2009	Phase III, randomized 1:1, open-label European multicenter (EBMT) 2001–2009	Phase II, randomized 1:1, open-label North American multicenter 2005–2011	Phase II, retrospective historical SSc control group Italian, single-center 2003–2011
Comparisons	HSCT vs 6-mo intravenous CY	HSCT vs 12-mo intravenous CY	HSCT vs 12-mo intravenous CY	HSCT vs historical cohort
Inclusion criteria	<60 y of age Disease duration ≤4 y Diffuse SSc mRSS ≥15 Internal organ involvement	18–65 y of age Disease duration ≤2–4 y[a] Diffuse SSc mRSS ≥15 Internal organ involvement	18–69 y of age Disease duration ≤4 y Diffuse SSc mRSS ≥16 Internal organ involvement	Diffuse SSc Disease duration ≤4 y mRSS ≥14 Clinical activity score (ESSG) ≥3
Exclusion criteria	PAPm >25mmHg or PAPsys >40mmHg LVEF <40% Creatinine >177μmol/L >6 Intravenous CY courses	PAPm>50mmHg LVEF <45% Creatinine >40mL/min Cumulative IV CY dose >5g or Cumulative oral CY dose >3g	Mean PAP >30mmHg LVEF<50% FVC <45% predicted DLCO <40% predicted Creatinine clearance <40mL/min Cumulative IV CY dose >3g/m^2 or Oral CY dose >4 months, or >6 Intravenous CY courses	PAH LVEF <45% DLCO <50% predicted, Prior renal crisis
Number of participants	19 (10 HSCT + 9 CY arm)	156 (79 HSCT + 77 CY arm)	75 (33 HSCT + 32 CY arm)	18 HSCT + 36 SSc controls
Median age	45 y	43.8 y	45.9 y	41 y
Disease duration	13.6 mo	16.8 mo	27 mo	24 mo
Transplant regimen	CY200 + rabbit ATG	CY200 + rabbit ATG	TBI + CY120 + equine ATG	CY200 + rabbit ATG
Total body irradiation	No	No	Yes	No
CD34+ selection	No	Yes	Yes	Yes
Follow-up after AHSCT	2.6 y (median)	5.8 y (median)	4.5 y (minimum)	5 y (minimum)
Overall survival	100% at median 2.6 y for both groups	80% in HSCT vs 65% in CY at 4 y 75% in HSCT vs 60% in CY at 8 y	86% in HSCT vs 51% in CY at 6 y	89% in HSCT vs 39% in SSc controls at 5 y
Transplant-related mortality	0%	10.6% (8/79 HSCT)	3% (1/33 HSCT)	5.6% (1/18 HSCT)

(*Continued*)

Table 2 (Continued).

	Burt et al 2011 (ASSIST)[16]	**van Laar et al 2014 (ASTIS)**[17]	**Sullivan et al 2018 (SCOT)**[18]	**Del Papa et al. 2017**[38]
Event-free survival	80% in the HSCT group at 2 y 11% in the CY group at 2 y	81% in HSCT vs 74% in CY at 4 y	79% in HSCT vs 50% in CY at 4.5 y 74% in HSCT vs 47% in CY at 6 y	Not described
Progression-free survival	100% in HSCT vs 11% CY at 1 y 88% in HSCT at 2.6 y	77% in HSCT vs 65% in CY at 5.8 y	Not described	Not available. Higher survival in HSCT than in the SSc control group
Disease progression	0 in HSCT vs 89% (8/9) in CY at 1 y	11% in HSCT vs 35% in CYC at 5.8 y	18% in HSCT vs 41% in CY at 6 years	Not available. Lower disease progression in HSCT than in controls
mRSS	Improvement up to 2 y in HSCT Worsening in CY group	Improvement at 2 y in HSCT HSCT better than CY at 2 y	Not described	Improvement from baseline to 12 mo after HSCT and stabilization thereafter
FVC/DLCO	FVC improved more in HSCT than in CY DLCO remained stable and not different between groups	FVC improved more in HSCT than CY DLCO remained stable and not different between groups	FVC improved/stabilized in more patients from HSCT than from CY group	Stabilization of FVC and DLCO No difference between HSCT and controls

Notes: [a]The protocol was amended in 2004 to shorten maximum duration of disease for enrolment to 2 years, instead of the previous 4 years. Progression-free survival (PFS): proportion of patients who were alive and with no worsening of disease when compared to baseline; event-free survival: proportion of patients who were alive and with no worsening of disease from best improvement after transplant.
Abbreviations: SSc, systemic sclerosis; HSCT, hematopoietic stem cell transplantation; mRSS, modified Rodnan's skin score; ESSG, European Scleroderma Study Group scoring system; LVEF, left ventricle ejection fraction; PAP, pulmonary artery pressure; PAPsys, systolic PAP; PAPm, mean PAP; CY, cyclophosphamide; CY200, CY at a dose of 200mg/kg; ATG, anti-thymocyte globulin; TBI, total body irradiation; PAH, pulmonary artery hypertension; FVC, forced vital capacity; DLCO, diffusing lung capacity for carbon monoxide; y, years; mo, months.

Therefore, the expertise of the transplant team in selecting the appropriate patients, and thereby excluding those with too advanced organ damage, and in managing intra-transplant events and complications, is essential for good patient outcomes. An EBMT study from 2010 retrospectively analyzed data from multiple autoimmune disease transplants, showing that less experienced transplant centers, ie, those with lower number of transplanted patients, had higher transplant-related deaths. These results indicate that there is an important learning curve associated with outcomes, especially considering that SSc is a disease difficult to manage during AHSCT.[48]

Tissue damage is accumulated over time in SSc.[49] Patients earlier in disease course have less internal organ impairment and should thus present less transplant-related toxicity and perhaps better disease control after AHSCT. In line with this, recent prospective studies have included patients with disease duration limited to 2 to 4 years.[16–18,38] Indeed, a very recent prospective study conducted by the EBMT showed, by multivariate analysis, that mRSS above 24 at baseline and older age at transplantation were significantly associated with lower progression-free survival, corroborating that patients should be enrolled earlier in disease course.[30] The contribution of this selection strategy to the final patient outcome is unknown, but an ongoing study that aims to enroll patients for AHSCT as upfront treatment and, therefore, shortly after diagnosis, should answer some of the pending questions.[50]

Disease Progression

Studies are heterogeneous on reports of disease control after AHSCT. Nevertheless, most studies demonstrate positive effects of AHSCT on patient outcomes. Disease progression, defined as worsening manifestations from baseline, varied between the phase I/II non-randomized studies, even between those with similar duration of follow-up. Disease progression rates varied from 11.9% at 2 years to 30.4% at 4.4 years after ASHCT.[30,31] For studies

with longer follow-up, the 8-year progression rate ranged from 10.5% to 24%,[29,37] reaching 42% at 11 years.[34] These different rates are probably secondary to differences in patient selection, different criteria for progression and duration of follow-up.

The three randomized studies – ASSIST, ASTIS and SCOT – showed better disease control in transplanted versus conventionally treated (control) patients.[16–18] This difference in event-free survival between transplanted and control groups is more pronounced in the SCOT (74% in transplanted vs 47% in controls), than in the ASTIS trial (77% in transplanted vs 65% in controls), probably owing to the higher transplant-related mortality of ASTIS and to slightly different criteria for disease progression between studies. In the SCOT trial, the progression rate was not described in traditional terms, as patient outcomes were measured by a specific global rank composite score (GRCS). This score included a hierarchy of events reflecting disease improvement or worsening, resulting in a final score that enabled comparisons between participants of the study. Unfortunately, the evaluating method does not allow outcomes to be directly compared to those from other studies. Nevertheless, a recent study retrospectively evaluated the French cohort of the ASTIS trial, consisting of 49 patients, for GRCS results, showing superiority of transplanted versus conventionally treated patients.[51] In this study, GRCS was 9 for transplanted versus −19 for conventionally treated patients at 60 months (p=0.018), while in SCOT, respective results were 20 and −8 at 48 months (p=0.008).

Skin Outcomes

Clinical trials on AHSCT for SSc show a significant reduction in skin thickening, assessed by the modified Rodnan skin score (mRSS).[52] Improvement in skin involvement is considered when mRSS decreases by more than 25%.[16,18,30] Collectively, phase I/II studies show improvement of mRSS early after AHSCT, usually more pronounced within the first year after transplantation, and tending to stabilize thereafter.[16,30,32,37] Longer follow-up studies show sustained mRSS improvement for at least 60 and 96 months, respectively.[29,37] In a post-hoc analysis of a phase I/II non-randomized study, patients who underwent AHSCT with and without graft selection were compared. CD34+ graft selection had an overall positive influence on patient outcomes but was specifically associated with improvements in mRSS.[29]

In the randomized controlled studies, the differences in mRSS between patients undergoing AHSCT and those treated with cyclophosphamide (control) are significant, usually with improving curves in transplanted and progressively worsening in control patients. In ASSIST, mRSS improved from 30 at baseline, to 16 at 12 months, and 9 at 24 months after AHSCT, while scores worsened in the control group. Patients from the control group were allowed to cross over to the transplantation group after 1 year of follow-up, and these also had improvements in mRSS.[16] The ASTIS and SCOT trials also show superior efficacy of transplanted over control patients regarding skin involvement, with sustained outcomes at 24 and 54 months, respectively.[17,18]

In a non-randomized, but comparative study, both mRSS and European Scleroderma Study Group (ESSG) scores showed a strongly significant reduction since 12 months after AHSCT. When groups were compared at 3-years, the probability that mRSS could decline to below 14 was above 90% in the AHSCT group, while only 60% in the control group.[38]

Improvement of the skin involvement may be also indirectly assessed by functional evaluations, such as joint range-of-motion measurements, hand grip strenght, finger-to-palm distance, mouth opening, and functional questionnaires for hand (Cochin) and upper limbs (DASH, Disabilities of the arm, shoulder and hand). A recent study showed improvements of these functional parameters at 6 and 12 months after AHSCT, when compared to baseline.[53]

Although the mRSS is the universally used method to quantify skin thickening in SSc, it bears intrinsic limitations and depends on evaluator expertise and opinion that affect reproducibility and consistency of the method.[54] Therefore, the reliability of mRSS to evaluate skin outcomes after AHSCT has been questioned. Nevertheless, skin biopsies, the gold-standard method to quantify cutaneous involvement in SSc, correlate well with mRSS, as the degree of fibrosis decreases while mRSS scores decline after AHSCT.[14,55]

Pulmonary Outcomes

Most phase I/II studies showed stabilization or slight improvement in FVC after transplantation, and DLCO stabilization.[30–33] One study showed significant improvement for both FVC and DLCO over 5 years and another study showed that FVC and DLCO improved only in the subgroup of patients with progressive lung disease as an

indication for AHSCT.[36,37] The ASSIST and ASTIS randomized trials showed that lung function outcomes in transplanted patients were superior to those from the control group treated with cyclophosphamide.[16,17] In ASSIST, in addition to the favorable difference between groups, CVF and computed tomography volumes increased in transplanted patients at 24 months of follow-up.[16] The SCOT study does not describe pulmonary function data in a format that enables comparisons, but there is a higher absolute number of patients having improved FVC after AHSCT than after cyclophosphamide pulses.[18]

A retrospective study from the EBMT did not associate graft selection with clinical outcomes, including pulmonary function test results.[28] A more recent study, however, prospectively compared two groups of SSc patients, randomized for AHSCT with manipulated or non-manipulated graft.[29] The authors showed that although overall survival was similar between the two groups, patients who received CD34+ selected grafts had better progression-free survival over an 8-year follow-up. Forced vital capacity also progressively increased over the years in the CD34+ selected patients, while remained mostly stable in the non-selected group, with a tendency to better outcomes in the selected versus non-selected group.

Physical capacity, assessed by the 6-minute walk test (6MWT), improved after AHSCT, as an indirect evidence of better pulmonary function, although the cardiovascular and musculoskeletal systems may have participated.[53] Another method to evaluate how AHSCT affects interstitial lung disease is high-resolution quantitative computed tomography. Studies have shown improvement of lung volumes and/or pulmonary tissue quality (evaluated by density), after transplantation, associated with pulmonary function outcomes.[16,56–58]

Quality of Life

Quality of life is an important indicator of transplant outcomes, as it reflects the patient's perspective and evaluates how patients are affected by the procedure in the context of their daily lives and environment. Quality of life assessments have been included in several clinical trials, but only one study has specifically addressed this aspect of evaluation in SSc patients undergoing AHSCT.[59] This retrospective study compared Short-Form 36 (SF36) questionnaire results from 41 SSc patients who underwent AHSCT and 65 conventionally treated (control) SSc patients, over a 7-year follow-up. Patient groups were different for physical components of quality of life,

favoring transplant, but no difference was detected between groups regarding the mental components of quality of life. In addition, Health Assessment Questionnaire (HAQ) scores were considerably lower (indicating better function) at all times in patients treated with AHSCT compared to the control group.[59]

Recently, a prospective, open and multicenter study assessed Scleroderma HAQ (sHAQ) scores in 15 patients who underwent AHSCT, showing significant improvement at 1 year and and 2 years of follow-up.[30] Another single-center study also prospectively evaluated quality of life in 28 SSc patients treated with AHSCT, showing improvement of the physical components of SF36 at 6 and 12 months, and of the mental components of SF36 at 12 months.[53] Finally, a systematic review from 2020 analyzed three randomized and five uncontrolled clinical trials that contained information about quality of life in transplanted SSc patients, showing that SF36 results were heterogeneous across studies, but with overall improvements in the physical components of quality of life. For the mental components, however, data were inconsistent.[60]

Fertility After AHSCT

Fertility is usually preserved in SSc patients, as there are no differences in rates of conception between SSc women and the general healthy population.[61,62] In the subset of cyclophosphamide-treated patients, however, fertility rates may be compromised, due to gonadal toxicity of the treatment, especially in women.[63] Pregnancies, on the other hand, may have worse outcomes in SSc women than in the non-SSc patients.[61] An Italian study has retrospectively evaluated 109 pregnancies in SSc women, showing a higher rate of preterm deliveries, intrauterine growth restriction and very low-weight babies than in the general obstetric population.[62] Male fertility is much less investigated and few studies have specifically addressed male impotency in SSc.[64,65]

In the setting of AHSCT, reduced fertility is a frequently reported complication, both in men and women, although SSc data are reported combined with other autoimmune diseases. Multicenter data from the EBMT that were retrospectively analyzed showed that among 324 adult female patients with autoimmune diseases that underwent AHSCT, 15 of them had 22 pregnancies along post-transplantation follow-up. Five of these patients had SSc as baseline disease, and all had received high-dose cyclophosphamide as part of the transplant-conditioning regimen. One of the SSc patients had three

pregnancies, including one miscarriage. There were no deaths during pregnancy, but one patient died shortly after delivery due to worsening of skin and pulmonary fibrosis.[66]

A single center from Germany described 15 patients (11 female), who had been previously treated with autologous AHSCT for severe autoimmune diseases, out of which 3 had SSc. All but one patient had received cyclophosphamide as conventional treatment prior to AHSCT and all received high-dose cyclophosphamide as part of transplant conditioning regimen. One female patient with SSc was considered to have impaired fertility since before AHSCT, remaining amenorrheic and not becoming pregnant after AHSCT. Another female SSc patient became temporarily amenorrheic shortly after AHSCT, recovering regular menses a few months later. This woman became spontaneously pregnant twice after AHSCT, with successful preterm deliveries at 34 and 35 weeks due to premature labour and breech presentation, respectively.[67]

Secondary Autoimmune Diseases After AHSCT

A debated concern in the field of AHSCT for autoimmune diseases is whether the profound manipulation of the immune system in genetically predisposed patients may lead to development of secondary autoimmune diseases.[68] Therefore, continuous long-term surveillance of post-transplant immune status is recommended for patients who undergo AHSCT.

A study from the EBMT has reported a cumulative incidence of secondary autoimmune diseases of 9.8% over a 5-year follow-up after AHSCT, among 347 patients who underwent AHSCT with an autoimmune disease as primary indication.[69] Most secondary autoimmune diseases were organ or tissue-specific, with variable severity. Two, out of the 29 patients who developed secondary autoimmune diseases after AHSCT, died as direct consequence of antiphospholipid syndrome (cerebral ischemia) and hemorrhage (acquired hemophylia), respectively. Multivariate analysis identified systemic lupus erythematosus as primary indication for AHSCT and use of anti-thymocyte globulin associated with graft selection in the conditioning regimen as risk factors for development of secondary autoimmune disease.[69]

Additional secondary autoimmune diseases have been reported in smaller case series. One, out of 26 transplanted SSc patients, presented morphea as a secondary

autoimmune manifestation after AHSCT.[31] In a cohort of 14 Japanese SSc patients treated with AHSCT, one patient developed multiple overlapped autoimmune disorders including Grave's disease, immune thrombocytopenia, systemic lupus erythematosus and antiphospholipid syndrome, and a second patient developed Sjogren's syndrome.[34] In a case series of 6 SSc patients who underwent AHSCT with thiotepa as part of the conditioning regimen, two female patients developed symptomatic Grave's disease with detectable autoantibodies against thyroid stimulating hormone (TSH) receptor, at 13 and 19 months after AHSCT. A third male patient developed antibodies against SSA and polyclonal hypergammaglobulinaemia 6 months after transplantation.[24] Finally, a French long-term outcome study reported secondary autoimmune diseases in 5 (8.9%) out of 56 transplanted SSc patients; thyroiditis, autoimmune hemolytic anemia, myasthenia gravis, sarcoidosis and anti-phospholipid syndrome.[35]

Secondary Malignancies After AHSCT

Systemic sclerosis is associated with increased relative risk of cancer, mainly lung, liver, hematologic and bladder, although with a low absolute risk.[70] Multiple mechanisms may contribute to such outcomes, including genetic background, defective immunological surveillance, pro-inflammatory status, epithelial hyperplasia and prior exposure to carcinogens, including those associated with cytotoxic treatment.[71]

Data on secondary malignancies in SSc after AHSCT are limited and variable. In addition, it is difficult to establish how much is influenced by the transplant procedure itself or by previous immunosuppressive and cytotoxic treatment, as well as prior viral infections. The ASTIS trial reported only one patient developed Epstein–Barr virus-related post-transplant lymphoproliferative disease shortly after AHSCT. Other five non-transplanted SSc patients from the cyclophosphamide (control) group also developed malignancies.[17] In the SCOT study, 3 of 33 SSc patients from the transplant group, and one from the conventional-treatment control group developed cancer. The authors believe that the regimen including total body irradiation may have contributed to the increased incidence of malignancy in the transplanted patients.[18] In a retrospective evaluation, the French Society for Bone Marrow Transplantation and Cellular Therapy reported 4 (7%) of 56 SSc patients who underwent AHSCT and developed cancer (oesophagus epidermoid carcinoma,

unspecified carcinoma, lung epidermoid carcinoma, spino-cellular carcinoma) over a median follow-up of 83 months.[35] Collectively, these results indicate that these patients may require longer follow-up and more detailed inspection for possible malignancies. Further studies should define if other factors, such as smoking or previous treatments, impose additional risks.

Future Developments

Although the field of AHSCT for SSc has grown over the years, translating into benefits for the patient, further improvements are still warranted. Strategies to discriminate subjects that may achieve best results after AHSCT may contribute to better outcomes and to consolidate the procedure as a therapeutic alternative for SSc. In this context, a study analyzing biological samples from participants of the SCOT trial clustered patients into groups, according to different gene expression profiles associated with different pathogenic mechanisms of disease. When each of these groups were analysed for clinical outcomes after AHSCT, patients with a more fibroproliferative profile showed the most significant long-term benefit, indicating a potential biomarker for patient selection before AHSCT.[72] More recently, this same research group was able to associate histological findings of fibroblast polarization with the previously defined fibroproliferative gene profile, and to further correlate them with severity of skin involvement assessed by mRSS.[73] In addition, ongoing prospective clinical trials aim to evaluate the role of post-transplant maintenance therapy with immunosuppressors in decreasing disease reactivation and progression after AHSCT.[74,75] Furthermore, more specific approaches to lessen transplant-related toxicity have been investigated. A recent strategy, still mostly limited to oncology and hematology fields, describes the use of antibody-targeted destruction of specific cell types.[76,77] Conjugation of toxins to anti-CD45 antibody, for instance, may concentrate depletion in hematopoietic cells and, thus, spare non-hematopoietic cells. This may be a future strategy for AHSCT in SSc patients, aiming to reduce transplant-related toxicity. Combined, clinical and translational activities are essential to develop the field, and to have patient welfare as the most important goal.

Disclosure

The authors report no conflicts of interest for this work.

References

1. Matucci-Cerinic M, Miniati I, Denton CP. Eular compendium on rheumatic diseases. In: Bijlsman JWJ, editor. *Systemic Sclerosis*. 2009:290–296.
2. Elhai M, Meune C, Boubaya M, et al. Mapping and predicting mortality from systemic sclerosis. *Ann Rheum Dis*. 2017;76 (11):1897–1905. doi:10.1136/annrheumdis-2017-211448
3. McMahan ZH, Volkmann ER. An update on the pharmacotherapeutic options and treatment strategies for systemic sclerosis. *Expert Opin Pharmacother*. 2020;21(16):2041–2056. doi:10.1080/14656566.2020.1793960
4. Elhai M, Meune C, Avouac J, Kahan A, Allanore Y. Trends in mortality in patients with systemic sclerosis over 40 years: a systematic review and meta-analysis of cohort studies. *Rheumatology*. 2012;51(6):1017–1026. doi:10.1093/rheumatology/ker269
5. Topal AA, Dhurat RS. Scleroderma therapy: clinical overview of current trends and future perspective. *Rheumatol Int*. 2013;33 (1):1–18.
6. Tyndall AJ, Bannert B, Vonk M, et al. Causes and risk factors for death in systemic sclerosis: a study from the EULAR scleroderma trials and research (EUSTAR) database. *Ann Rheum Dis*. 2010;69 (10):1809–1815. doi:10.1136/ard.2009.114264
7. Fransen J, Popa-Diaconi D, Hesselstrand R, et al. Clinical prediction of 5-year survival in systemic sclerosis: validation of a simple prognostic model in EUSTAR centres. *Ann Rheum Dis*. 2011;70 (10):1788–1792. doi:10.1136/ard.2010.144360
8. Domsic RT, Medsger TA Jr. Connective tissue disease: predicting death in SSc: planning and cooperation are needed. *Nat Rev Rheumatol*. 2011;7(11):628–630. doi:10.1038/nrrheum.2011.152
9. Tyndall A. Hematopoietic stem cell transplantation for systemic sclerosis: review of current status. *BioDrugs*. 2019;33(4):401–409. doi:10.1007/s40259-019-00364-3
10. Marmont AM. Stem cell transplantation for autoimmune disorders. Coincidental autoimmune disease in patients transplanted for conventional indications. *Best Pract Res Clin Haematol*. 2004;17 (2):223–232. doi:10.1016/j.beha.2004.04.004
11. Binks M, Passweg JR, Furst D, et al. Phase I/II trial of autologous stem cell transplantation in systemic sclerosis: procedure related mortality and impact on skin disease. *Ann Rheum Dis*. 2001;60 (6):577–584. doi:10.1136/ard.60.6.577
12. Farge D, Marolleau JP, Zohar S, et al. Autologous bone marrow transplantation in the treatment of refractory systemic sclerosis: early results from a French multicentre Phase I-II study. *Br J Haematol*. 2002;119(3):726–739. doi:10.1046/j.1365-2141.2002.03895.x
13. Farge D, Passweg J, van Laar JM, et al. Autologous stem cell transplantation in the treatment of systemic sclerosis: report from the EBMT/EULAR Registry. *Ann Rheum Dis*. 2004;63(8):974–981. doi:10.1136/ard.2003.011205
14. Nash RA, McSweeney PA, Crofford LJ, et al. High-dose immunosuppressive therapy and autologous hematopoietic cell transplantation for severe systemic sclerosis: long-term follow-up of the US multicenter pilot study. *Blood*. 2007;110(4):1388–1396. doi:10.1182/blood-2007-02-072389
15. Oyama Y, Barr WG, Statkute L, et al. Autologous non-myeloablative hematopoietic stem cell transplantation in patients with systemic sclerosis. *Bone Marrow Transplant*. 2007;40(6):549–555. doi:10.1038/sj.bmt.1705782
16. Burt RK, Shah S, Dill K, et al. Autologous non-myeloablative haemopoietic stem-cell transplantation compared with pulse cyclophosphamide once per month for systemic sclerosis (ASSIST): an open-label, randomised Phase 2 trial. *Lancet*. 2011;378 (9790):498–506. doi:10.1016/S0140-6736(11)60982-3

17. Van Laar JM, Farge D, Sont JK, et al. Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. *JAMA*. 2014;311(24):2490–2498. doi:10.1001/jama.2014.6368

18. Sullivan KM, Goldmuntz EA, Keyes-Elstein L, et al. Myeloablative autologous stem-cell transplantation for severe scleroderma. *N Engl J Med*. 2018;378(1):35–47. doi:10.1056/NEJMoa1703327

19. Kowal-Bielecka O, Fransen J, Avouac J, et al. Update of EULAR recommendations for the treatment of systemic sclerosis. *Ann Rheum Dis*. 2017;76(8):1327–1339. doi:10.1136/annrheumdis-2016-209909

20. Farge D, Burt RK, Oliveira MC, et al. Cardiopulmonary assessment of patients with systemic sclerosis for hematopoietic stem cell transplantation: recommendations from the European Society for blood and marrow transplantation autoimmune diseases working party and collaborating partners. *Bone Marrow Transplant*. 2017;52 (11):1495–1503. doi:10.1038/bmt.2017.56

21. Snowden JA, Saccardi R, Allez M, et al. Haematopoietic SCT in severe autoimmune diseases: updated guidelines of the European Group for blood and marrow transplantation. *Bone Marrow Transplant*. 2012;47(6):770–790. doi:10.1038/bmt.2011.185

22. Snowden JA, Sharrack B, Akil M, et al. Autologous haematopoietic stem cell transplantation (aHSCT) for severe resistant autoimmune and inflammatory diseases - a guide for the generalist. *Clin Med*. 2018;18(4):329–334. doi:10.7861/clinmedicine.18-4-329

23. McSweeney PA, Nash RA, Sullivan KM, et al. High-dose immunosuppressive therapy for severe systemic sclerosis: initial outcomes. *Blood*. 2002;100(5):1602–1610. doi:10.1182/blood.V100.5.1602. h81702001602_1602_1610

24. Henes CJ, Koetter I, Horger M, et al. Autologous stem cell transplantation with thiotepa-based conditioning in patients with systemic sclerosis and cardiac manifestations. *Rheumatology*. 2014;53 (5):919–922. doi:10.1093/rheumatology/ket464

25. Burt RK, Farge D. Autologous HSCT is efficacious, but can we make it safer? *Nat Rev Rheumatol*. 2018;14(4):189–191. doi:10.1038/ nrrheum.2018.34

26. Martínez C, Urbano-Ispizua A, Rozman C, et al. Immune reconstitution following allogeneic peripheral blood progenitor cell transplantation: comparison of recipients of positive CD34+ selected grafts with recipients of unmanipulated grafts. *Exp Hematol*. 1999;27 (3):561–568. doi:10.1016/S0301-472X(98)00029-0

27. Kohno K, Nagafuji K, Tsukamoto H, et al. Infectious complications in patients receiving autologous CD34-selected hematopoietic stem cell transplantation for severe autoimmune diseases. *Transpl Infect Dis*. 2009;11(4):318–323. doi:10.1111/j.1399-3062.2009.00401.x

28. Oliveira MC, Labopin M, Henes J, et al. Does ex vivo CD34+ positive selection influence outcome after autologous hematopoietic stem cell transplantation in systemic sclerosis patients? *Bone Marrow Transplant*. 2016;51(4):501–505. doi:10.1038/bmt.2015.299

29. Ayano M, Tsukamoto H, Mitoma H, et al. CD34-selected versus unmanipulated autologous haematopoietic stem cell transplantation in the treatment of severe systemic sclerosis: a post hoc analysis of a phase I/II clinical trial conducted in Japan. *Arthritis Res Ther*. 2019;21(1):30. doi:10.1186/s13075-019-1823-0

30. Henes J, Oliveira MC, Lapobin M, et al. Autologous stem cell transplantation for progressive systemic sclerosis: a prospective non-interventional study from the European Society for blood and marrow transplantation autoimmune disease working party. *Haematologica*. 2021;106 (2):375–383. doi:10.3324/haematol.2019.230128

31. Henes JC, Schmalzing M, Vogel W, et al. Optimization of autologous stem cell transplantation for systemic sclerosis- a single-center longterm experience in 26 patients with severe organ manifestations. *J Rheumatol*. 2012;39(2):269–275. doi:10.3899/jrheum.110868

32. Burt RK, Oliveira MC, Shah S, et al. Cardiac involvement and treatment-related mortality after non-myeloablative haemopoietic stem-cell transplantation with unselected autologous peripheral blood for patients with systemic sclerosis: a retrospective analysis. *Lancet*. 2013;381(9872):1116–1124. doi:10.1016/S0140-6736(12)62114-X

33. Helbig G, Widuchowska M, Koglega A, et al. Safety profile of autologous hematopoietic stem cell mobilization and transplantation in patients with systemic sclerosis. *Clin Rheumatol*. 2018;37 (6):1709–1714. doi:10.1007/s10067-017-3954-5

34. Nakamura H, Odani T, Yasuda S, et al. Autologous haematopoietic stem cell transplantation for Japanese patients with systemic sclerosis: long-term follow-up on a Phase II trial and treatment- related fatal cardiomyopathy. *Mod Rheumatol*. 2018;28(5):879–884. doi:10.1080/14397595.2017.1416920

35. Guillaine-Jugnot P, Badoglio M, Labopin M, et al. Autologous haematopoietic stem cell transplantation (AHSCT) in autoimmune disease adult patients in France: analysis of the long-term outcome from the French Society for Bone Marrow Transplantation and Cellular Therapy (SFGM-TC). *Clin Rheumatol*. 2019;38(5):1501–1511. doi:10.1007/s10067-019-04435-2

36. van Bijnen S, Vries-Bouwstra J, van den Ende CH, et al. Predictive factors for treatment-related mortality and major adverse events after autologous haematopoietic stem cell transplantation for systemic sclerosis: results of a long-term follow-up multicentre study. *Ann Rheum Dis*. 2020;79(8):1084–1089. doi:10.1136/annrheumdis-2020-217058

37. Henrique-Neto A, Vanconcelos MYK, Dias JBE, et al. Hematopoietic stem cell transplantation for systemic sclerosis: Brazilian experience. *Adv Rheumatol*. 2021;61(1):9. doi:10.1186/s42358-021-00166-8

38. Del Papa N, Onida F, Zaccara E, et al. Autologous hematopoietic stem cell transplantation has better outcomes than conventional therapies in patients with rapidly progressive systemic sclerosis. *Bone Marrow Transplant*. 2017;52(1):53–58. doi:10.1038/bmt.2016.211

39. Burt RK, Oliveira MC, Shah SJ. Cardiac assessment before stem cell transplantation for systemic sclerosis. *JAMA*. 2014;312(17):1803. doi:10.1001/jama.2014.12566

40. Burt RK, Shah SJ, Gheorghiade M, Ruderman E, Schroeder J. Hematopoietic stem cell transplantation for systemic sclerosis: if you are confused, remember: "it is a matter of the heart". *J Rheumatol*. 2012;39(2):206–209. doi:10.3899/jrheum.111302

41. Hosing C, Nash R, McSweeney P, et al. Acute kidney injury in patients with systemic sclerosis participating in hematopoietic cell transplantation trials in the United States. *Biol Blood Marrow Transplant*. 2011;17(5):674–681. doi:10.1016/j.bbmt.2010.08.003

42. Craciunescu OI, Steffey BA, Kelsey CR, et al. Renal shielding and dosimetry for patients with severe systemic sclerosis receiving immunoablation with total body irradiation in the scleroderma: cyclophosphamide or transplantation trial. *Int J Radiat Oncol Biol Phys*. 2011;79(4):1248–1255. doi:10.1016/j.ijrobp.2010.05.036

43. Dhesi S, Chu MP, Blevings G, et al. Cyclophosphamide- induced cardiomyopathy: a case report, review and recommendations for management. *J Investig Med High Impact Case Rep*. 2013;1(1):2324709613480346.

44. Allanore Y, Meune C, Vonk MC, et al. Prevalence and factors associated with left ventricular dysfunction in the EULAR Scleroderma Trial and Research group (EUSTAR) database of patients with systemic sclerosis. *Ann Rheum Dis*. 2010;69 (1):218–221. doi:10.1136/ard.2008.103382

45. Sandmeier B, Jäger VK, Nagy G, et al. Autopsy versus clinical findings in patients with systemic sclerosis in a case series from patients of the EUSTAR database. *Clin Exp Rheumatol*. 2015;33(4 Suppl 91):S75–S79.

46. Burt RK, Han X, Quigley K, et al. Cardiac safe hematopoietic stem cell transplantation for systemic sclerosis with poor cardiac function: a pilot safety study that decreases neutropenic interval to 5 days. *Bone Marrow Transplant*. 2021;56(1):50–59. doi:10.1038/s41409-020-0978-2

47. Snowden JA, Badoglio M, Labopin M, et al.; for European Society for Blood and Marrow Transplantation (EBMT) Autoimmune Diseases Working Party (ADWP); EBMT Paediatric Working Party (PWP); Joint Accreditation Committee of the International Society for Cellular Therapy (ISCT); EBMT (JACIE). Evolution, trends, outcomes, and economics of hematopoietic stem cell transplantation in severe autoimmune diseases. *Blood Adv*. 2017;1(27):2742–2755. doi:10.1182/bloodadvances.2017010041.

48. Farge D, Lapobin M, Tyndall A, et al. Autologous hematopoietic stem cell transplantation for autoimmune diseases: an observational study on 12 years' experience from the European Group for Blood and Marrow Transplantation Working Party on Autoimmune Diseases. *Haematologica*. 2010;95(2):284–292. doi:10.3324/haematol.2009.013458

49. Denton CP, Khanna D. Systemic sclerosis. *Lancet*. 2017;390 (10103):1685–1699. doi:10.1016/S0140-6736(17)30933-9

50. Spierings J, van Rhenen A, Welsing PM, et al. A randomised, open-label trial to assess the optimal treatment strategy in early diffuse cutaneous systemic sclerosis: the UPSIDE study protocol. *BMJ Open*. 2021;11(3):e044483. doi:10.1136/bmjopen-2020-044483

51. Ait Abdallah N, Wang M, Lansiaux P, et al. Long term outcomes of the French ASTIS systemic sclerosis cohort using the global rank composite score. *Bone Marrow Transplant*. 2021;56(9):2259–2267. doi:10.1038/s41409-021-01355-1

52. Clements PJ, Hurwitz EL, Wong WK, et al. Skin thickness score as a predictor and correlate of outcome in systemic sclerosis: high-dose versus low-dose penicillamine trial. *Arthritis Rheum*. 2000;43 (11):2445–2454. doi:10.1002/1529-0131(200011)43:11<2445::AID-ANR11>3.0.CO;2-Q

53. Costa-Pereira KR, Guimaraes AL, Moraes DA, et al. Hematopoietic stem cell transplantation improves functional outcomes of systemic sclerosis patients. *J Clin Rheumatol*. 2020;26(7SSuppl 2):S131–S138. doi:10.1097/RHU.0000000000001117

54. Khanna D, Furst DE, Clements PJ, et al. Standardization of the modified Rodnan skin score for use in clinical trials of systemic sclerosis. *J Scleroderma Relat Disord*. 2017;2(1):11–18. doi:10.5301/jsrd.5000231

55. Daikeler T, Kump E, Stern M, et al. Autologous hematopoietic stem cell transplantation reverses skin fibrosis but does not change skin vessel density in patients with systemic sclerosis. *Pathol Biol*. 2015;63(4–5):164–168. doi:10.1016/j.patbio.2015.07.006

56. Wada DT, Almeida FA, Moraes DA, et al. Automatic quantitative computed tomography evaluation of the lungs in patients with systemic sclerosis treated with autologous stem cell transplantation. *J Clin Rheumatol*. 2020;26(7s Suppl 2):S158–S164. doi:10.1097/RHU.0000000000001242

57. Kloth C, Thaiss WM, Preibsch H, et al. Quantitative chest CT analysis in patients with systemic sclerosis before and after autologous stem cell transplantation: comparison of results with those of pulmonary function tests and clinical tests. *Rheumatology*. 2016;55 (10):1763–1770. doi:10.1093/rheumatology/kew259

58. Ciaff J, van Leeuwen NM, Boonstra M, et al. Evolution of interstitial lung disease one year after hematopoietic stem cell transplantation or cyclophosphamide for systemic sclerosis. *Arthritis Care Res*. 2020. doi:10.1002/acr.24451

59. Maltez N, Puyade M, Wang M, et al. Association of autologous hematopoietic stem cell transplantation in systemic sclerosis with marked improvement in health-related quality of life. *Arthritis Rheumatol*. 2021;73(2):305–314. doi:10.1002/art.41519

60. Puyade M, Maltez N, Lansiaux P, et al. Health-related quality of life in systemic sclerosis before and after autologous haematopoietic stem cell transplant- a systematic review. *Rheumatology*. 2020;59 (4):779–789. doi:10.1093/rheumatology/kez300

61. Steen VD, Medsger TA Jr. Fertility and pregnancy outcomes in women with systemic sclerosis. *Arthritis Rheum*. 1999;42 (4):763–768. doi:10.1002/1529-0131(199904)42:4<763::AID-ANR21>3.0.CO;2-V

62. Taborelli M, Ramoni V, Brucato A, et al. Brief report: successful pregnancies but a higher risk of preterm births in patients with systemic sclerosis: an Italian multicenter study. *Arthritis Rheum*. 2012;64(6):1970–1977. doi:10.1002/art.34350

63. Harward LE, Mitchell K, Pieper C, Copland S, Criscione-Schreiber LG, Clowse ME. The impact of cyclophosphamide on menstruation and pregnancy in women with rheumatologic disease. *Lupus*. 2013;22 (1):81–86. doi:10.1177/0961203312468624

64. Nowlin NS, Brick JE, Weaver DJ, et al. Impotence in scleroderma. *Ann Intern Med*. 1986;104(6):794–798. doi:10.7326/0003-4819-104-6-794

65. Krittian SM, Saur SJ, Schloegl A, et al. Erectile function and connective tissue diseases. Prevalence of erectile dysfunction in German men with systemic sclerosis compared to other connective tissue diseases and healthy subjects. *Clin Exp Rheumatol*. 2021;39(Suppl 131):52–56.

66. Snarski E, Snowden JA, Oliveira MC, et al. Onset and outcome of pregnancy after autologous haematopoietic SCT (AHSCT) for autoimmune disease: a retrospective study of the EBMT aUtoimmune Diseases Working Party (ADWP). *Bone Marrow Transplant*. 2015;50 (2):216–220. doi:10.1038/bmt.2014.248

67. Massenkeil G, Alexander T, Rosen O, et al. Long-term follow-up of fertility and pregnancy in autoimmune diseases after autologous haematopoietic stem cell transplantation. *Rheumatol Int*. 2016;36 (11):1563–1568. doi:10.1007/s00296-016-3531-2

68. Somers EC, Thomas SL, Smeeth L, Hall AJ. Are individuals with an autoimmune disease at higher risk of a second autoimmune disorder? *Am J Epidemiol*. 2009;169(6):749–755. doi:10.1093/aje/kwn408

69. Daikler T, Labopin M, Di Gioia M, et al. Secondary autoimmune disease occurring after HSCT for an autoimmune disease: a retrospective study of the EBMT autoimmune disease working party. *Blood*. 2011;118 (6):1693–1698. doi:10.1182/blood-2011-02-336156

70. Onishi A, Sugiyama D, Kumagai S, Morinobu A. Cancer incidence in systemic sclerosis: meta-analysis of population-based cohort studies. *Arthritis Rheum*. 2013;65(7):1913–1921. doi:10.1002/art.37969

71. Wooten M. Systemic sclerosis and malignancy: a review of the literature. *South Med J*. 2008;101(1):59–62. doi:10.1097/SMJ.0b013e31815838ce

72. Franks JM, Martyanov V, Wang Y, et al. Machine learning predicts stem cell transplant response in severe scleroderma. *Ann Rheum Dis*. 2020;79(12):1608–1615. doi:10.1136/annrheumdis-2020-217033

73. Showalter K, Spiera R, Magro C, et al. Machine learning integration of scleroderma histology and gene expression identifies fibroblast polarization as a hallmark of clinical severity and improvement. *Ann Rheum Dis*. 2021;80(2):228–237. doi:10.1136/annrheumdis-2020-217840

74. EBMT ADWP prospective non-interventional Study: post-AHSCT management in SSC patients (NISSC-2). Available from: https://clinicaltrials.gov/ct2/show/NCT03444805. NLM identifier: NCT03444805. Accessed September 6, 2021.

75. Scleroderma Treatment With Autologous Transplant (STAT) Study. Available from: https://clinicaltrials.gov/ct2/show/NCT01413100. NLM identifier: NCT01413100. Accessed September 6, 2021.

76. Tsuchikama K, An Z. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. *Protein Cell*. 2018;9(1):33–46. doi:10.1007/s13238-016-0323-0

77. Palchaudhuri R, Saez B, Hoggatt J, et al. Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin. *Nat Biotechnol*. 2016;34(7):738–745. doi:10.1038/nbt.3584

Outcomes of Patients Who Undergo Transfusion of Fresh Frozen Plasma: A Prospective, Observational, Multicentre Cohort Study in Hiroshima, Japan

Aya Sugiyama, Teruhisa Fujii, Yoshiko Okikawa, Fumie Sasaki, Masazumi Okajima, Hidekuni Hidaka, Koji Iwato, Kazuyoshi Sato, Akira Kokubunji, Noboru Takata, Masahiro Yamamoto & Junko Tanaka

Published online: 12 Nov 2021.

Submit your article to this journal 🗗

Article views: 197

View related articles 🗗

View Crossmark data 🗗

Citing articles: 5 View citing articles 🗗

ORIGINAL RESEARCH

Outcomes of Patients Who Undergo Transfusion of Fresh Frozen Plasma: A Prospective, Observational, Multicentre Cohort Study in Hiroshima, Japan

Aya Sugiyama[1]
Teruhisa Fujii[2,3]
Yoshiko Okikawa[4]
Fumie Sasaki[5]
Masazumi Okajima[3,6]
Hidekuni Hidaka[3,7]
Koji Iwato[3,8]
Kazuyoshi Sato[3,9]
Akira Kokubunji[3,10]
Noboru Takata[3,11]
Masahiro Yamamoto[3,12]
Junko Tanaka[1,3]

[1]Department of Epidemiology, Infectious Disease Control and Prevention, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan; [2]Division of Transfusion Medicine, Hiroshima University Hospital, Hiroshima, Japan; [3]Joint Committee for Blood Transfusion Therapy in Hiroshima Prefecture, Hiroshima, Japan; [4]Department of Oncology, Hiroshima City Hiroshima Citizens Hospital, Hiroshima, Japan; [5]Division of Laboratory, Hiroshima City Hiroshima Citizens Hospital, Hiroshima, Japan; [6]Department of Surgery, Hiroshima City Hiroshima Citizens Hospital, Hiroshima, Japan; [7]Division of Surgical Services, Fukuyama City Hospital, Hiroshima, Japan; [8]Department of Hematology, Hiroshima Red Cross Hospital & Atomic-Bomb Survivors Hospital, Hiroshima, Japan; [9]Division of Laboratory, Shobara Red Cross Hospital, Shobara, Japan; [10]Department of Medical Science and Technology, Faculty of Health Sciences, Hiroshima International University, Hiroshima, Japan; [11]Oda Internal Medical Clinic, Hiroshima, Japan; [12]Hiroshima Red Cross Blood Centre, Hiroshima, Japan

Correspondence: Teruhisa Fujii
Division of Transfusion Medicine, Hiroshima University Hospital, 1-2-3, Kasumi, Minami-ward, Hiroshima, 734-8551, Japan
Tel +81-82-257-5581
Email terchan@hiroshima-u.ac.jp

Purpose: Given the chronic shortage of blood for transfusion in Japan, promotion of appropriate use of fresh frozen plasma (FFP) urgently needs to be addressed by the national blood project in Japan. Whether FFP transfusions are administered appropriately in Japan is currently unclear. In this study, we aimed to investigate the outcomes of patients who undergo FFP transfusion and the appropriateness of use of FFP.

Patients and Methods: This multicentre, prospective, observational cohort study was conducted from September 2017 to April 2019 at the 15 medical institutions in Hiroshima Prefecture that are the top providers of FFP. All patients who underwent FFP transfusion during the study period were included, relevant data being extracted from the medical records. The indications for FFP transfusion were classified in accordance with the Guidelines of the Ministry of Health, Labour and Welfare of Japan. Factors associated with patient outcomes at day 28 after FFP transfusion were subjected to multivariable logistic regression analysis.

Results: In total, data of 1299 patients were eligible for analysis. At least 63.8% of indications for FFP were in accordance with the guideline for FFP transfusions. The mortality rate at day 28 after FFP transfusion was 16.2%. Older age (65–74 years: adjusted odds ratio [AOR]=4.3, ≥75 years: AOR=4.1), non-perioperative use (AOR=4.5), coagulopathy associated with liver damage (AOR=2.7), large volume of FFP transfused (AOR=2.5), and lack of improvement in blood coagulation following FFP transfusion were independently and significantly associated with death within 28 days after FFP transfusion.

Conclusion: Our findings do not support the simple conclusion that FFP transfusions contribute to prognosis. However, given that coagulopathy in patients with end-stage liver disease is infrequently improved by FFP transfusion, "inappropriate" use of FFP should be avoided. It is important to promote appropriate use of FFP so as not to waste blood resources.

Keywords: prognosis, coagulopathy, inappropriate use, compliance with guideline

Introduction

Though lower than in the USA and Germany, consumption of fresh frozen plasma (FFP) per 1000 people in Japan is still 1.4 times higher than in France and the UK.[1] Within Japan, the difference in FFP consumption per bed is up to four times greater in the prefecture with the highest consumption than in that with the lowest consumption. Consumption in Hiroshima Prefecture has been relatively high. However, the number of blood donations in Japan is continually decreasing because the birth rate is declining and the population is ageing.[2] Therefore, given the

chronic shortage of blood for transfusion, promotion of appropriate use of FFP urgently needs to be addressed by the national blood project in Japan.

The Guideline for the Use of Blood Products formulated by the Ministry of Health, Labour and Welfare of Japan in 2005 states that a primary purpose of FFP transfusion is coagulant factor replacement in the absence of concentrated product.[3] It also states that, to prevent overuse, neither prophylactic use of FFP nor its use in patients with terminal illness is indicated, despite the fact that FFP has historically often been transfused for these purposes. After the guideline had been updated in 2017 (Table 1),[4] it came close to replicating international guidelines because it incorporated available evidence on blood transfusion.[5–8] The former guideline provides values for prothrombin time, activated partial thromboplastin time (APTT), and fibrinogen concentration that should trigger FFP transfusion,[3] whereas the revised guideline states that these values should not be used as triggers because they do not reliably predict a bleeding tendency. According to the Japanese guidelines, FFP transfusion is indicated to promote recovery from bleeding tendencies associated with coagulopathy or coagulation factor deficiency (eg, liver disease, disseminated intravascular coagulation, dilutional coagulopathy caused by massive red blood cell transfusion or fluid infusion), active bleeding alone not being a prerequisite for FFP transfusion. However, because many Japanese clinicians are not familiar with the revised guideline, some continue to transfuse FFP unnecessarily without determining the causes of the bleeding tendency.

Almost all of the many studies that have investigated the appropriate use of blood component products in Japan have reported only differences in, or annual changes in, the amounts used.[8–10] Therefore, whether FFP is transfused in accordance with the guidelines or in eligible patients only remains unclear. Moreover, few studies have provided evidence for the efficacy of FFP transfusion. Although the massive transfusion of FFP mentioned in some guidelines was recently reported to be effective,[11,12] the prognosis of patients who undergo FFP transfusion for other reasons is unknown.[13–15]

The Joint Committee for Blood Transfusion Therapy in Hiroshima Prefecture, which consists of representatives from the local government and major medical institutions, therefore conducted a multicentre, prospective, observational study on outcomes of patients who undergo FFP

transfusion. Use of FFP in accordance with the Japanese guideline was also analysed as a secondary endpoint.

Patients and Methods

This multicentre, prospective, observational cohort study was conducted from September 2017 to April 2019 at the 15 medical institutions in Hiroshima Prefecture that are the top providers of FFP. All patients who received FFP transfusion in those institutions during the study period were included. They were registered at each institution, the goal being to enrol 1000 patients in total. In order to meet the purpose of this study and at the same time to minimize the on-site burden related to data collection, the following 11 items were extracted from the medical records: 1. age; 2. sex; 3. primary disease; 4. date and number of days of FFP transfusion; 5. whether FFP was used perioperatively (related to surgery) and, if so, the type of surgery performed; 6. the indications for it, and dose of FFP; 7. coagulation test results before and after FFP use (prothrombin time–international normalised ratio [PT-INR], APTT, and fibrinogen concentration); 8. adverse reactions after FFP use; 9. presence and amount of red blood cell (RBC) transfusion; 10. duration of hospitalisation or discharge date; and 11. survival outcome 28 days after the first day of FFP transfusion. The indications for FFP transfusion were classified into the following categories in accordance with the Guidelines for the Use of Blood Products (revised in 2017 by the Ministry of Health, Labour and Welfare of Japan) (Table 1):[4] coagulopathy associated with liver disease, coagulopathy associated with disseminated intravascular coagulation (DIC), dilutional coagulopathy as a result of massive transfusion and fluid infusion, coagulation factor replacement in the absence of concentrated product, acute correction of warfarin effects, and plasma exchange. Although the indications for FFP were determined by the clinicians in each institution, they were reviewed during analysis because some were obviously wrongly classified. If FFP had been used in the absence of any of the indications in the guideline, its use was defined as "inappropriate". Massive transfusion was defined as transfusion of ≥10 units of RBCs within 24 hours. Each recruiting institution sent their data to the central institution, Hiroshima University Hospital, after anonymisation. All data were compiled and analysed by the Department of Epidemiology, Infectious Disease Control and Prevention, Graduate School of Biomedical and Health Sciences, Hiroshima University. In the case of patients who had received multiple transfusions during the same episode, the data were merged and the FFP doses summed. When the same patient was

Table 1 Guidelines for FFP Transfusion Established by Ministry of Health, Labour and Welfare of Japan (2017)

Trigger values of coagulation tests (as reference)	\<PT\> \<APTT\>	(i) INR 2.0 or higher, or (ii) 30% or lower Above twice the upper limit of the standard value at each medical institution	
	\<Fibrinogen\>	150 mg/dL or less, or when there is a risk of further deterioration	
Indications for FFP	1) Coagulation factor supplementation		
		a) Complex coagulopathy	i. Liver disease with bleeding tendency ii. L-asparaginase administration related ii. Disseminated intravascular coagulation (DIC) iv. Dilutional coagulopathy caused by massive transfusion and fluid infusion
		b) Coagulation factor replacement in the absence of concentrated product (when bleeding or before invasive procedures)	
		c) Correction of warfarin effect	
	2) Plasma exchange (thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome)		
Inappropriate indications	1. Expansion of circulatory volume		
	2. For nutritional purposes		
	3. For promotion of wound healing		
	4. Administration to terminal patients		
	5. Prophylactic transfusion ✓ In case of surgery or trauma that does not require a large amount of blood transfusion ✓ Chronic liver disease ✓ Severe burns ✓ Acute pancreatitis		

Abbreviations: FFP, fresh frozen plasma; PT, prothrombin time; INR, international normalised ratio; APTT, activated partial thromboplastin time.

enrolled more than once, each patient was included in the analysis if the purpose of FFP transfusion was different. If the purpose was the same but the reason for hospitalisation was different, we included each instance. During the study period, there were 1499 FFP transfusion events at the 15 recruiting medical institutions. After merging repeated use of FFP during the same episodes, 1299 patients were eligible for analysis.

Statistical Analysis

Coagulation test values of patients before and after FFP transfusion were compared using a paired t-test. Outcomes with and without massive transfusion of FFP ($\geq$2400 mL) were compared by the χ^2 test. After exclusion of cases of plasma exchange, factors associated with mortality 28 days after the first FFP transfusion were analysed using the χ^2 test and logistic regression for univariate and multivariable analysis. In the multivariable analysis, sex, age, and amount of transfused FFP were forced entry predictors, the other 11 variables being selected using the

stepwise method ($p<0.25$). Statistical analysis was performed with JMP 14.2.0 (SAS Institute, Cary, NC, USA), and the significance level was set at 0.05.

Ethics

There were no burdens or anticipated risks to patients as a result of the medical record review. In addition, decisions to transfuse FFP were made by the attending physician. The data were anonymised at each hospital before being sent to the central institution, and the created database was stored in a password-protected computer with no external connection. Careful consideration was given to the handling of personal information. An opt-out procedure was used for informed consent. The study was approved by the Ethics Committee of Hiroshima University (Approval No. E-976).

Results

The most common recipient age was within the seventh decade of life, accounting for 30.5% of the total sample (Figure 1) and 62.0% of the patients were men. The total
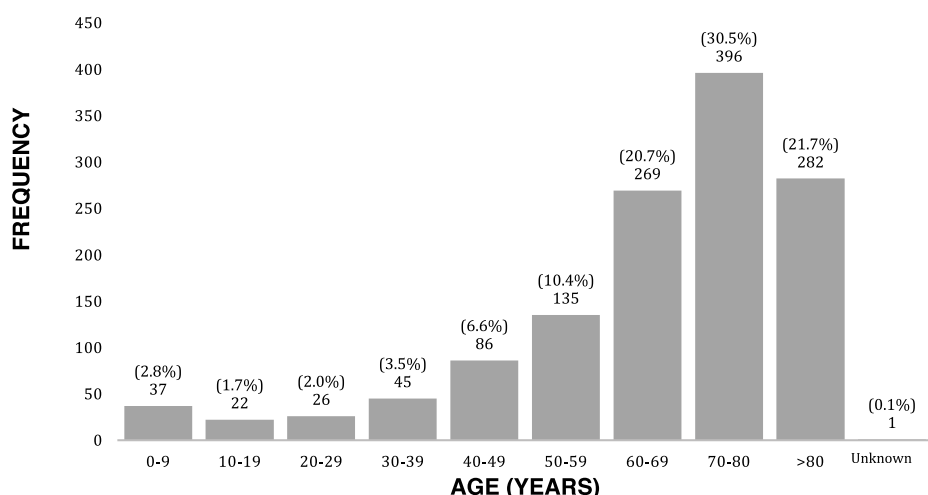
Figure 1 Age distribution of patients who underwent FFP transfusion.

dose of FFP was 16,700 units (one unit is equivalent to 120 mL in Japan; thus, 16,700 units = 2,004,000 mL). The median (min−max) FFP transfusion volume per patient was six (1–428) units. FFP was transfused perioperatively in 63.0% of cases, cardiovascular surgery being the most common form of surgery at 32.5%.

The rates of performing coagulation tests before FFP transfusion were 90.3% for PT-INR, 85.4% for APTT, and 65.9% for fibrinogen. The post-transfusion test rates were 86.0% for PT-INR, 79.2% for APTT, and 61.7% for fibrinogen. Trigger values stated in the former Japanese guideline were met for all three coagulation tests before FFP transfusion in 39.4% (512/1299) of cases, the values being confirmed to have improved after FFP transfusion in 18.2% (236/1299).

The indications for FFP transfusion are shown in Figure 2. "Inappropriate" was the most frequent, accounting for 473 cases (36.2%), followed by dilutional coagulopathy (424 cases, 32.6%), coagulopathy associated with DIC (155 cases, 11.9%), and coagulopathy associated with liver disease (155 cases, 11.9%). The main underlying diseases in the 473 patients who received FFP inappropriately were aortic dissection (100/473, 21.3%), ischaemic heart disease (39/473, 8.3%), and valvular disease (34/473, 7.2%).

Outcomes 28 days after transfusion were 75.2% alive, 16.2% dead, and 8.5% unknown. There were no severe adverse effects related to FFP transfusion, such as transfusion-related acute lung injury or transfusion-associated circulatory overload. The results of coagulation tests before and after FFP transfusion by patient outcome 28

days after transfusion are shown in Table 2. There were significant improvements in all coagulation test values in the surviving patients (p<0.0001 for all), but no significant improvement in the patients who had died (PT-INR, p=0.1954; APTT, p=0.1891; fibrinogen, p=0.6863). The total number of days of FFP transfusion was also compared. Overall, FFP was transfused on significantly more days in the deceased group than in the surviving patients (p<0.0001). Twenty or more units (≥2400 mL) of FFP were transfused in 15.2% (197/1299) of all patients. The main reasons for using such large amounts of FFP were dilutional coagulopathy (38.1%), coagulopathy associated with DIC (17.8%), and plasma exchange (17.3%). The mortality rate was significantly higher among patients who underwent FFP transfusion of ≥20 than <20 units (32.0% vs 13.4%, respectively; p<0.0001, χ^2 test).

Excluding the 34 patients who received FFP for plasma exchange, factors associated with death 28 days after FFP transfusion were analysed using multivariable logistic regression. Older age (65–74 years: adjusted odds ratio [AOR], 3.5; 95% confidence interval [95% CI], 1.0–11.9; p=0.0463 and ≥75 years: AOR, 3.4; 95% CI, 1.0–11.0; p=0.0452), non-perioperative use of FFP (AOR, 4.6; 95% CI, 2.6–8.2; p<0.0001), coagulopathy associated with liver damage (AOR, 2.7; 95% CI, 1.6–6.2; p=0.0190), transfusion of seven or more units of FFP (AOR, 2.4; 95% CI, 1.1–4.9; p=0.0175), post-transfusion PT-INR of ≥2.0 (AOR, 12.8; 95% CI, 4.8–34.1; p<0.0001), and post-transfusion APTT of ≥75 seconds (AOR, 9.5; 95% CI, 3.1–29.0; p<0.0001) were independently and significantly associated factors (Table 3).
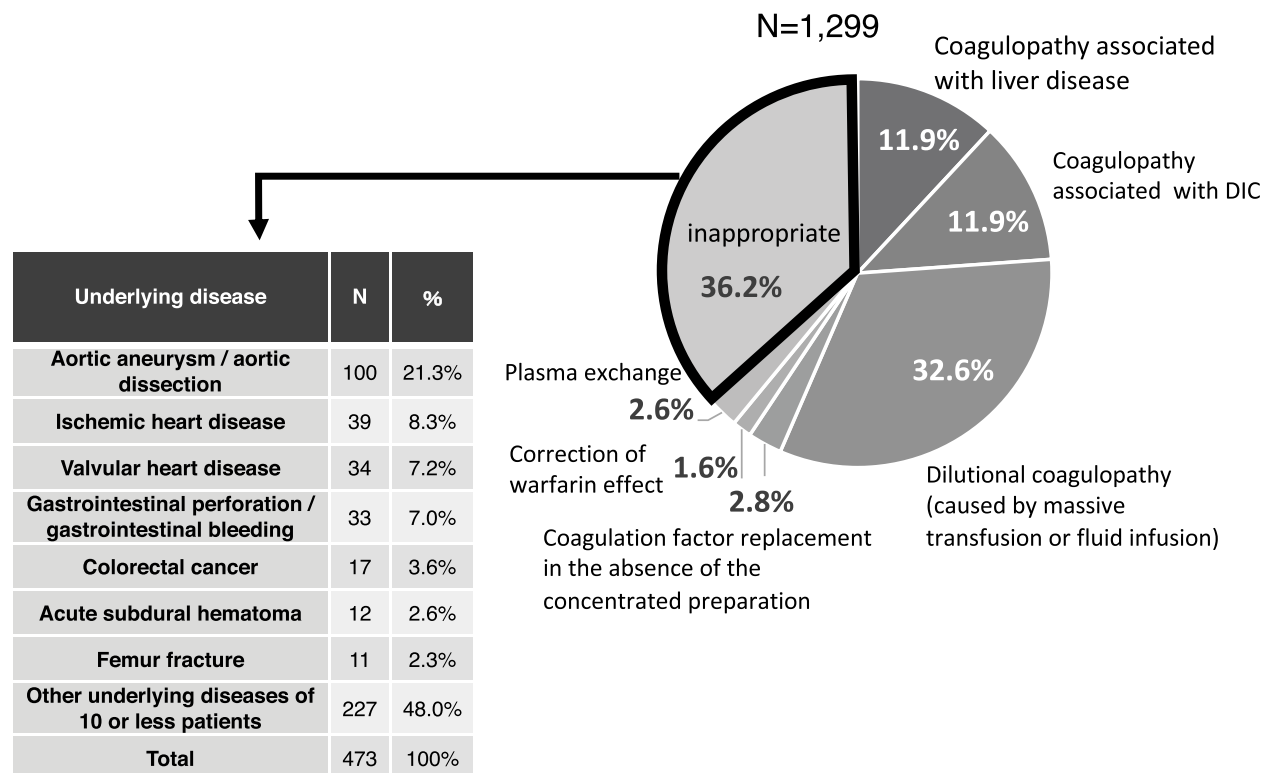
N=1,299

Coagulopathy associated with liver disease

11.9%

Coagulopathy associated with DIC

11.9%

inappropriate

36.2%

32.6%

Plasma exchange

2.6%

Correction of warfarin effect

1.6%

2.8%

Coagulation factor replacement in the absence of the concentrated preparation

Dilutional coagulopathy (caused by massive transfusion or fluid infusion)

Underlying disease	N	%
Aortic aneurysm / aortic dissection	100	21.3%
Ischemic heart disease	39	8.3%
Valvular heart disease	34	7.2%
Gastrointestinal perforation / gastrointestinal bleeding	33	7.0%
Colorectal cancer	17	3.6%
Acute subdural hematoma	12	2.6%
Femur fracture	11	2.3%
Other underlying diseases of 10 or less patients	227	48.0%
Total	473	100%

Figure 2 Distribution of indications for FFP transfusion, and underlying disease in which the reason for FFP use was "inappropriate".

Discussion

A major strength of this study is that all cases at 15 major medical institutions were registered prospectively for 1.5 years and that 1299 patients were analysed. In this study, the mortality rate 28 days after FFP transfusion was 16.2%. We also found that a significant proportion of FFP use (36.2%) was outside the indications in the Japanese guidelines (Table 1). The rate of inappropriate use of FFP has varied from 21% to 78% in surveys conducted in other regions.[16–23] In comparison, the rate in this study was not very high. Conversely, 63.8% of transfused FFP was used appropriately according to the guideline. Considering the reported 70% compliance rate with the guideline in general medical practice,[24,25] the use of FFP in Hiroshima appears to be largely in compliance with it.[4]

Table 2 Coagulation Test Values Before and After FFP Transfusion by Patients' Outcomes at Day 28

		N	Before FFP Transfusion	After FFP Transfusion	P*
PT-INR	Total	1051	1.3 (0.1–14.6)	1.2 (0.2–23.9)	<0.0001
	Alive	826	1.3 (0.1–14.1)	1.2 (0.2–23.9)	<0.0001
	Dead	151	1.6 (0.9–14.6)	1.5 (0.9–5.6)	0.1954
APTT	Total	947	36.1 (7.8–321)	34.7 (1.2–378)	<0.0001
	Alive	746	34.9 (7.8–200)	33.5 (1.2–378)	<0.0001
	Dead	132	51.2 (24.5–321)	44.2 (9.9–250)	0.1891
Fib	Total	654	206.6 (1–965)	236.9 (1.2–1023)	<0.0001
	Alive	541	213.7 (1–965)	246 (1.2–901)	<0.0001
	Dead	94	173.4 (25–796)	184.9 (41–1023)	0.6863

Notes: Data are shown as median (min-max). *Paired *t*-test between before and after FFP transfusion.
Abbreviations: FFP, fresh frozen plasma; SD, standard deviation; PT-INR, prothrombin time–international normalised ratio; APTT, activated partial thromboplastin time; Fib, fibrinogen.

Table 3 Univariate and Multivariate Analysis on Factors Associated with the Outcome at Day 28 After FFP Transfusion

	Outcome at 28 Days		Univariable Analysis*		Multivariable Analysis#	
	Dead N (%)	Alive N (%)	OR [95% CI]	p-value	AOR [95% CI]	p-value
Age (years)						
≤39	12(10.2)	106(89.8)	1		1	
40–64	48(17.1)	233(82.9)	1.8[0.9–3.6]	0.2337	1.7 [0.5–5.7]	0.4151
65–74	50(15.8)	266(84.2)	1.7[0.9–3.2]	0.4029	3.5 [1.0–11.9]	0.0463
Over 75	89(20.3)	349(79.7)	2.3[1.2–4.3]	0.0333	3.4 [1.0–11.0]	0.0452
Sex						
Female	66(15.2)	368(84.8)	0.8[0.6–1.1]	0.1520	1.2 [0.7–2.0]	0.5223
Male	133(18.5)	586(81.5)	1		1	
Perioperative use of FFP						
No	128(31.9)	273(68.1)	4.8[3.4–6.6]	<0.0001	4.6 [2.6–8.2]	<0.0001
Yes	66(9.0)	671(91.0)	1		1	
Indication for FFP						
Coagulopathy caused by liver disease	50(33.6)	99(66.4)	6.5[3.9–10.7]	<0.0001	2.7[1.2–6.2]	0.0190
Coagulopathy associated with DIC	48(33.1)	97(66.9)	6.4[3.8–10.5]	<0.0001	2.1[0.9–5.2]	0.0922
Dilutional coagulopathy	59(15.7)	316(84.3)	2.4[1.5–3.8]	<0.0001	1.4[0.6–2.9]	0.4094
Coagulation factor replacement	6(17.1)	29(82.9)	2.7[1.0–6.9]	0.0368	1.3[0.2–7.2]	0.7691
Warfarin effects correction	6(30.0)	14(70.0)	5.5[2.0–15.4]	0.0003	5.8[1.0–32.3]	0.0613
Inappropriate use	31(7.2)	399(92.8)	1		1	
Pre-dose PT-INR						
≥2	59(35.1)	109(64.9)	3.2[2.2–4.6]	<0.0001	-	
<2	130(14.6)	761(85.4)	1			
Pre-dose APTT(seconds)						
≥75	49(48.0)	53(52.0)	5.6[3.7–8.7]	<0.0001	1.5[0.7–3.2]	0.2760
<75	127(14.1)	775(85.9)	1		1	
Pre-dose Fibrinogen (mg/dl)						
≤150	61(26.8)	167(72.3)	2.2[1.5–3.2]	<0.0001		
>150	78(14.3)	468(85.7)	1			
Amount of transfused FFP (Units)						
≥7	127(22.8)	431(77.2)	2.1[1.5–2.9]	<0.0001	2.4[1.1–4.9]	0.0175
<7	73(12.3)	523(87.8)	1		1	
Amount of transfused RBC (Units)						
≥7	92(20.7)	352(79.3)	1.5[1.1–2.1]	0.0090	1.4[0.7–2.9]	0.3116
<7	102(14.7)	590(85.3)	1		1	
Ratio FFP/RBC						
>1.5	59(23.5)	192(76.5)	1.8[1.2–2.6]	0.0017	1.2[0.6–2.3]	0.6373
≤1.5	99(14.8)	572(85.3)	1		1	
Post-dose PT-INR						
≥2	44(58.5)	30(40.5)	11.7[7.0–19.4]	<0.0001	12.8[4.8–34.1]	<0.0001
<2	104(11.2)	829(88.9)	1		1	
Post-dose APTT (seconds)						
≥75	27(57.5)	20(42.3)	9.6[5.2–17.7]	<0.0001	9.5[3.1–29.0]	<0.0001
<75	109(12.3)	776(87.7)	1		1	

(Continued)

970

Journal of Blood Medicine 2021:12

Table 3 (Continued).

	Outcome at 28 Days		Univariable Analysis*		Multivariable Analysis#	
	Dead N (%)	Alive N (%)	OR [95% CI]	p-value	AOR [95% CI]	p-value
Post-dose Fibrinogen (mg/dl)						
≤150	41(36.0)	73(64.0)	4.3[2.7–6.8]	<0.0001		
>150	74(11.6)	566(88.4)	I			
Total number of days of FFP administration (days)						
≥3	52(29.9)	122(70.1)	2.4[1.7–3.5]	<0.0001		
<2	147(15.1)	828(84.9)	I			

Notes: Excluding the cases of plasma exchange (N=34), factors associated with the mortality outcome at 28 days after FFP transfusion were analysed. *Chi-square test with post-hoc multiple comparisons by Bonferroni correction were used to compare groups. #Logistic regression analysis with the stepwise method: sex, age, and the amount of transfused FFP were forced entry predictors, and the other 11 variables were selected using the stepwise method (p<0.25). R^2 = 0.3084, model p-value < 0.0001, n = 1265. **Abbreviations**: OR, odds ratio; AOR, adjusted odds ratio; CI, confidence interval; FFP, fresh frozen plasma; DIC, disseminated intravascular coagulation; APTT, activated partial thromboplastin time; PT-INR, prothrombin time and international normalized ratio; RBC, red blood cells.

Seventy-one (5.5%) patients received FFP for ≥3 days, and 28 (2.2%) for ≥10 days. Some of these patients may have had end-stage liver disease with coagulopathy because of impaired production of blood coagulation factors. Coagulopathy in patients with end-stage liver disease is difficult to eliminate, even with several FFP transfusions. This accounts for the tendency of clinicians to continue FFP transfusion over a long period, resulting in transfusion of large total volumes of FFP. In our study, patients who were still alive at 28 days had received FFP for significantly fewer days than those who had died. The mortality rate was also significantly higher in patients who had received ≥20 units of FFP than in those who had received <20 units. We cannot simply conclude that a longer duration or a large amount of FFP transfusion leads to a poor prognosis. However, these findings do indicate that FFP is still given to patients with terminal conditions and poor prognoses, and that such use of FFP is inefficient.

The second edition of the Japanese guideline states that blood coagulation tests are not necessary before FFP transfusion;[4] this differs from the recommendation in the first edition. However, in this study we found that, in most institutions, coagulation tests were performed to assess the patients' haemostatic status, assist decisions on whether to administer FFP, and determine the likely efficacy of FFP transfusion. Admittedly, the trigger values for the coagulation tests are only reference values. However, FFP may have been administered "inappropriately" to patients in whom one or more coagulation test results did not reach the trigger values. There were no significant differences in

any coagulation test values between before and after FFP transfusion in the patients who subsequently died. This lack of improvement may indicate that patients whose conditions are very serious and who are at higher risk of dying have chronic rather than acute defects in coagulation.

The original purpose of FFP transfusion was not to improve patients' prognoses but to improve coagulation ability, stop bleeding, and reduce RBC transfusion volumes. However, because FFP should be administered only to eligible patients to prevent wasting of resources, determining survival rates after FFP transfusion is still important in Japan. Although 36.2% of FFP use was outside the indications and considered "inappropriate", this relatively low rate suggests that all "inappropriate" use of FFP was not fruitless. Some of these FFP transfusions may have improved coagulation ability and contributed to restoring good haemostasis, leading to a good prognosis. Even though the mortality rate in this study was 16.2%, it cannot be concluded that FFP transfusion contributed to patients' prognoses.

We used multivariable logistic regression to analyse factors associated with death at day 28 after FFP transfusion. The factors independently and significantly associated with death at day 28 after FFP transfusion were older age, non-perioperative use of FFP, coagulopathy associated with liver damage, FFP transfusion of seven or more units, post-transfusion PT-INR of ≥2, and post-transfusion APTT of ≥75 seconds. Patients with end-stage liver disease often have chronic coagulopathy that does not improve with FFP transfusion.

This study had several limitations. First, although bleeding volume is a mortality-related factor, it could not be included in the multivariable analysis. In addition, we did not include the patients' underlying diseases in the multivariable analysis because they were too diverse. As to specific causes of death, we confirmed that no deaths were due to bleeding. However, we did not further investigate the causes of death.

This is the first study to comprehensively determine the actual circumstances and outcomes of FFP transfusion in major medical institutions in Hiroshima Prefecture. Surveys need to be performed in other prefectures and nationwide and the results compared with those of this study. Our findings indicate that it is necessary to continue to promote appropriate FFP use to minimise wasting of blood resources.

Conclusion

In this multicentre prospective cohort study, which was performed to determine the actual circumstances and outcomes of FFP transfusion in Hiroshima Prefecture, we found that FFP was transfused appropriately in at least 63.8% of cases. The mortality rate 28 days after transfusion was 16.2%. The factors independently and significantly associated with mortality were older age, non-perioperative use of FFP, coagulopathy associated with liver damage, FFP transfusion of seven or more units, post-transfusion PT-INR of ≥2, and post-transfusion APTT of ≥75 seconds.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas. Especially, Aya Sugiyama and Junko Tanaka had a great contribution to analysing the data. Aya Sugiyama and Teruhisa Fujii mainly wrote the manuscripts, and the rest of all authors critically reviewed it. All authors gave final approval of the manuscript version to be published and agreed to be accountable for every aspect of the work.

Funding

Disclosure

Dr Masazumi Okajima reports grants from Ministry of Health, Labour and Welfare of Japan, during the conduct of the study; personal fees from Chugai Pharmaceutical Co., Ltd., Taiho Pharma, Johnson and Johnson K. K., Medicaroid Corporation, Eli Lily Japan K. K., Olympus Corporation, and Covidien Japan Inc., outside the submitted work. The authors declare that they have no other conflicts of interest in this work.

References

1. Ministry of Health. Labour and welfare of Japan: annual report on national blood project in 2016. Available from: https://www.mhlw.go.jp/stf/seisaku nitsuite/bunya/0000154567.html. Accessed December 9, 2020.

2. Ministry of Health. Labour and welfare of Japan: annual report on national blood project in 2020. Available from: https://www.mhlw.go.jp/stf/seisaku nitsuite/bunya/0000063233.html. Accessed August 4, 2021.

3. Ministry of Health. Labour and welfare of Japan: the guideline for the use of blood products. First edition. Available from: https://www.mhlw.go.jp/new-info/kobetu/iyaku/kenketsugo/5tekisei3b.html. Accessed December 7, 2020.

4. Ministry of Health. Labour and welfare of Japan: the guideline for the use of blood products (revised in 2017). Available from: https://www.mhlw.go.jp/file/06-Seisakujouhou-11120000-Iyakushokuhinkyoku/0000161115.pdf. Accessed December 9, 2020.

5. Liumbruno G, Bennardello F, Lattanzio A, et al. Recommendations for the transfusion of plasma and platelets. *Blood Transfus*. 2009;7:132–150.

6. O'Shaughnessy DF, Atterbury C, Bolton Maggs P, et al. Guidelines for the use of fresh-frozen plasma, cryoprecipitate and cryosupernatant. *Br J Haematol*. 2004;126:11–28. doi:10.1111/j.1365-2141.2004.04972.x

7. Roback JD, Caldwell S, Carson J, et al. Evidence-based practice guidelines for plasma transfusion. *Transfusion*. 2010;50:1227–1239. doi:10.1111/j.1537-2995.2010.02632.x

8. Makino S, Konno H, Okamoto Y, et al. Current status and future issues in blood transfusion therapy in Japan: a nationwide survey. *Jpn J Transfus Cell Ther*. 2020;66:619–628. doi:10.3925/jjtc.66.619

9. Makino S, Tanaka A, Takahashi K, et al. Nationwide questionnaire survey of transfusion medicine in fiscal year 2008- Analysis of transfusion management systems and the appropriateness of transfusion therapy. *Jpn J Transfus Cell Ther*. 2010;56:515–521. doi:10.3925/jjtc.56.515

10. Kanno H, Okamoto H, Kitazawa J, et al. Nationwide questionnaire survey of transfusion medicine in fiscal year 2017. *Jpn J Transfus Cell Ther*. 2018;64:752–760. doi:10.3925/jjtc.64.752

11. Borgman MA, Spinella PC, Holcomb JB, et al. The effect of FFP: RBC ratio on morbidity and mortality in trauma patients based on transfusion prediction score. *Vox Sang*. 2011;101:44–54. doi:10.1111/j.1423-0410.2011.01466.x

12. Zehtabchi S, Nishijima DK. Impact of transfusion of fresh-frozen plasma and packed red blood cells in a 1:1 ratio on survival of emergency department patients with severe trauma. *Acad Emerg Med*. 2009;16:371–378. doi:10.1111/j.1553-2712.2009.00386.x

13. Zhang LM, Li R, Zhao XC, et al. Increased transfusion of fresh frozen plasma is associated with mortality or worse functional outcomes after severe traumatic brain injury: a retrospective study. *World Neurosurg*. 2017;104:381–389. doi:10.1016/j.wneu.2017.04.140

14. Shiba H, Ishida Y, Haruki K, et al. Negative impact of fresh-frozen plasma transfusion on prognosis after hepatic resection for liver metastases from colorectal cancer. *Anticancer Res*. 2013;33:2723–2728.

15. Müller MC, Arbous MS, Spoelstra-de Man AM, et al. Transfusion of fresh-frozen plasma in critically ill patients with a coagulopathy before invasive procedures: a randomized clinical trial. *Transfusion*. 2015;55:26–35. doi:10.1111/trf.12750

16. Kakkar N, Kaur R, Dhanoa J. Improvement in fresh frozen plasma transfusion practice: results of an outcome audit. *Transfus Med*. 2004;14:231–235. doi:10.1111/j.0958-7578.2004.00505.x

17. Luk C, Eckert KM, Barr RM, et al. Prospective audit of the use of fresh-frozen plasma, based on Canadian Medical Association transfusion guidelines. *CMAJ*. 2002;166:1539–1540.

18. Stanworth SJ, Grant-Casey J, Lowe D, et al. The use of fresh-frozen plasma in England: high levels of inappropriate use in adults and children. *Transfusion*. 2011;51:62–70. doi:10.1111/j.1537-2995.2010.02798.x

19. Tinmouth A, Thompson T, Arnold DM, et al. Utilization of frozen plasma in Ontario: a provincewide audit reveals a high rate of inappropriate transfusions. *Transfusion*. 2013;53:2222–2229.

20. Moiz B, Arif FM, Hashmi KZ. Appropriate and inappropriate use of fresh frozen plasma. *J Pak Med Assoc*. 2006;56:356–359.

21. Qiang JK, Thompson T, Callum J, et al. Variations in RBC and frozen plasma utilization rates across 62 Ontario community hospitals. *Transfusion*. 2019;59(2):545–554. doi:10.1111/trf.15070

22. Shinagare SA, Angarkar NN, Desai SR, et al. An audit of fresh frozen plasma usage and effect of fresh frozen plasma on the pre-transfusion international normalized ratio. *Asian J Transfus Sci*. 2010;4(2):128–132. doi:10.4103/0973-6247.67024

23. Goel R, Patel EU, White JL, et al. Factors associated with red blood cell, platelet, and plasma transfusions among inpatient hospitalizations: a nationally representative study in the United States. *Transfusion*. 2019;59(2):500–507. doi:10.1111/trf.15088

24. Arts DL, Voncken AG, Medlock S, Abu-Hanna A, van Weert HC. Reasons for intentional guideline non-adherence: a systematic review. *Int J Med Inform*. 2016;89:55–62. doi:10.1016/j.ijmedinf.2016.02.009

25. Cordasco KM, Yuan AH, Danz MJ, et al. Veterans health administration primary care provider adherence to prescribing guidelines for systemic hormone therapy in menopausal women. *J Healthc Qual*. 2019;41:99–109. doi:10.1097/JHQ.0000000000000183

Differentiation of Yin, Yang and Stasis Syndromes in Severe Aplastic Anemia Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation and Their Correlation with Iron Metabolism, cAMP/cGMP, 17-OH-CS and Thyroxine

Huijin Hu, Tao Chen, Wenbin Liu, Yiping Shen, Qiushuang Li, Yuhong Zhou, Baodong Ye & Dijiong Wu

Dovepress

ORIGINAL RESEARCH

Differentiation of Yin, Yang and Stasis Syndromes in Severe Aplastic Anemia Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation and Their Correlation with Iron Metabolism, cAMP/cGMP, 17-OH-CS and Thyroxine

Huijin Hu[1],*
Tao Chen[2],*
Wenbin Liu[1]
Yiping Shen[1]
Qiushuang Li [iD][3]
Yuhong Zhou[1]
Baodong Ye [iD][1]
Dijiong Wu [iD][1]

[1]Department of Hematology, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, People's Republic of China; [2]Department of Hematology, Xiaoshan Traditional Chinese Medical Hospital, Hangzhou, Zhejiang, People's Republic of China; [3]Center of Clinical Evaluation and Analysis, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, People's Republic of China

*These authors contributed equally to this work

Correspondence: Dijiong Wu; Baodong Ye
Department of Hematology, The First Affiliated Hospital of Zhejiang Chinese Medical University, #54 Youdian Road, Hangzhou, Zhejiang, 310006, People's Republic of China
Fax +8657187073569
Email wudijiong@zcmu.edu.cn; 13588453501@163.com

Objective: To better understanding and differentiation of traditional Chinese medicine (TCM) syndromes in severe aplastic anemia (SAA) patients undergoing hematopoietic stem cell transplantation (Allo-HSCT) and their correlation with iron metabolism, cAMP/cGMP, 17-OH-CS and thyroxine.

Methods: Eighteen patients with SAA who underwent HSCT were enrolled. The syndrome was evaluated before conditioning and days after stem cell reinfusion (−10d, −1d, +7d, +30d, +60d, and +90d). The correlation of TCM syndrome (Yin, Yang, and stasis) to cyclic nucleotides, 17-OH-CS, thyroxine, and iron metabolism were analyzed and compared to data from normal subjects.

Results: More "Yin deficiency" (n=11, 11/18) syndrome was observed before HSCT, and nearly 61% was complicated with "blood stasis". After conditioning, the proportion of "kidney Yin and Yang deficiency" increased to 61.6%. Fourteen days after HSCT, the syndrome developed into "Spleen-Kidney Yang Deficiency," and the stasis score decreased. On +90day, majority patients were diagnosed with "Kidney Yang Deficiency" (35.7%) or "Spleen-Kidney Yang Deficiency" (28.6%), and 88.9% were diagnosed without stasis. The correlation analysis showed that cGMP might represent "Deficient Yang" as well as low total triiodothyronine (T3) and free T3 (FT3). There was also a positive relation between labile plasma iron (LPI), hepcidin, soluble transferrin receptor (sTfR), and "Yin deficiency", and the last two factors, along with marrow nitric oxide synthase were also positively related to "Stasis" syndrome.

Conclusion: During HSCT, the syndrome evolved from "kidney Yin and Yang deficiency" to "kidney Yang deficiency" or "spleen–kidney Yang deficiency", and the "stasis" along with "Yin deficiency" syndromes were quickly relieved within 90 days. The changes of cyclic nucleotides, 17-OH-CS, thyroxine, and iron metabolism indexes can be applied for better differentiation of TCM syndrome.

Keywords: aplastic anemia, traditional Chinese medicine syndrome, iron metabolism, syndrome evolution, hematopoietic stem cell transplantation

Background

Aplastic anemia (AA) is a bone marrow failure syndrome often present with pancytopenia. Patients with AA are at high risk of infection and hemorrhage.[1] The incidence of AA in China is ~7.4/10[6], which is almost three times higher

compared to Europe and North America.[2,3] Severe AA (SAA) accounts for ~18%,[4] with a characteristic of rapid disease progression and high mortality if left untreated. According to the guidelines and clinical practice,[5] allogeneic hematopoietic stem cell transplantation (allo-HSCT) and immune suppression therapy (IST) are the first treatment options for SAA, while disease-free survival (DFS) of HSCT is around 50–80%.[6,7]

Since 1964, traditional Chinese medicine (TCM) had been applied in the management of AA in China. The beneficial effect of TCM has also been confirmed in the treatment of aplastic anemia.[8] And the combination of TCM with androgen or IST (cyclosporine with or without anti-thymoglobulin antibody) may significantly improve the overall response rate in patients with AA.[9–12] In addition, our previous study revealed that the combination of TCM during the HSCT might reduce graft failure, transplant-related mortality and improve the 5 years overall survival.[13] Yet, there are still limited data available on how and when the TCM should be applied. In addition, the principle of TCM during different stages of stem cell transplantation remains unclear.

The basic TCM syndrome is based on "qi and blood", "Yin and Yang", "deficiency and excess", and "exterior and interior", also known as "eight principles". "Yin and Yang" differentiation is the general and main principle. To better understanding the material basis of TCM syndrome, many studies have disclosed the correlation of syndrome differentiation with objective indicators, including endocrine hormone (adrenocorticosteroids, thyroid hormones, prostaglandins), plasma cyclic nucleotides (cAMP, cGMP, and their dynamic balance), as well as energy metabolism situation,[14,15] which have been applied as the evaluation index for TCM treatment.[16,17] However, TCM syndrome is complex, and the syndrome evolution is not always clear. During the HSCT, we found that the changing of TCM syndrome occurred much more rapidly, and the deviation of "Yin" and "Yang" were more obvious, thus suggesting that HSCT can also be an ideal disease model for observing TCM syndrome evolution and verifying the material basis of "Yin", "Yang", as well as "blood stasis" syndrome in our AA patients. Herein, we investigated the evolution and characteristics of TCM syndromes in SAA patients undergoing HSCT, and also analyzed the correlation with the objective indicators, which may profit from developing a TCM treatment proposal for HSCT.

Materials and Methods
Participants and Recruitment
A total of 18 patients with SAA who underwent HSCT between December 2015 and January 2017 were recruited in the First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China. Twelve of them were male (12/18), with a median age of 28.5 (11–53) years, and 12 of them progressed from non-SAA. All the patients met the diagnostic and severity assessment criteria for SAA.[18] The syndrome differentiation was made according to the "Revised Edition of Guidelines for The Clinical Diagnosis and Treatment of Aplastic Anemia (Sui Lao)" issued by the National Administration of Traditional Chinese Medicine in 2013.[19] Moreover, 21 healthy subjects without obvious syndrome deviation (equilibrium in Yin and Yang) were included as normal control for peripheral serum and urine detection, 11 males (11/21), with a median age of 39 (21–53) years. In addition, five bone marrow samples from normal bone marrow transplantation donors were also prepared as control bone marrow samples.

Sources of Stem Cell
Donors were selected based on the best available human leukocyte antigen (HLA) match at the time of transplantation. HLA-A, B, C DRB1, and DQB1 loci were confirmed by the high-resolution molecular method. Six (6/18) patients were grafted from matched sibling donors, 7 (7/18) from matched unrelated donors from the China Marrow Donor Program (CMDP), 4 (4/18) from haploidentical donors, and 1 (1/18) from a mismatched related donor.

Conditioning Regimen
All 18 patients received the same conditioning regimen: fludarabine (Flu) $30mg/m^2 \times 5days$, rabbit anti-thymoglobulin antibody (ATG) $2.5mg/kg \times 4days$, cyclophosphamide (CY) 100–120mg/kg in total (given in 2 or 3 days).

GVHD Prophylaxis
GVHD prophylaxis consisted of mycophenolate mofetil (MMF) $1200–1500mg/m^2$, cyclosporine (target trough concentration 200–300ng/mL) per day, and methotrexate (MTX $15mg/m^2$ on day +1, and $10mg/m^2$ on day +3, +6, and +11).

976

Journal of Blood Medicine 2021:12

Aplastic Anemia (AA) TCM Syndrome Diagnosing and Scoring

Diagnosing TCM syndrome in AA was done according to "Revised Edition of Guidelines for The Clinical Diagnosis, and Treatment of Aplastic Anemia (Sui Lao)",[19] which included heat-toxin congestion and excessiveness syndrome, Yin deficiency and blood heat syndrome (spleen) kidney yang deficiency syndrome, (liver) kidney Yin deficiency syndrome, and kidney Yin and Yang deficiency syndrome. symptoms of the major syndrome included a pale face, dizziness, palpitations, and shortness of breath; symptoms of the minor syndrome included feverish palms and soles, hectic fever and night sweating, dipsia, yellow urine, cold body, anorexia, and diarrhea. All symptoms were scored according to the severity (light, moderate or severe): 0, 2, 4, and 6 points, or 0, 1, 2, and 3 points[10] (Table 1). The assessment and scoring of TCM syndrome were performed 10 days before stem cell reinfusion (−10d), the day before stem cell reinfusion (−1d), 7 days after stem cell reinfusion (+7d), and on +14d, +30d, +60d, and +90d.

Blood Stasis Syndrome Evaluation and Scoring

The diagnosis of blood stasis syndrome included the inspection of the tongue, facial and skin, pain assessment, degree of hemorrhage evaluation, assessing consciousness, mental and bodily sensation, pulse manifestation, and abdominal mass evaluation. According to the severity (light, moderate or severe), syndromes were scored with 0, 1, 2, and 3 points (Table 2). With an overall score over 4, the diagnosis of blood stasis syndrome was confirmed. The assessment and scoring of stasis syndrome were performed on −10d, −1d, +7d, +14d, +30d, +60d, and +90d.

Collection and Preparing of Clinical Specimens

The peripheral blood (10mL), urine (5mL), and bone marrow (10mL) were collected from each patient and volunteer. After centrifugation, serum, urine, and marrow mononuclear cells were prepared and frozen in −70°C for further use.

ELISA Assay

Approximately 0.5 mL serum was taken from each group for single detection. Serum cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), thyroxine (total triiodothyronine, free triiodothyronine, total tetraiodothyronine, and free tetraiodothyronine), iron load indicator (ferritin and labile plasma iron), and its regulator iron metabolism indicators (hepcidin, soluble transferrin receptor, and ferroportin) were detected after each time

Table 1 Scoring System for Basic TCM Syndrome

Symptom		Scoring			
		None (0)	Mild (2 for Major Syndromes, and 1 for Minor)	Moderate (4 for Major Syndromes, and 2 for Minor)	Severe (6 for Major Syndromes, and 3 for Minor)
Major syndromes	Pale face		Slightly	Moderate, pale without vitality	Severe, pale with a white paper looking
	Dizziness		Occasionally	Frequently	Persistently and hard to relieve
	Fatigue		Slightly, can still work on daily life	Moderate, hard to work	Severe, can only stay in bed
	Palpitations and shortness of breath		Occasionally	Frequently	Recurrently and hard to relieve
Minor syndromes	Feverish palms and soles		Slightly feverish in night	Moderate feverish with vexation	Feel burning, and away from clothing
	Hectic fever and night sweating		Occasionally, mostly on head	Recurrently, on chest and waist	Frequently, showing as wet body
	Dipsia		Occasionally	Frequently, tolerable	Frequently, intolerable
	Yellow urine		Primrose yellow	Medium yellow	Dark yellow
	Cold body		Cold in hands and feet	Cold limbs	Clod body, cannot relieve with warming
	Anorexia		Slightly, appetite decreases 1/3 to 2/3	Bad, appetite decreases above 2/3	Severe anorexia, even no diet
	Diarrhea		Once daily	Two to three times daily	More than three times daily

Table 2 Scoring System for Stasis Syndrome

	Grading and Scoring			
	Normal (0)	**Mild (1)**	**Medium (2)**	**Severe (3)**
Inspection of the tongue	Pink tongue, without ecchymosis and sublingual varices	Dark red tongue, with petechiae	Dark purple tongue, with petechiae and ecchymosis	Bluish purple tongue, or with sublingual varices
Inspection of the facial	No purplish black at face, lips, gums or eyes	Dark red less than two sites	Dark red or bluish purple more than two sites	Purple black more than two sites
Skin manifestation	No rough skin nor scales	Rough hand and foot, but without scales	Rough hand and foot, with scales	Rough skin throughout the body with scales
Pain	No fixed pain, prickling or cramping	Occasionally pain, and always relieved within half an hour	Pain sometimes, always relieved within three hours, and no need for drug	Persistent pain, and painkillers needed
Bleeding	No bleeding, hypodermic ecchymosis, nor menstrual disorders	Small petechiae in one site, or with occasionally dysmenorrhea and dark menstruation	Petechiaes more than two sites, or always with dysmenorrhea and dark menstruation	Petechiaes more than three sites, or suffer from abdominal pain during menstruation, and even amenorrhea
Conscious	No vertigo or forgetful	Occasionally happen	Happened sometimes, and more frequently after physical work	Always happen, and even during rest
Mentality	No mental abnormality	Occasionally happen, and relieved without intervene	Happened sometimes, and can be stopped by persuading	Always happen, and can only stopped by constraint or medication
Physical sensation	No numbness or hemiplegia	Occasionally happen, and relieved without intervene	Numbness cannot be alleviate by self, with mild hemiplegia	Numbness Limbs and with hemiplegia
Pulse manifestation	Normal pulse	Hesitant pulse	Thread and hesitant pulse	No pulse or irregularly pulse
Lump in abdomen	No organ enlargement, mass, tissue hyperplasia, new organisms, etc.	Slightly stiff and painful with pressing	Moderate stiff and painful	Painful and reject pressing

point (totally seven times). The levels of bone marrow vascular endothelial growth factor (VEGF), nitric oxide synthase (NOS), and reactive oxygen species (ROS) were detected before HSCT and monthly after HSCT (totally four times). All detection was performed according to the manufacturer's protocol of the ELISA kit.

Statistical Analysis

The compiled data were first inputted into the Excel spreadsheet and were then read into SPSS15.0 biometric statistical software program for further analysis. Normal data were expressed as mean±standard deviation, and non-normal data were expressed as medians (interquartile range). Variance and pairwise comparisons were used for

normal data, whereas non-normal data were subjected to non-parametric tests. The Kruskal–Wallis H-test was used for pairwise comparisons and the Mann–Whitney U-test for multiple comparisons. Binary variable regression models were used to analyze the correlation among objective factors and TCM syndromes. A P value <0.05 was considered to be statistically significant.

Results

Syndrome Differentiation Based on "Yin–Yang" and "Zang–Fu Viscera"

Patients were primarily diagnosed with "Yin" or "Yang" deficiency syndrome, and further syndrome differentiation was performed based on "Zang–Fu viscera". Yin deficiency

syndrome was differentiated into "kidney Yin deficiency", "liver–kidney Yin deficiency" and "kidney Yin and Yang deficiency" syndromes, and Yang deficiency syndrome was differentiated into "kidney Yang deficiency", "spleen–kidney Yang deficiency", "kidney Yin and Yang deficiency" syndromes, respectively. After careful assessment of the TCM syndrome and scoring, we found that before HSCT (−10d), the syndrome was more likely "Yin deficiency" or "Yang deficiency", rather than "Yin and Yang deficiency". During the conditional stage (−10d to −1d), more patients manifested "kidney Yin and Yang deficiency" syndrome. Moreover, after stem cell engraftment (+14d), more "spleen–kidney Yang deficiency" syndrome was seen. Finally, 2 months after stem cell reinfusion, the TCM syndrome became stable and mainly manifested as "kidney Yang deficiency" or "spleen–kidney Yang deficiency" syndrome (Table 3, Figure 1).

Blood Stasis Syndrome Evaluation During HSCT

The scoring of blood stasis syndrome was performed at the corresponding time point during HSCT. A total of 61% (11/18) patients were complicated with stasis syndrome before HSCT (score >4). During the transplantation conditioning, the stasis score further increased and peaked 7 days after stem cell reinfusion (+7d); however, no significant differences were observed (P>0.05). After the stem cell engraftment (around +14d), the stasis score decreased (P<0.01) and was kept at the same level (score ≤4) during +60d~+90d. At +90d, the stasis score decreased to the minimum value, and 88.9% (16/18) had no symptoms of stasis (Table 4, Figure 2).

Typical Evolution of Tongue Manifestation During HSCT

Tongue manifestations have a crucial role in TCM syndrome differentiation. During the syndrome assessment, we also recorded and compared the tongue manifestation. Three typical evolution (Type I:2A-2E; Type II:2F-2J; Type III:2K-2O) of tongue manifestation during the process of HSCT are shown in Figure 3. The evolution of TCM syndrome of Type I went from the firstly "spleen–kidney Yang deficiency" stage to "liver–kidney Yin deficiency with blood stasis" stage, "spleen–kidney Yang deficiency with blood stasis" stage, and finally of relatively stable "kidney Yang deficiency" stage. In Type II, the syndrome evolution was as follows: "kidney Yang deficiency", to a syndrome of "Yin deficiency with blood stasis", to "spleen–kidney Yang

Table 3 The Distribution of TCM Syndrome in AA Patients Undergoing HSCT

| Time Points (HSCT Following Up)	Yang Deficiency (n)			Yin Deficiency (n)		
	Kidney Yang Deficiency	Spleen–Kidney Yang Deficiency	Kidney Yin and Yang Deficiency	Kidney Yin Deficiency	Liver–Kidney Yin Deficiency	Kidney Yin and Yang Deficiency
10 days before stem cell reinfusion (d-10)	1	6	2	4	5	2
The day before stem cell reinfusion (d-1)	2	1	11	4	0	11
7 days after stem cell reinfusion (d+7)	0	7	8	2	1	8
14 days after stem cell reinfusion (d+14)	2	5	5	1	5	5
30 days after stem cell reinfusion (d+30)	1	11	3	—	2	3
60 days after stem cell reinfusion (d+60)	5	10	0	—	2	0
90 days after stem cell reinfusion (d+90)	5	4	1	3	1	1
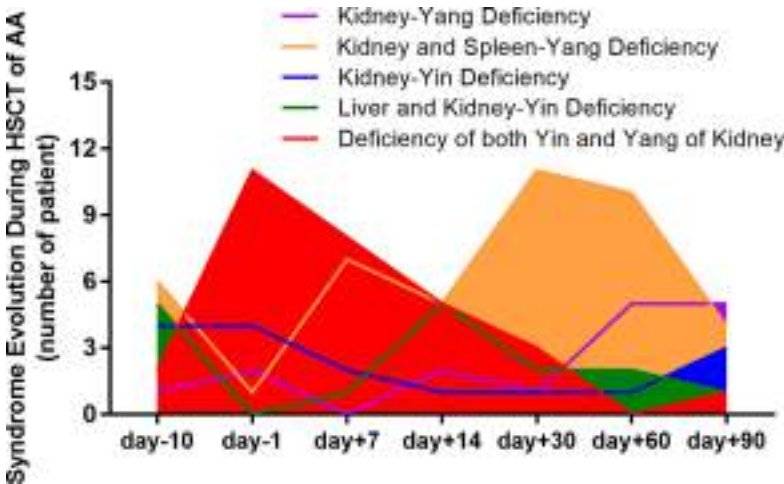
Figure I Evolution of TCM syndromes in severe aplastic anemia (SAA) patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) based on "Yin–Yang" and "Zang–Fu viscera" syndrome differentiation.

deficiency" and finally "kidney Yang deficiency" stage. In Type III, the syndrome evolution was as follows: "kidney Yin deficiency", to "Yin deficiency fire hyperactivity", to "Yin and Yang deficiency", and finally "kidney Yang deficiency" stage.

Changes of Serum cAMP and cGMP During the HSCT Process

The expression level of serum cAMP in the patient was relatively equal to the normal control, while the cGMP was significantly higher ($P<0.01$). During the process of HSCT, the cAMP remarkably decreased from day -1, and then reached the lowest level at day -60 ($P<0.01$). It then relatively increased at day -90. No differences were observed among day $+30$, $+60$, and $+90$ ($P>0.05$). For the cGMP, similar changes were observed. The cGMP decreased after the conditioning regimen and tended to stabilize after day $+14$, but the level was still higher than the normal control ($P<0.01$) (Figure 4).

Table 4 Comparison of Stasis Score Among Different Time Point After During HSCT (Wilcoxon), P value

	Day 14	Day+30	Day+60	Day+90
Day-10	NS	0.011	0.001	0.002
Day-1	NS	0.005	0.001	0.001
Day+7	0.013	0.003	0.001	0.000
Day 14	/	0.05	0.015	0.007
Day+30	0.05	/	NS	0.047
Day+60	0.015	NS	/	NS
Day+90	0.007	0.047	NS	/

Changes of Urine 17-OH-CS During HSCT Process

The urine level of 17-OH-CS in AA before HSCT was significantly higher than the normal group ($P<0.01$). After transplantation conditioning, the 17-OH-CS gradually decreased over time; the lowest level was observed after day $+60$, after which it remained stable until $+90$ (Figure 4).

Changes of Serum Thyroxine During HSCT Process

The serum level of total triiodothyronine (TT3), tetraiodothyronine (TT4), and free T3 (FT3), T4 (FT4) were detected during the HSCT. The TT3 and FT3 were significantly lower in AA before HSCT; the level decreased over time (from day-1 to day+30) after transplantation. Sixty to 90 days after stem cell reinfusion, the TT3 and FT3 remarkably increased ($P<0.05$) and reached the same level detected before HSCT (Figure 5A). For the TT4 and FT4, the expression level did not have a great change during the HSCT, but they decreased on day $+30$ and day $+60$ ($P<0.05$ or $P<0.01$) when compared to the level at the early stage of HSCT (day -10 to day $+7$) (Figure 5B).

Comparison of Iron Load and Its Regulator During HSCT Process

The serum level of iron load indexes, ferritin and labile plasma iron (LPI), as well as iron-absorbing regulators, hepcidin and soluble transferrin receptor (sTfR), were significantly higher in AA patients than the normal

980
Journal of Blood Medicine 2021:12
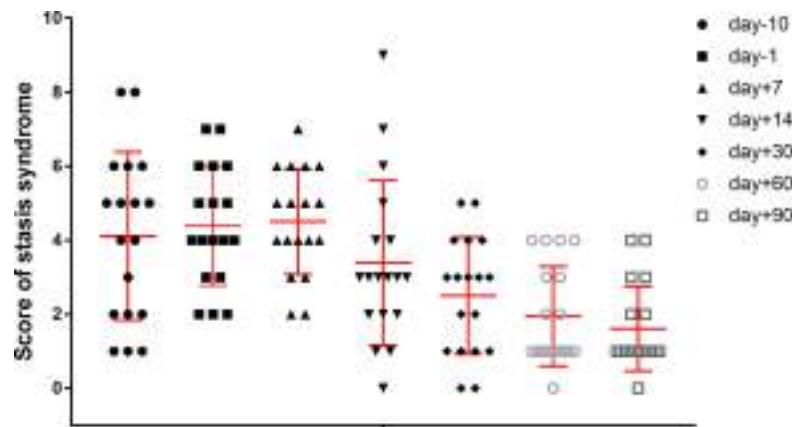
Figure 2 Evolution of "blood stasis" syndrome in SAA patients during HSCT. The stasis syndrome was differentiated, scoring and compared during different stage of HSCT. A total of 61% (11/18) patients were complicated with stasis syndrome before HSCT, and increased after conditioning. After the stem cell engraftment (around +14d), the stasis score decreasing and 88.9% (16/18) had no symptoms of stasis at +90d.

Figure 3 Typical evolution of tongue manifestation during HSCT. Type I: went from the firstly "spleen–kidney Yang deficiency" stage to "liver–kidney Yin deficiency with blood stasis" stage, "spleen–kidney Yang deficiency with blood stasis" stage, and finally of relatively stable "kidney Yang deficiency" stage (2A-2E); Type II: went from "kidney Yang deficiency", to a syndrome of "Yin deficiency with blood stasis", to "spleen–kidney Yang deficiency" and finally "kidney Yang deficiency" stage (2F-2J); and Type III: went from "kidney Yin deficiency", to "Yin deficiency fire hyperactivity", to "Yin and Yang deficiency", and finally "kidney Yang deficiency" stage (2K-2O).

population before HSCT, while ferroportin (FPN) was much lower ($P<0.05$ or $P<0.01$). Moreover, after transplantation, the ferritin and LPI gradually decreased over time and then became stable after +60d. The LPI reached the lowest point on day +60 (Figure 6A). The change in hepcidin was much slower than ferritin, which obviously decreased 30 days after stem cell reinfusion ($P<0.05$ or $P<0.01$), while FPN gradually increased after day+7 ($P<0.05$ or $P<0.01$) (Figure 6B).

Changes of Bone Marrow VEGF, NOS, and ROS During HSCT Process

The impairment of iron deposition is mainly mediated by oxygen-free radical damage. In this study, the marrow
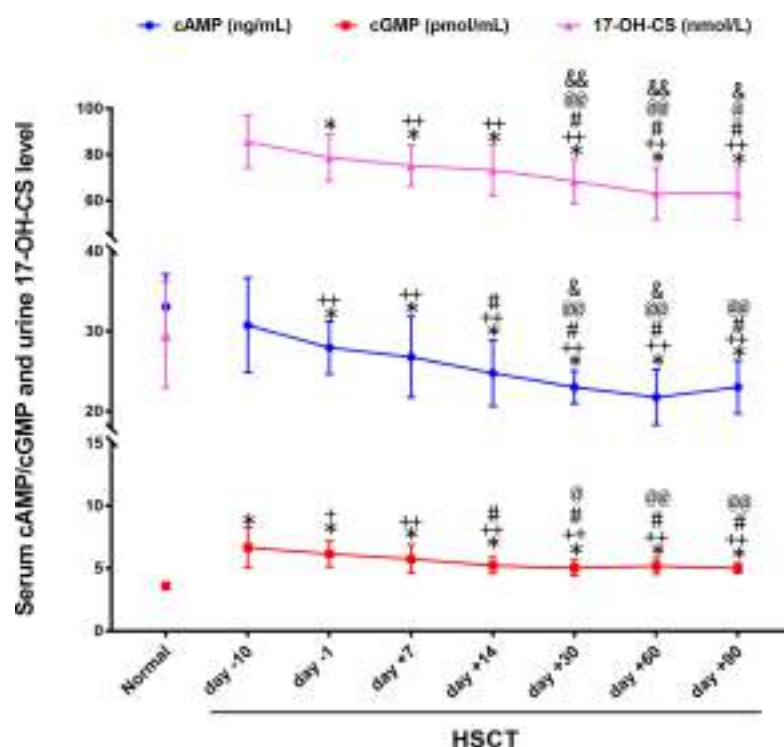
Figure 4 Changes of serum cAMP, cGMP and urine 17-OH-CS during the HSCT process. Data are presented as mean ± SD; *$P < 0.01$ (as compared with the normal population);$^+P < 0.05$, and $^{++}P < 0.01$ (as compared with −10d); $^#P < 0.01$ (as compared with −1d); $^@P < 0.05$, $^{@@}P < 0.01$ (as compared with +7d); $^&P < 0.05$, and $^{&&}P < 0.01$ (as compared with +14d).

serum level of VEGF, NOS, and ROS was detected. The expression of VEGF in bone marrow from AA patients was significantly lower, while NOS and ROS were much higher than the normal subjects (all $P<0.01$). After HSCT, the VEGF remarkably increased after +60days, and NOS and ROS decreased after +30days ($P<0.01$) (Figure 7).

Correlation Analysis Between TCM Syndrome Evolution and Serum Indicators

To analyze the indicator of "Yin" and "Yang" syndrome, we deleted the patient without obvious "Yin" and "Yang" deviation during the HSCT. Patients with "Deficiency of both Yin and Yang" were assigned into "Yin" deficiency and "Yang" deficiency group. The Spearman coefficient analysis was used to assess the cross-correlation of "Yin", "Yang" deficiency syndrome, "blood stasis" syndrome with metabolism indicators (cAMP, cGMP, 17-OH-CS, and thyroxine) as well as iron-related indicators (ferritin, LPI, Hepcidin, sTfR, and FPN). Results showed that "Yang deficiency" was significantly negatively correlated with TT3, FT3, and TSH, while "Yin deficiency"

syndrome had a strong positive correlation with cGMP, 17-OH-CS, LPI, hepcidin, and sTfR (Tables 5 and 6). Considering "blood stasis" syndrome, it showed a remarkable positive correlation to cAMP, cGMP, 17-OH-CS, hepcidin, sTfR, and a negative correlation to FPN (Tables 7 and 8).

Discussion

Our data suggested that the main syndrome of a newly diagnosed patient with SAA was "Yin deficiency with fire hyperactivity" or "heat toxic exacerbation",[20] while those with non-severe chronic AA presented with "kidney deficiency with blood stasis".[10] For patients who were planning to receive stem cell transplantation, the severe infection was kept under control, and the "Heat Toxic" syndrome was alleviated before HSCT. In this study, we found that the 9/18 patients were diagnosed with "Yang deficiency" and 11/18 with "Yin deficiency" before conditioning (two of them deficient in both Yin and Yang). For patients with "Yang deficiency", 66.7% were diagnosed with "spleen–kidney Yang deficiency", while in "Yin deficiency" group, "kidney Yin deficiency" (36%) and "liver–kidney Yin deficiency" (45%) occupied the
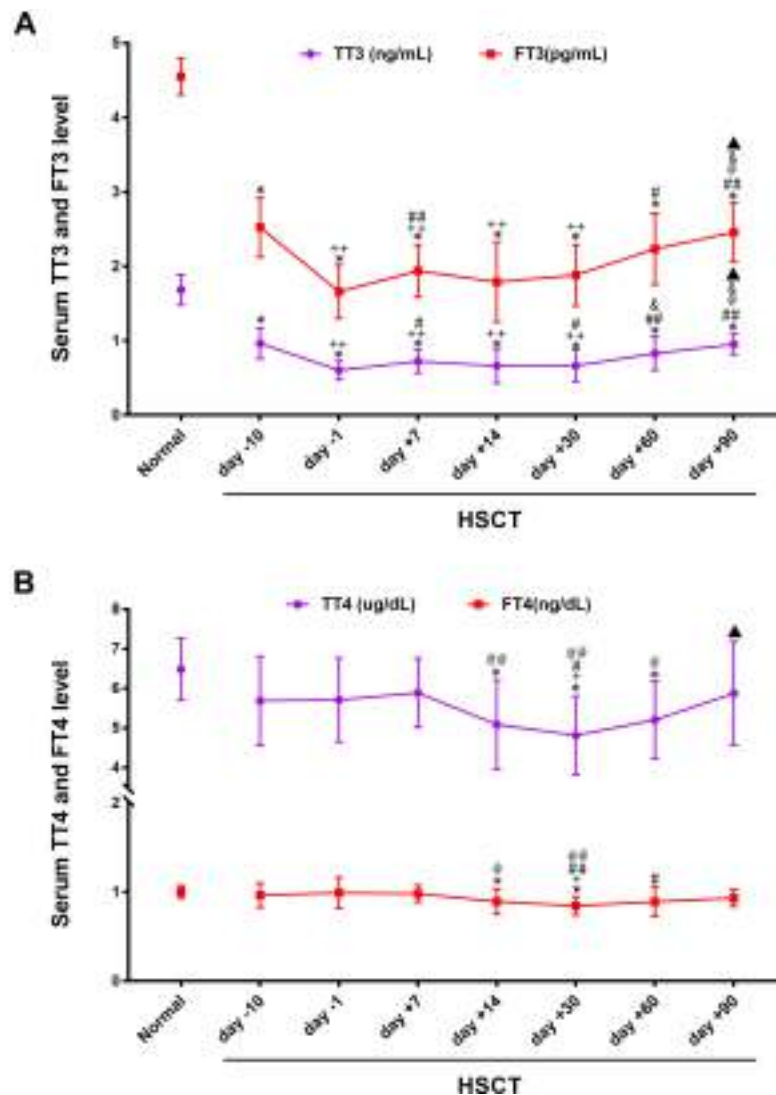
Figure 5 Changes of serum thyroxine during HSCT process. The serum level of total triiodothyronine (TT3), free T3 (FT3) (**A**) and tetraiodothyronine (TT4), free T4 (FT4) (**B**) were detected during the HSCT. Data are presented as mean ± SD; *$P < 0.01$ (as compared with the normal population); +$P < 0.05$, and ++$P < 0.01$ (as compared with −10d); #$P < 0.05$, ##$P < 0.01$ (as compared with −1d); @$P < 0.05$, @@$P < 0.01$ (as compared with +7d); &$P < 0.01$ (as compared with +14d); ▲$P < 0.01$ (as compared with +30d).

majority. After conditioning, the proportion of "Kidney Yin and Yang deficiency" increased to 61.6% and decreased after stem cell transfusion. Fourteen days after stem cell transfusion, the TCM syndrome gradually developed into "spleen–kidney Yang deficiency". The proportion reached the peak at +30day (61.1%) and became stable after +60day. On +90day, the majority of patients were diagnosed with "kidney Yang deficiency" (35.7%) or "spleen–kidney Yang deficiency" (28.6%), and four patients manifested with no obvious "Yin" or "Yang" deviation. Our research presented a whole syndrome map during HSCT for a patient without severe complications.

The cyclic nucleotides (cAMP and cGMP) act as intercellular second messengers by stimulating hormones, neurotransmitters, and inflammatory mediators.[21] cAMP is considered as "Yang", and cGMP as "Yin" in "Yang deficiency" model, which can be restored by warming and recuperating kidney yang.[22–24] However, cAMP and cGMP function may differ in different syndrome or disease models. It is reported that in patients with "Yang deficiency" physique, the serum cGMP increased and cAMP/cGMP decreased after Yang nourishment, while no changes in cAMP were observed.[25] Besides cyclic nucleotides, the hypothalamic-pituitary-adrenal (HPA) axis dysfunction has a crucial role in the pathological
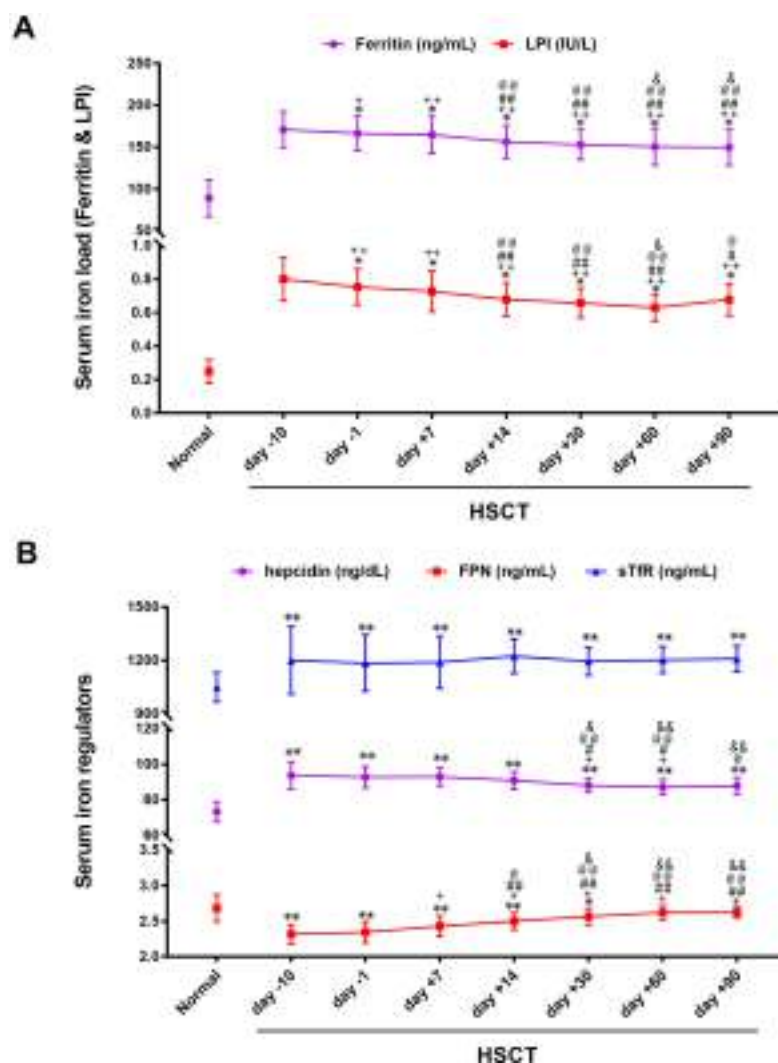
Figure 6 Comparison of iron load and its regulator during HSCT process. The serum level of iron load indexes, ferritin and labile plasma iron (LPI) (**A**), as well as iron-absorbing regulators, hepcidin and soluble transferrin receptor (sTfR) (**B**) were detected. Data are presented as mean ± SD; *$P < 0.05$, **$P < 0.01$ (as compared with the normal population);[+]$P < 0.05$, and [++]$P < 0.01$ (as compared with −10d); [#]$P < 0.05$, [##]$P < 0.01$ (as compared with −1d); [@]$P < 0.05$, [@@]$P < 0.01$ (as compared with +7d); [&]$P < 0.05$, and [&&]$P < 0.01$ (as compared with +14d).

basis of "Kidney Yang Deficiency".[23,26] Our results showed that the SAA patient had a significantly higher cGMP level and lower cAMP than the normal subjects; cGMP and cAMP remarkably decreased after conditioning. After stem cell reinfusion, the cGMP became stable since +14day, and cAMP recovered around +90day. In addition, patients also had a higher level of 17-OH-CS, which decreased during the HSCT, and became stable around +60-+90day. The correlation analysis showed a positive relation between cGMP, 17-OH-CS, and "Yin Deficiency", which means that cGMP may form the basis of "Yang" here, and the "Yang" that cGMP represented actually was "deficient Yang" ascribed to the "Yin deficiency". The increased expression of 17-OH-CS before

HSCT means that the activated adrenocortical function may also be due to hyperactivation of "deficient Yang". The extremely low TT3 and FT3 in SAA before HSCT and their negative correlation with "Yang deficiency" suggested "Yang deficiency" before HSCT. All these changes indicated that both "Yin" and "Yang" were deficient in SAA; the "Yin deficiency" or "Yang deficiency" syndrome was actually the manifestation of deviated deficiency based on "Yin and Yang Deficiency".

Owing to dysfunctional iron metabolism and excess transfusion, iron overload (IO) is one of AA's most encountered complications, even in newly diagnosed patients.[27,28] Analogism is regarded as a major theoretical construction tool of TCM, which has an important role in
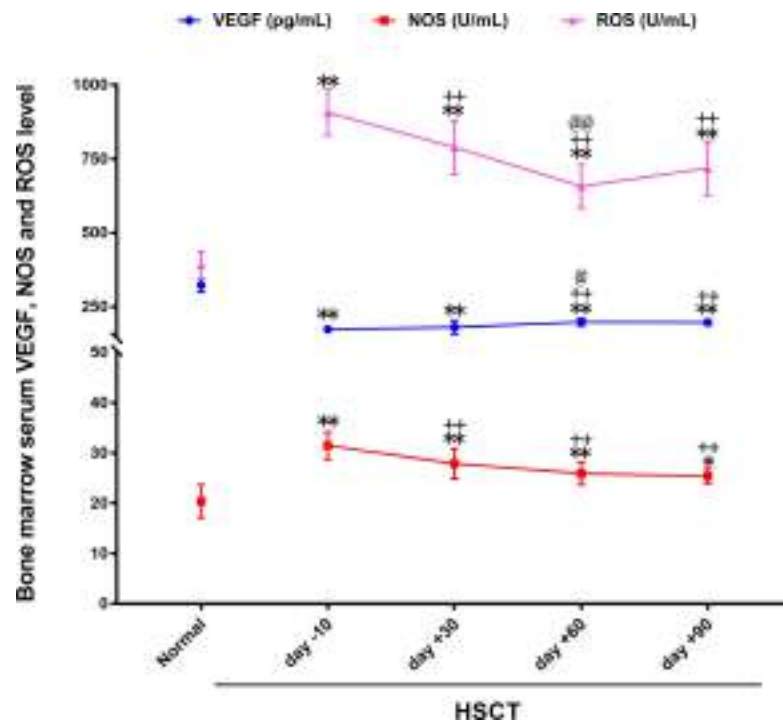
Figure 7 Changes of bone marrow VEGF, NOS, and ROS during HSCT process. The marrow serum level of VEGF, NOS, and ROS were detected. Data are presented as mean ± SD; *$P < 0.05$, **$P < 0.01$ (as compared with the normal population); ++$P < 0.01$ (as compared with −10d); @$P < 0.05$, and @@$P < 0.01$ (as compared with +7d).

concept design and theory systematization. In patients with heavy IO, hyperpigmentation occurs; some may even suffer from abdominal pain, which was similar to the patient with "Stasis" syndrome. Our previous study revealed a close relationship between IO and "Stasis" syndrome in chronic AA. Also, the iron chelation therapy contributed to the alleviation of "Stasis" syndrome and promoted the hematopoiesis,[29,30] which is also in accordance with the TCM theory of "removing blood stasis for promoting tissue regeneration". Our result showed that nearly 61% of the patients were diagnosed with "blood stasis" syndrome before HSCT, and the stasis scoring increased after conditioning (until +7d). However, after +14day, the stasis score decreased, and 88.9% patients were diagnosed without stasis.

There was also a notable higher level of ferritin and LPI in AA patients, which suggested a higher iron load. Hepcidin is a major communicator between liver iron stores and the intestinal iron absorption and transport mechanisms that can negatively regulate the iron assimilation, and induce FPN endocytosis, phosphorylation, and catabolism. The expression of hepcidin was regulated by sTfR mediated HJV-BMP-SMAD pathway,[31,32] and was upregulated in AA or myelodysplastic syndrome (MDS)

with IO.[33,34] Higher hepcidin, sTfR, and decreased FPN were also found in our cases. After HSCT, hepcidin started to significantly decrease 30 days after stem cell reinfusion, and FPN recovered to a normal level around +60day, while no obvious changes were observed on sTfR. In addition, the correlation analysis showed a strong positive relation of LPI, hepcidin, sTfR to "Yin Deficiency", while FPN was negatively related with "Yin deficiency" syndrome. We also found a positive correlation between hepcidin and sTfR, and a negative correlation between FPN and "stasis" syndrome. All these indicated that IO might contribute to the formation of "stasis", and that "Yin deficiency and blood stasis" may the tendentiousness syndrome in IO.

Besides the direct influence of iron overload to "Stasis", the impaired angiogenesis induced by oxygen free radical damage may also contribute to the stasis formation in AA patient.[35] This study showed a significantly lower VEGF and extremely higher NOS and ROS in bone marrow from patients with SAA compared to the normal controls, and these indexes were corrected 30 to 90 days after HSCT. There was positive correlation of bone marrow NOS level to "Stasis" syndrome, and a negative correlation of VEGF to "Yin Deficiency" syndrome, which means that the oxygen free radical damage and impaired

Table 5 Correlation Analysis Between "Yin" and "Yang" Deficiency Syndrome and Serum Indicators (I)

	Yang Deficiency (Ratio)	Yin Deficiency (Ratio)	cAMP	cGMP	17-OH	TT3	FT3	TT4	FT4	Ferritin	LPI	Hepcidin	sTfR	FPN
Normal	0.00	0.00	33.076	3.601	29.579	1.683	4.552	6.485	1.000	9.17	0.25	73.23	73.23	2.68
Day-10	0.50	0.61	30.765	6.682	85.623	0.965	2.523	5.685	0.963	4.69	0.80	93.60	93.60	2.32
Day-1	0.78	0.83	27.949	6.164	78.7402	0.602	1.658	5.708	0.991	4.59	0.75	92.72	92.72	2.34
Day+7	0.83	0.61	26.775	5.751	75.249	0.723	1.938	5.889	0.982	4.80	0.73	92.84	92.84	2.43
Day+14	0.67	0.61	24.763	5.240	73.226	0.663	1.791	5.087	0.892	4.76	0.68	90.74	90.74	2.50
Day+30	0.83	0.33	23.012	5.042	68.391	0.665	1.881	4.814	0.844	4.63	0.66	88.02	88.02	2.56
Day+60	0.77	0.23	21.744	5.195	63.044	0.829	2.235	5.203	0.890	4.21	0.63	87.04	87.04	2.62
Day+90	0.56	0.56	22.879	4.983	61.222	0.949	2.428	5.996	0.944	4.61	0.68	87.79	87.79	2.62

Table 6 Correlation Analysis Between "Yin" and "Yang" Deficiency Syndrome and Serum Indicators (II)

		cAMP	cGMP	17-OH	TT3	FT3	TT4	FT4	Ferritin	LPI	Hepcidin	sTfR	FPN
Yang Deficiency	Coefficient of association	−0.407	0.252	0.311	−0.731*	−0.731*	−0.467	−0.371	−0.275	0.156	0.299	0.299	−0.311
	Sig	0.158	0.274	0.453	0.020	0.020	0.122	0.183	0.255	0.356	0.236	0.236	0.226
Yin Deficiency	Coefficient of association	0.220	0.830**	0.854**	−0.610	−0.610	−0.122	0.220	−0.098	0.927**	0.854**	0.854**	−0.854**
	Sig	0.301	0.005	0.007	0.054	0.054	0.387	0.301	0.409	0.000	0.003	0.003	0.003

Notes: Binary variable regression models were used to analyze the correlation among objective factors and "Yin" and "Yang" deficiency syndromes. *$P<0.05$; **$P<0.01$.

Table 7 Correlation Analysis Between "Blood Stasis" Syndrome and Serum Indicators (I)

Average Score of "Stasis Syndrome"		cAMP	cGMP	17-OH	TT3	FT3	TT4	FT4	Ferritin	LPI	Hepcidin	sTfR	FPN
Day-10	4.111	30.765	6.682	85.623	0.965	2.523	5.685	0.963	4.69	0.80	93.60	93.60	2.32
Day-1	4.389	27.949	6.164	78.7402	0.602	1.658	5.708	0.991	4.59	0.75	92.72	92.72	2.34
Day+7	4.500	26.775	5.751	75.249	0.723	1.938	5.889	0.982	4.80	0.73	92.84	92.84	2.43
Day+14	3.389	24.763	5.240	73.226	0.663	1.791	5.087	0.892	4.76	0.68	90.74	90.74	2.50
Day+30	2.500	23.012	5.042	68.391	0.665	1.881	4.814	0.844	4.63	0.66	88.02	88.02	2.56
Day+60	1.944	21.744	5.195	63.044	0.829	2.235	5.203	0.890	4.21	0.63	87.04	87.04	2.62
Day+90	1.611	22.879	4.983	61.222	0.949	2.428	5.996	0.944	4.61	0.68	87.79	87.79	2.62

Table 8 Correlation Analysis Between "Blood Stasis" Syndrome and Serum Indicators (II)

		cAMP	cGMP	17-OH	TT3	FT3	TT4	FT4	Ferritin	LPI	Hepcidin	sTfR	FPN
Stasis syndrome	Coefficient of association	0.821*	0.821*	0.857*	−0.357	−0.357	0.143	0.714	0.536	0.750	0.857*	0.857*	−0.857*
	Sig	0.023	0.023	0.014	0.432	0.432	0.760	0.071	0.215	0.052	0.014	0.014	0.014

Notes: Binary variable regression models were used to analyze the correlation among objective factors and "blood stasis" deficiency syndromes. *$P<0.05$.

angiogenesis may also be the pathologic basis of "Yin deficiency and blood stasis" syndrome.

In conclusion, our study revealed the syndrome evolution pattern in AA patients undergoing HSCT, and found the possible basis of "Yin deficiency", "Yang deficiency", and "stasis" syndromes, which may promote the diagnosis and differentiation of the syndrome. For clinical practice, our results suggest it is necessary to take care of "Yin" during the whole stage of HSCT, as herbs with "hot" nature should be avoided in case of exhaustion of "Yin" regardless of their beneficial effect on hematopoiesis at the early stage, and "warm" herbs should be preferred instead. In addition, it is also necessary to understand "Stasis" in AA, where the abnormal iron metabolism may have a crucial role in the "blood stasis" syndrome formation, and therapeutic methods with blood-activating and stasis-resolving may be of considerable importance during the process of HSCT.

Abbreviations

AA, aplastic anemia; HSCT, hematopoietic stem cell transplantation; NOS, nitric oxide synthase; IST, immune suppression therapy; DFS, disease free survival; TCM, traditional Chinese medicine; CMDP, China Marrow Donor Program; Flu, fludarabine; ATG, anti-thymoglobulin antibody; CY, cyclophosphamide; MMF, mycophenolate mofetil; MTX, methotrexate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; TT3, total triiodothyronine; FT3, free triiodothyronine; TT4, total tetraiodothyronine; FT4, free tetraiodothyronine; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; LPI, labile plasma iron; sTfR, soluble transferrin receptor; FPN, ferroportin; HPA, hypothalamic-pituitary-adrenal; IO, iron overload; MDS, myelodysplastic syndrome.

Data Sharing Statement

Additional data can be requested by contacting the corresponding author (wudijiong@zcmu.edu.cn) on the email address provided.

Ethics Approval and Consent to Participate

This study was approved by the ethical committee of First Affiliated Hospital of Zhejiang Chinese Medical University and registered with the Chinese Clinical Trials Registry, # ChiCTR-ONC-1600803030. All the patients enrolled were gave informed consent and agreed to participate in the study. Informed consent was signed by all participants or their legal guardian (for patients under 18 years old). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Funding

Disclosure

The authors report no conflicts of interest in this work.

References

1. Young NS, Calado RT, Scheinberg P. Current concepts in the patho-physiology and treatment of aplastic anemia. *Blood*. 2006;108 (8):2509–2519. doi:10.1182/blood-2006-03-010777
2. Ye L, Zhang F, Kojima S. Current insights into the treatments of severe aplastic anemia in China. *Int J Hematol*. 2020;112 (3):292–299. doi:10.1007/s12185-020-02955-1
3. Young NS, Kaufman DW. The epidemiology of acquired aplastic anemia. *Haematologica*. 2008;93(4):489–492. doi:10.3324/haematol. 12855
4. Ahmed P, Chaudhry QUN, Satti TM, et al. Epidemiology of aplastic anemia: a study of 1324 cases. *Hematology*. 2020;25(1):48–54. doi:10.1080/16078454.2019.1711344
5. Killick SB, Bown N, Cavenagh J, et al. Guidelines for the diagnosis and management of adult aplastic anaemia. *Br J Haematol*. 2016;172 (2):187–207. doi:10.1111/bjh.13853
6. Kamranzadeh Fumani H, Jalili M, Rad S, et al. Outcome of peripheral blood allogeneic hematopoietic stem cell transplantation as a treatment option in patients with severe aplastic anemia between 40 and 50 years. *Hematol Oncol Stem Cell Ther*. 2020;30111–30115. doi:10.1016/j.hemonc.2020.06.004
7. Xu ZL, Zhou M, Jia JS, et al. Immunosuppressive therapy versus haploidentical transplantation in adults with acquired severe aplastic anemia. *Bone Marrow Transplant*. 2019;54(8):1319–1326. doi:10.10 38/s41409-018-0410-3
8. Red Blood Cell Disease Group CSoHCMA. Chinese expert consensus on the diagnosis and treatment of aplastic anemia (2017). *Zhonghua Xue Ye Xue Za Zhi*. 2017;38:1–5. Chinese. doi:10.3760/ cma.j.issn.0253-2727.2017.01.001
9. Zhu N, Wu D, Ye B. The progress of traditional Chinese medicine in the treatment of aplastic anemia. *J Transl Int Med*. 2018;6 (4):159–164. doi:10.2478/jtim-2018-0031
10. Wu D, Shen Y, Ye B, et al. Efficacy and advantages of modified traditional Chinese medicine treatments based on "kidney reinforcing" for chronic aplastic anemia: a randomized controlled clinical trial. *J Tradit Chin Med*. 2016;36(4):434–443. doi:10.1016/S0254-6272(16)30059-0

11. Ma R. Treatment of bone marrow failure syndrome with integrated traditional and Western medicine. *Chin J Integr Med*. 2007;13 (2):85–88. doi:10.1007/s11655-007-0085-x

12. Zheng BR, Shen JP, Zhuang HF, et al. Treatment of severe aplastic anemia by immunosuppressor anti-lymphocyte globulin/anti-thymus globulin as the chief medicine in combination with Chinese drugs. *Chin J Integr Med*. 2009;15(2):145–148. doi:10.1007/s11655-009-0141-9

13. Ye BD, Zhang X, Shao KD, et al. Combined use of Chinese medicine with allogeneic hematopoietic stem cell transplantation for severe aplastic anemia patients. *Chin J Integr Med*. 2014;20(12):903–909. doi:10.1007/s11655-014-2026-9

14. Kuang AK. Preliminary observation on endocrine and immune functions and concentration of cyclic nucleotides in plasma in "deficiency of yang" (author's transl). *Zhonghua Nei Ke Za Zhi*. 1979; 18:105–108. Chinese. PMID: 555897

15. Taylor MK, Hernandez LM, Kviatkovsky SA, et al. The "yin and yang" of the adrenal and gonadal systems in elite military men. *Stress*. 2017;20(3):258–264. doi:10.1080/10253890.2017.1333594

16. Yu W, Zhao H, Zong X, et al. The effects of Radix Astragali water abstract on energy metabolism in rat yang-deficiency cold syndrome model through PPAR signaling pathway. *Evid Based Complement Alternat Med*. 2018;2018:9194362. doi:10.1155/2018/9194362

17. Wang Q, Ren XJ, Yao SL, et al. Clinical observation on the endocrinal and immune functions in subjects with yin-deficiency constitution. *Chin J Integr Med*. 2010;16(1):28–32. doi:10.1007/s11655-010-0028-9

18. Camitta BM. Pathogenesis and treatment of aplastic anemia. *Rinsho Ketsueki*. 1984;25:459–469.

19. Wu D, Ye B, Ji C, et al. Research status of clinical practice guidelines for aplastic anemia and amendment and thinking of TCM guidelines. *J Tradit Chin Med*. 2014;55(3):274–278. doi:10.1016/S0254-6272(14)60090-X

20. Wu D, Hu Z, Shen Y, et al. Approach of staging evidences of acute aplastic anemia. *China J Tradit Chin Med Pharm*. 2010;25:1099–1101.

21. Castro A, Jerez MJ, Gil C, et al. Cyclic nucleotide phosphodiesterases and their role in immunomodulatory responses: advances in the development of specific phosphodiesterase inhibitors. *Med Res Rev*. 2005;25(2):229–244. doi:10.1002/med.20020

22. Zeng Y, Li T, Zhang X, et al. Effects of Haima Duobian pill in a rat model of kidney yang deficiency syndrome. *Evid Based Complement Alternat Med*. 2021;2021:6696234. doi:10.1155/2021/6696234

23. Zhang L, Lang J, Jin L, et al. Effect of Hirsutella sinensis fungus on the hypothalamic-pituitary-adrenal axis in Lewis rats with kidney-yang deficiency syndrome. *Evid Based Complement Alternat Med*. 2020;2020:5952612.

24. Li GH, Wang QL, Liu FQ. [Experimental study of warming and recuperating kidney yang by you-gui-yin]. *Zhong Xi Yi Jie He Za Zhi*. 1990;10:547–548, 518. Chinese. PMID: 2176578

25. Chen J, Feng G. Effects of the warming box-moxibustion on the symptom and the ratio of serum cAMP/cGMP in the subjects with Yang Deficiency Constitution. *JJ Tradit Chin Med Univ Hunan*. 2016;36:64–67.

26. Ji B, Li YY, Yang WJ, et al. Jinkui Shenqi Pills Ameliorate asthma with "Kidney Yang Deficiency" by enhancing the function of the hypothalamic-pituitary-adrenal axis to regulate T helper 1/2 imbalance. *Evid Based Complement Alternat Med*. 2018;2018:7253240. doi:10.1155/2018/7253240

27. Messa E, Biale L, Castiglione A, et al. Erythroid response during iron chelation therapy in a cohort of patients affected by hematologic malignancies and aplastic anemia with transfusion requirement and iron overload: a FISM Italian multicenter retrospective study. *Leuk Lymphoma*. 2017;58(11):2752–2754. doi:10.1080/10428194.2017.1312385

28. Jin P, Wang J, Li X, et al. Evolution of iron burden in acquired aplastic anemia: a cohort study of more than 3-year follow-up. *Int J Hematol*. 2015;101(1):13–22. doi:10.1007/s12185-014-1708-6

29. Wu D, Luo Y, Liu W, et al. Correlation of iron overload and the manifestation of "Blood Stasis Syndrome" in chronic "Suilao" Patient: a single-center clinical study. *J Zhejiang Chin Med Univ*. 2016;40:265–269.

30. Wu D, Ye B, Luo Y, et al. Effects of deferoxamine on 42 patients with blood diseases and blood transfusion related iron overload. *J Pract Med*. 2013;29:142–144.

31. Schwartz AJ, Das NK, Ramakrishnan SK, et al. Hepatic hepcidin/intestinal HIF-2alpha axis maintains iron absorption during iron deficiency and overload. *J Clin Invest*. 2019;129(1):336–348. doi:10.1172/JCI122359

32. Wu D, Wen X, Liu W, et al. Comparison of the effects of deferasirox, deferoxamine, and combination of deferasirox and deferoxamine on an aplastic anemia mouse model complicated with iron overload. *Drug Des Devel Ther*. 2018;12:1081–1091. doi:10.2147/DDDT.S161086

33. Gattermann N. Iron overload in myelodysplastic syndromes (MDS). *Int J Hematol*. 2018;107(1):55–63. doi:10.1007/s12185-017-2367-1

34. Dijiong W, Xiaowen W, Linlong X, et al. Iron chelation effect of curcumin and baicalein on aplastic anemia mouse model with iron overload. *Iran J Basic Med Sci*. 2019;22:660–668.

35. Wu DJ, Ye BD, Hu ZP, et al. Bone marrow angiogenesis in patients presenting with differential Chinese medicine syndrome: correlation with the clinico-pathological features of aplastic anemia. *Chin J Integr Med*. 2013;19(12):905–912. doi:10.1007/s11655-013-1652-y
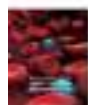
Final Results of the Prospective ADVATE® Immune Tolerance Induction Registry (PAIR) Study with Plasma- and Albumin-Free Recombinant Factor VIII

Amy D Shapiro, Alejandro Fernandez, Jerome Teitel, Jaco Botha & Kate Khair

Published online: 20 Nov 2021.

Submit your article to this journal

Article views: 115

View related articles

View Crossmark data

ORIGINAL RESEARCH

Final Results of the Prospective ADVATE® Immune Tolerance Induction Registry (PAIR) Study with Plasma- and Albumin-Free Recombinant Factor VIII

Amy D Shapiro [ID] [1]
Alejandro Fernandez [2]
Jerome Teitel [ID] [3]
Jaco Botha [2]
Kate Khair [ID] [4]

[1]Indiana Hemophilia & Thrombosis Center, Indianapolis, IN, USA; [2]Takeda Pharmaceutical International AG, Zürich, Switzerland; [3]St. Michael's Hospital, University of Toronto, Toronto, ON, Canada; [4]Centre for Outcomes and Experience Research in Children's Health Illness and Disability (ORCHID), Great Ormond Street Hospital, London, UK

Introduction: Neutralizing antibodies to coagulation factor VIII (FVIII) remain a major complication associated with FVIII replacement therapy.

Aim: To assess safety and efficacy of immune tolerance induction (ITI) therapy with ADVATE® (antihemophilic factor [recombinant] [rAHF]) in patients who participated in the Prospective ADVATE Immune Tolerance Induction Registry (PAIR) study.

Methods: The PAIR study was an international, multicenter, open-label, prospective, observational study in patients with hemophilia A and inhibitors, prescribed rAHF ITI therapy in clinical practice. The primary endpoint was adverse event (AE) reporting; the secondary endpoints included incidence of central venous access device-related complications and success rates of ITI therapy. Maintenance of immune tolerance was monitored for 12 months post-ITI therapy.

Results: Of 44 patients, 36 completed ITI therapy, including 31 completing the 12-month follow-up. Most patients received rAHF 90–130 IU/kg/day (59.1%) and a mean of 6.0 doses/ week; the median duration of rAHF ITI therapy during the PAIR study was 600 days. Overall, 284 AEs were reported; 56 AEs were serious, of which none were considered rAHF-related. Of 228 nonserious AEs, 14 (in six patients) were deemed rAHF-related: increase of FVIII inhibitors titer due to anamnestic response, nausea, catheter site pain, pyrexia, urticaria, upper respiratory tract infection, arthralgia, and hemarthrosis. None were severe or led to ITI discontinuation. Eighteen patients experienced ≥1 central venous access device-related complication, and 21 of 36 completers achieved a negative inhibitor titer. The Kaplan–Meier estimate of success for achievement of first negative titer at 18 months of ITI therapy was 68.3% (95% confidence interval 51.8–83.6%) among completers. Of patients with partial or complete success post-ITI, 87% (20/23) maintained immune tolerance at 12-month follow-up.

Conclusion: Data suggest that rAHF ITI therapy in the PAIR study was effective, with no unexpected safety signals reported.

Keywords: hemophilia A, immune tolerance, post-marketing product surveillance, therapeutics, adverse effects

Introduction

Development of neutralizing inhibitory antibodies (inhibitors) remains a major risk associated with coagulation factor VIII (FVIII) replacement therapy. Estimates suggest that 8–44% of patients with severe hemophilia A and 3–13% of patients with mild or moderate hemophilia A develop inhibitory antibodies,[1–4] with occurrence

Correspondence: Jaco Botha
Takeda Pharmaceutical International AG, Thurgauerstrasse 130, Glattpark-Opfikon, Zürich, 8152, Switzerland
Tel +41 79 961 8561
Email jaco.botha@takeda.com

dependent on genetic- and patient-related factors, such as mutations, severity of hemophilia, ethnicity, age at first treatment, and family history of hemophilia.[1,5–8] In the absence of intervention, inhibitors compromise the effectiveness of FVIII therapy and lead to increased morbidity and mortality due to poor bleed control.[9,10]

For patients with hemophilia A and inhibitors, induction of FVIII immune tolerance aims to restore FVIII pharmacokinetics (PK) and responsiveness to FVIII therapy, which is the standard of care.[11,12] The success rate of immune tolerance induction (ITI) using various dosing regimens has been reported to range between 60 and 80% in patients with severe hemophilia A and inhibitors to FVIII.[13–15] In a "good-risk" subgroup of ITI patients (inhibitor titer <10 Bethesda units (BU)/mL and historic peak titer of <200 BU/mL) and severe hemophilia A, data from a randomized, prospective study indicate that success rates were similar between low-dose (50 IU/kg FVIII, three times a week) and high-dose (200 IU/kg FVIII, daily) regimens; however, patients receiving low-dose ITI therapy took longer to achieve tolerance and had higher bleed rates compared with the high-dose group.[14] The optimal ITI treatment and dosage regimen is unknown, and information on factors that may predict ITI therapy success or failure emanates from ITI therapy studies.[16–21]

Previous reports in small patient cohorts suggest that ITI therapy with ADVATE® (antihemophilic factor [recombinant] [rAHF] ; Baxalta US Inc., a Takeda company, Lexington, MA, USA) is effective and confirm an overall established record of safety.[22,23] The international Prospective ADVATE Immune Tolerance Induction Registry (PAIR) study was designed to characterize the safety and tolerability of rAHF as a primary FVIII therapeutic agent in ITI therapy in patients with hemophilia A and inhibitors in clinical practices. The main objectives of the study were to assess the incidence of serious and nonserious adverse events (AEs) related to rAHF, incidence of central venous access device (CVAD)–related infections, and the success rates in patients with hemophilia A and inhibitors receiving ITI therapy with rAHF.

Here, we report the final analysis of the PAIR study, encompassing 7 years of prospective surveillance.

Materials and Methods
Study Design and Conduct
The PAIR study was an international, multicenter, open-label, prospective, observational study in patients with hemophilia A and inhibitors prescribed rAHF ITI therapy

in clinical practice. The PAIR study was conducted at 27 sites in 10 countries (Belgium, Canada, Denmark, France, Germany, Greece, Italy, Spain, United Kingdom, and United States). Recruitment began in July 2007, and the study was completed in July 2015.

Patients
Patients diagnosed at any age with hemophilia A of any severity, previously diagnosed with an inhibitor to FVIII (low-titer [<5 BU] and high-titer [≥5 BU]), and due to start rAHF ITI therapy as part of routine clinical practice were eligible for this study. The choice of rAHF ITI therapy was made before PAIR study participation. Previous failure of ITI therapy on rAHF and history of hypersensitivity reactions to FVIII were exclusion factors. The protocol for this study defined severe hemophilia A as a baseline level of FVIII ≤1% and non-severe hemophilia A as FVIII >1%, reflecting the classification of hemophilia severity at the time of the study. A FVIII baseline level of <1% has since been adopted for severe hemophilia A.[12]

Procedures
The rAHF ITI therapy dosing regimen and monitoring schedule were at the treating physician's discretion, based on individual patient's requirements. ITI therapy regimens described in the peer-reviewed literature[14,16,19–21,24,25] or similar to those described in the International ITI Protocol[26] were recommended.

The observation period for each patient was from the time ITI therapy was initiated through 33 months of ITI therapy, with an additional 12-month post-observation follow-up after ITI therapy was considered successful or partially successful. If ITI therapy (partial) success was not achieved within 33 months of ITI therapy initiation, participation in the study ended.

Study-specific patient diaries were used to record the following variables: AEs, ITI infusion administration details, treatment and cause of new bleeding episodes, response to bleed treatment, and concomitant medications taken (including vaccinations). Diary data were recorded by investigators on case report forms (CRFs).

Investigators monitored their patients for the occurrence of any AE. All AEs associated with rAHF during bleed management (not including bleed events during ITI therapy) were reported on the AE CRF. If an AE was serious, the event was recorded using separate serious AE (SAE) forms.

Laboratory testing for the quantitation of FVIII, FVIII inhibitor, and FVIII recovery and PK was performed at baseline, during ITI therapy, at the end of ITI therapy, and at a post-observation 12-month follow-up. All testing was conducted at the local laboratory of the participating site according to locally established procedures and methods. Laboratory tests were not mandated by the protocol, but investigators could utilize the procedure in the International Immune Tolerance Study[14] to determine the disappearance of the inhibitor.

The approach to the management of bleeding episodes (ie, breakthrough bleeding) was determined by the treating physician. Bleed events and hemostatic agents received at home or in the clinic were recorded on the Bleeding Episode Treatment Record CRF.

Study Endpoints and Outcome Measures

The primary endpoint was the incidence of AEs possibly related to rAHF during ITI therapy, categorized according to the *Medical Dictionary for Regulatory Activities* (MedDRA, version 17.0). The study comprised a retrospective and prospective period, defined respectively as the period of time during which rAHF ITI therapy was administered prior to, and following, PAIR study enrollment.

Secondary endpoints were the incidence of CVAD-related infections, general success rate of ITI therapy, and success rate in patients with severe hemophilia A (baseline level of FVIII ≤1%) and receiving rAHF as their primary ITI therapy, with no previous ITI therapy attempt and any titer inhibitor to FVIII.

Consensus recommendations of the 2006 International ITI Workshop define the response to ITI (ie, success) as a combination of a decrease and absence of inhibitors (usually less than 0.6 BU) and a normalization of the PK of FVIII.[13] Per the study protocol, precise ITI success was categorized as complete, partial, failure, unassessable, and relapse, based on inhibitor titers and PK assessments performed in the International Immune Tolerance Study.[14] However, due to the observational nature of this study and as PK assessments were not available in all centers, success in this analysis was defined only on inhibitor titer. General success was defined as achieving a FVIII inhibitor titer <0.6 BU or cut-off limit of inhibitor detection per local laboratory standard. Partial success occurred within 9–33 months of ITI treatment and was an inhibitor titer <5 BU (ie, conversion from high to low titer). Failure was ≥33 months of treatment with titer ≥5 BU, or 9–33 months of treatment with failure to

achieve a >20% reduction in titer. Relapse was a positive inhibitor titer during the 12-month follow-up after inhibitor disappearance.

Statistical Analyses

Safety data, including incidence of CVAD-related infections, were analyzed in the full analysis set (FAS), defined as all patients who enrolled and received at least one dose of rAHF ITI. The incidence of general success of rAHF ITI was analyzed using the completer analysis set (CAS), which comprised all patients who carried ITI therapy to completion, regardless of whether or not they were followed up for 12 months after completion. The nonparametric Kaplan–Meier product limit method was used to estimate time to ITI success (percentage of patients with inhibitor titers who achieved a negative FVIII inhibitor titer following ITI therapy) in the FAS, CAS, and per-protocol analysis set (PPS). The PPS was defined as the subset of patients in the CAS who completed ITI therapy with an assessable outcome (ie, all necessary inhibitor titer results were available in order to determine ITI therapy success or failure).

If the end date for a patient's current/initial ITI regimen was not collected, the end date was assumed to be 1 day before the first recorded start date of a change in therapy or date of therapy completion, whichever came first. If there was a discrepancy between unique titer measurements reported in multiple places on the CRF, the result found in the patient's titer log prevailed. To determine the end date of the initial ITI regimen, which was not collected on the CRF, if the initial ITI start date was not missing then the day before the start date of the current ITI regimen was used. If the initial ITI start date was missing, then the date of the end of the previous rAHF therapy was used.

Descriptive statistics were used to summarize continuous variables; frequencies were used for categorical variables. All data were analyzed with SAS® software package, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA).

Results

The results reported in in the main text of this article are based on the protocol definitions of severe hemophilia A (baseline level of FVIII ≤1%) and non-severe hemophilia A (baseline FVIII >1%). Results based on the updated definition of severe hemophilia (baseline level of FVIII <1%)[12] are provided in the supplementary materials where appropriate.

Patients

As of July 2015, all patients had completed study participation. Of the 44 patients who enrolled and were exposed to rAHF, 36 (81.8%) completed ITI therapy and 31 (70.5%) completed the 12-month follow-up (Figure 1).

The median age at enrollment was 23.5 months (Table 1). Most patients (86.4%, 38/44) had severe hemophilia A (baseline FVIII level ≤1%); 13.6% (6/44) had non-severe hemophilia A (baseline FVIII level >1%). At initiation of ITI therapy (ie, baseline), high-titer inhibitors were present in 15 of 44 patients (≥10 BU in seven patients), low titers were present in 23 of 44 patients, and titers were unreported in six patients with any hemophilia severity. The most common type of FVIII mutation was an intron 22 inversion (43.2%, 19/44). Most of the remaining patients reported either unknown (27.3%, 12/44) or other (22.7%, 10/44) mutations; missense and nonsense mutations were reported in 4.5% of patients (2/44) and 2.3% of patients (1/44), respectively.

Use of rAHF as ITI Therapy

The median duration of rAHF ITI therapy administration before PAIR study enrollment was 122 days (n = 34), and during the PAIR study it was 600 days (n = 44).

The most frequently initiated rAHF dose range (59.1%, 26/44 patients) was 90–130 IU/kg/day (Table 2), which was initiated in patients from Germany (n = 1), Denmark (n = 1), Spain (n = 1), Greece (n = 1), France (n = 3/5), Italy (n = 3/5), the United Kingdom (n = 6/9), and United States (n = 9/17). In Belgium, one patient was initiated on 90–130 IU/kg/day and one patient on ≥200 IU/kg/day rAHF. In Canada, two of two patients were initiated on <90 IU/kg/day rAHF. The mean (SD) dose per infusion of rAHF ITI therapy was 113.4 (66.6) IU/kg; patients with severe hemophilia A had a higher mean (SD) dose per infusion than those with non-severe hemophilia A (119.3 [69.4] versus 77.3 [26.9] IU/kg, respectively). Results using the current classification of severe hemophilia are provided in Supplementary Table 2. The total mean (median, range) number of rAHF doses per week was 6.0 (5.2, 0.6–14.0).

Following the initial dose, 47.7% of all patients (21/44) and 47.4% of patients with severe hemophilia (18/38) experienced at least one dose increase during the study. Overall, the percentage of patients receiving dose increases during the study was similar for those with low- (<5 BU, 53.3%) and high-inhibitor titers (≥5 BU, 44.8%) at baseline. Information on whether inhibitors
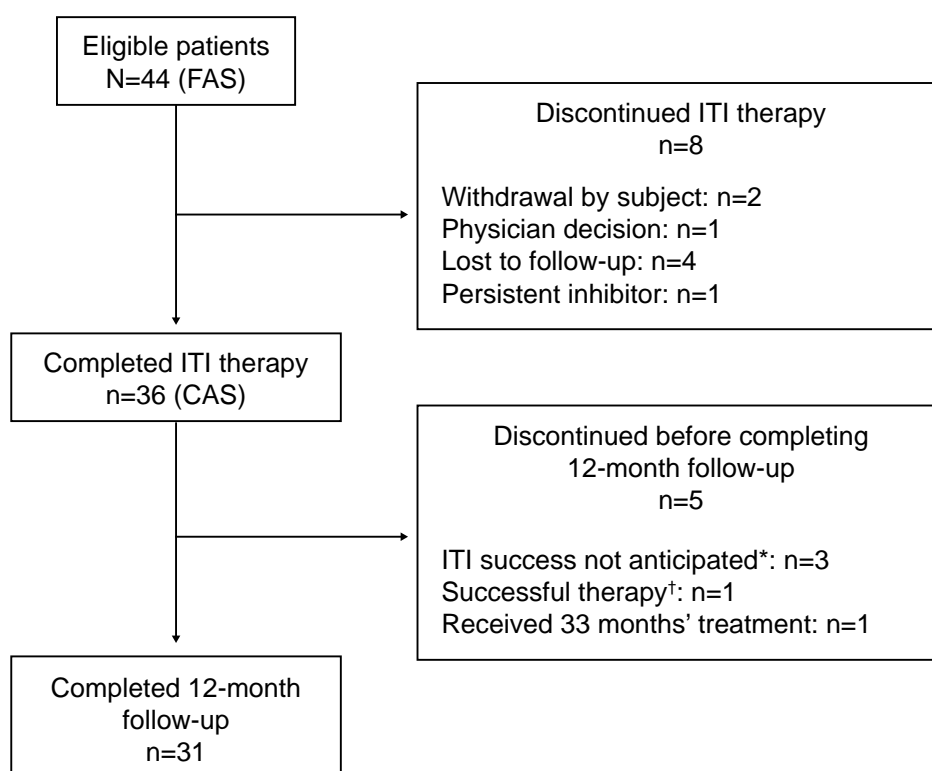

Figure 1 Flow of patients through the PAIR study. *Within 33 months of treatment. †As defined by the protocol.
Abbreviations: CAS, completer analysis set; FAS, full analysis set; ITI, immune tolerance induction; PAIR, Prospective ADVATE Immune Tolerance Induction Registry.

Table 1 Baseline Characteristics of Patients in the PAIR Study

Characteristic	FVIII ≤1% (n = 38)	All Patients (N = 44)
Age at ITI therapy start, months, median (range)	19.0 (1.0–320.0)	23.5 (1.0–676)
Race, n (%)		
White	24 (63.2)	29 (65.9)
Asian	1 (2.6)	1 (2.3)
Black	4 (10.5)	4 (9.1)
Hispanic	3 (7.9)	3 (6.8)
Other/missing	6 (15.8)	7 (15.9)
Family history of inhibitor, n (%)		
Yes	10 (26.3)	11 (25.0)
No	25 (65.8)	30 (68.2)
Unknown	3 (7.9)	3 (6.8)
Titer, BU, median (mean, range)		
At diagnosis	4.9 (19.1, 0.7–173.0)	4.9 (21.2, 0.5–173.0)
Peak before ITI therapy[a]	12.5 (44.0, 0.7–225.2)	12.5 (43.0, 0.7–225.2)
Immediately before ITI therapy	4.0 (8.5, 0–60.6)	3.95 (8.0, 0–60.6)

Notes: Results by current classification of severe hemophilia (FVIII <1%) are provided in Supplementary Table 1. [a]If peak titer before ITI therapy was not reported, the maximum of all titer measurements made before ITI therapy was used.
Abbreviations: BU, Bethesda unit; FVIII, coagulation factor VIII; ITI, immune tolerance induction; PAIR, Prospective ADVATE Immune Tolerance Induction Registry.

were low- or high-responding was not available in this study.

Safety

A total of 284 AEs were reported in 44 patients before (retrospective period) and after (prospective period) PAIR study enrollment; 56 AEs (19.7%) were serious and not considered related to rAHF ITI therapy (Table 3). Before study enrollment, 31 AEs were reported in 10 of 34 patients, of which eight were SAEs reported in five patients. During the study, 253 AEs were reported in 32 of 44 patients, of which 48 were SAEs reported in 21 patients. A total of 14 nonserious AEs considered related to rAHF ITI therapy occurred in six patients (13.6%), and none of these AEs were severe. These

were pyrexia (n = 1), urticaria (n = 1), nausea (n = 2), catheter site pain (n = 1), upper respiratory tract infection (n = 1), arthralgia (n = 2), hemarthrosis (n = 2), and increase of FVIII inhibitors titer due to anamnestic response (n = 4).

Of 44 patients, 18 (40.9%) had a CVAD placed before rAHF ITI therapy initiation, 10 patients (22.7%) had their first CVAD placed during ITI, six patients (13.6%) had their CVAD replaced during ITI, and 18 patients (40.9%) had a CVAD placed due to complications during ITI therapy. In total, 18 patients experienced at least one CVAD-related complication. The most frequently reported CVAD complications included 18 episodes of local site line infection in five patients (11.0%), nine episodes of line insertion site bleed in three patients (6.8%), and eight

Table 2 ITI Therapy Dosage Regimens Utilized in the PAIR Study

Dose in IU/kg/day, n (%)	FVIII ≤1% (n = 38)		All Patients (N = 44)	
	High Titer[a,b] (n = 26)	Low Titer[a,c] (n = 12)	High Titer[a,b] (n = 29)	Low Titer[a,c] (n = 15)
≥200	4 (15.4)	0	4 (13.8)	0
~131–199	2 (7.7)	0	3 (10.3)	0
90–130	16 (61.5)	6 (50.0)	18 (62.1)	8 (53.3)
<90	4 (15.4)	6 (50.0)	4 (13.8)	7 (46.7)

Notes: Results by current classification of severe hemophilia (FVIII <1%) are provided in Supplementary Table 3. [a]If peak titer before ITI therapy was not reported, the maximum of all titer measurements made before ITI therapy was used. [b]≥5 BU at baseline. [c]<5 BU at baseline.
Abbreviations: BU, Bethesda unit; FVIII, coagulation factor VIII; ITI, immune tolerance induction; IU, international unit; PAIR, Prospective ADVATE Immune Tolerance Induction Registry.

Table 3 Summary of AEs in the PAIR Study (FAS)

AEs	n (% of Total AEs)
All AEs	284 (100.0)
SAEs	
Total	56 (19.7)
Considered treatment-related	0
Nonserious AEs	
Total	228 (80.3)
Considered unrelated to treatment	214 (75.4)
Considered treatment-related	14 (4.9)
Discontinuations	n (% of Total Patients) (N = 44)
Patient withdrew	2 (4.5)
Physician decision	1 (2.3)
Lost to follow-up	4 (9.1)
Other	2 (4.5)

Abbreviations: AE, adverse events; FAS, full analysis set; PAIR, Prospective ADVATE Immune Tolerance Induction Registry; SAE, serious adverse event.

episodes of systemic/septic line infection in five patients (11.0%). Other CVAD-associated complications occurring in more than one patient were line insertions (five occurrences in four patients) and line removal (two occurrences in two patients).

Efficacy of ITI Therapy

Of the 36 patients who completed ITI treatment (ie, those in the CAS), 21 (58.3%) achieved a negative titer during the study, two (5.6%) converted from a high- to a low-titer inhibitor, and eight (22.2%) experienced treatment failure (Table 4). Six of 13 patients (46.2%) in the CAS with severe hemophilia (baseline level of FVIII ≤1%) and high titer at baseline achieved a negative titer. Of 23 patients with partial or complete success after ITI therapy, 20 (87%) maintained immune tolerance at 12-month follow-up.

The estimated cumulative success rate of rAHF ITI therapy at approximately 18 months (516 days) was 72.4% (95% confidence interval [CI] 55.5–87.0%) in the PPS (n = 31) and 68.3% (95% CI 51.8–83.6%) in the CAS (n = 36) (Figure 2). These rates were in patients with high- and low-titer inhibitors (PPS patients: 20 with high-titer and 11 with low-titer inhibitors; CAS patients: 23 with high-titer and 13 with low-titer inhibitors), and success was defined as the percentage of patients with inhibitors who achieved a negative FVIII inhibitor titer following ITI therapy. The median times to first and second negative titers for all patients were 4.3 months and 5.8 months, respectively. For those patients with a high-titer inhibitor at baseline, the median times to first and second negative titer test were 4.8 and 6.7 months, respectively. Specifically in children (<18 years of age), the estimated cumulative success rate of rAHF ITI therapy at approximately 18 months was 69.4% (95% CI 51.5–85.6%) in the PPS and 65.4% (95% CI 48.1–82.1%) in the CAS.

Maximum inhibitor titers ≥5 BU, ≥10 BU, and ≥100 BU were detected in 25, 22, and 17 patients, respectively, during ITI therapy. Of the patients who achieved a negative inhibitor titer (61.5%, 16/26), three patients (18.8%) reported a relapse at their 12-month follow-up appointment. The first of these patients was 56 years of age at ITI therapy initiation and had a peak inhibitor titer before ITI therapy of 1.6 BU and an inhibitor titer of 1.1 BU at the 12-month follow-up. The second patient was 3 years of age at ITI therapy initiation, with a peak inhibitor titer before ITI therapy of 196.0 BU; although relapse was indicated in the report, the most recent titer at 12 months was 0 BU. The third patient with relapse was 4 months old at ITI initiation and had a peak inhibitor titer of 4.3 BU before ITI therapy and an inhibitor titer of 0.5 BU at follow-up (equal to the local laboratory cut-off for inhibitor detection). The first patient had been receiving a follow-up prophylaxis regimen of 35 IU/kg every other day; the second patient had continued ITI therapy (50 IU/kg every day). The third patient had received 71 IU/kg twice a week and 89 IU/kg once a week.

Table 4 Outcomes in Patients Who Completed rAHF ITI Therapy (CAS)

Completers, n (%)	Severe Hemophilia (FVIII ≤1%) (n = 31)	Non-Severe Hemophilia (FVIII > 1%) (n = 5)	Total (n = 36)
General success	18 (58.1)	3 (60.0)	21 (58.3)
Partial success	2 (6.5)	0 (0)	2 (5.6)
Failure	7 (22.6)	1 (20.0)	8 (22.2)

Note: Results by current classification of severe hemophilia (FVIII <1%) are provided in Supplementary Table 4.
Abbreviations: CAS, completer analysis set; FVIII, coagulation factor VIII; ITI, immune tolerance induction; NA, not available; PK, pharmacokinetic; rAHF, antihemophilic factor (recombinant).
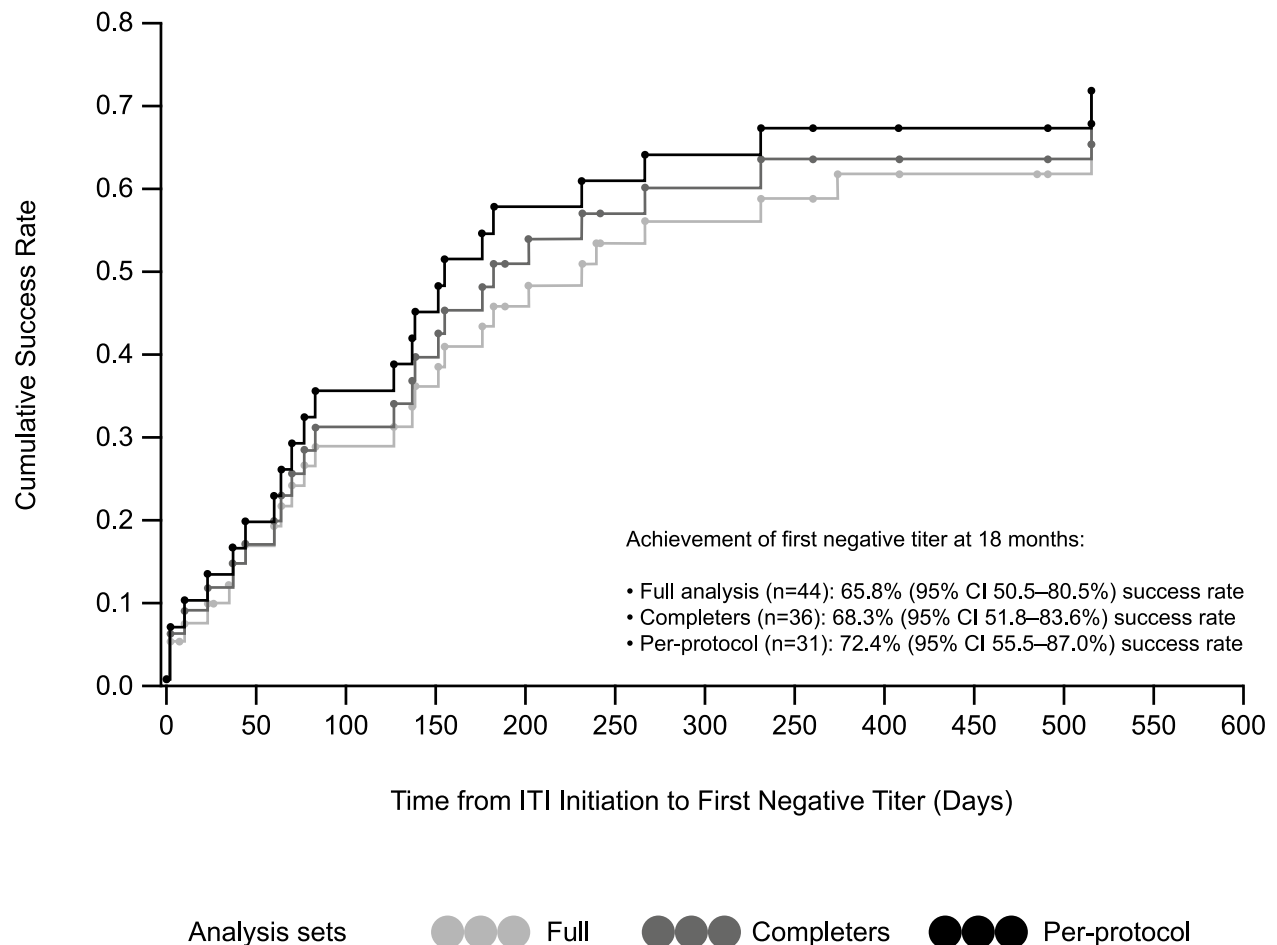
Figure 2 Kaplan–Meier success curves for achievement of first negative titer.
Abbreviations: CI, confidence interval; ITI, immune tolerance induction.

The median time from inhibitor diagnosis to ITI therapy initiation for all patients with available data (n = 42) was 2.5 months; the median time for patients with severe hemophilia (baseline FVIII level ≤1%) (n = 36) was 2.2 months, and for patients with FVIII level >1% (n = 6) it was 3.7 months.

The median time from inhibitor diagnosis to ITI therapy initiation was 0.5 months for patients with an ITI therapy outcome of general success (n = 20), 34.0 months for patients with partial success (n = 2, high to low titer conversion), and 12.48 months for patients with ITI therapy failure (n = 7).

Treatment of Bleeding Episodes

A total of 339 bleeding episodes occurred in 33 of 36 patients in the CAS on rAHF during ITI therapy. Most bleeding episodes (n = 308) occurred in 29 of 31 patients with severe hemophilia (FVIII ≤ 1%), and the remaining 31 bleeding episodes occurred in four of five patients with non-severe hemophilia (FVIII >1%). The results using the current classification of severe hemophilia are provided in Supplementary Table 5. The most frequent cause of bleeding episodes was trauma-related (156/339), whereas 94 of 339 bleeding episodes were spontaneous. The etiologies of a further 89 bleeding episodes were either unknown or missing.

Most bleeding episodes that occurred during ITI therapy were treated exclusively with recombinant activated factor VII (rFVIIa) products (n = 177), whereas three were exclusively treated with activated prothrombin complex concentrates (aPCC) and 37 with rAHF. For 84 bleeding episodes, treatment began with rAHF and alternated with rFVIIa products, and for five bleeding episodes, treatment began with rFVIIa products and alternated with rAHF.

Discussion

The PAIR study was an international, non-interventional prospective study of rAHF as ITI therapy in a real-world setting. The observational, non-interventional nature of the study enabled the assessment of safety, tolerability and efficacy of rAHF ITI therapy under routine conditions in common clinical practice. In this setting, patients had independently chosen to receive ITI therapy with rAHF and were treated by the investigators according to their clinical judgement and experience. The PAIR study collected and analyzed this real-life experience and summarized the most salient findings. Overall, the findings from the PAIR study add to previous experiences with rAHF ITI therapy in other clinical studies utilizing different designs and treatment regimens.[14,22,23,27]

The safety results were consistent with the known safety profile of rAHF, and there were no unknown or unexpected safety signals for treatment of bleeding episodes or for ITI therapy.[28–30] Most complications that occurred in the PAIR study related to CVAD use. These are known issues associated with the use of these devices, and education on best practices may help to mitigate them. Of note, there were no reports of CVAD-related thromboses, which are a relatively common complication of CVAD insertion.[31]

The present investigation supports the efficacy conclusion of previous ITI therapy studies.[22,23,32] rAHF was effective in a variety of ITI dosing regimens currently used in clinical practice. The estimated PPS and CAS cumulative success rates at 18 months were 72.4% and 68.3%, respectively, in patients who had both high- and low-titer inhibitors at baseline (information on whether inhibitors were low- or high-responding was not available). In the International Immune Tolerance Study, patients with severe hemophilia (defined as FVIII <1%) who were defined as good risk and had high-titer and high-responding inhibitors achieved a success rate of 69.7% (n = 46/66).[14] This compares with 46.2% of patients with severe hemophilia (baseline FVIII ≤1%) and high titer in the present study. However, this percentage is based on a sample of 13 patients. In addition, the apparently lower success rate may be a consequence of how severe hemophilia was defined in our study, as patients with moderate hemophilia generally have a lower success rate versus those with severe disease.

Separate retrospective analyses from two US centers suggested that an interval of <1 month from inhibitor detection to ITI therapy start was associated with improved outcome.[15] In the present study, although the numbers were small, 20 patients with general success had an inhibitor-diagnosis-to-ITI-therapy-initiation interval of 0.48 months versus 12.48 months for patients with ITI therapy failure (n = 7) and 33.95 months for patients with high to low titer conversion (n = 2). The overall rate of relapse in the present study (13%) is in line with that reported by previous ITI therapy studies (2.3% to 29.7%).[33–35]

The PAIR study was conducted in eight European countries, as well as the United States and Canada, without the limitation of eligibility criteria used in interventional clinical trials. The number of patients enrolled in all sites and followed up was adequate for a reliable assessment of ITI therapy across multiple geographies, though country-specific comparisons were not possible. The PAIR study provides important information on the administered doses of rAHF for ITI therapy in real-world conditions and may indicate patterns of dosing based on the state of disease. Due to the non-interventional design of the study, it is possible that not all data available were entered into the database. Of note, more than half of the patients had missing bleeding episode data. In addition, the subgroup data may have been skewed by the different sample sizes of patients with severe or moderately severe hemophilia and the fact that the patient age range was wide. Another potential limitation is that the study used the protocol definition of ≤1% for severe hemophilia, which differs from the current definition of <1%.[12] However, the results using both definitions are similar and do not change the conclusions in this report. Finally, the number of patients in the study was too low for conclusions about differences between countries.

Conclusions

The observational PAIR study provides important real-world data on a widely used recombinant factor VIII concentrate. The results of the PAIR study are consistent with rAHF as an effective therapy for ITI in patients with hemophilia A and inhibitors. No SAEs related to treatment were reported. Future prospective studies may further define factors associated with ITI therapy success and failure in patients with hemophilia A.

Abbreviations

AE, adverse event; BU, Bethesda unit; CAS, completer analysis set; CRF, case report form; CVAD, central venous access device; FAS, full analysis set; FVIII, coagulation

factor VIII; ITI, immune tolerance induction; IU, international unit; PAIR, Prospective ADVATE Immune Tolerance Induction Registry; PK, pharmacokinetic; PPS, per-protocol analysis set; rAHF, antihemophilic factor (recombinant); SAE, serious adverse event.

Study Registration

The PASS-INT-004 registry study titled "Prospective ADVATE immune tolerance induction registry (PAIR)" was a non-interventional post-authorization study initiated in 2007. At the time of initiation, there was no legal requirement to register such types of studies in the clinicaltrials.gov, EudraCT, and EUPAS registers.

Data Sharing Statement

The datasets, including the redacted study protocol, redacted statistical analysis plan, and individual participant data supporting the results reported in this article, will be made available within 3 months from initial request, to researchers who provide a methodologically sound proposal. The data will be provided after their de-identification, in compliance with applicable privacy laws, data protection, and requirements for consent and anonymization. Data requests should follow the process outlined in the Data Sharing section on:

www.takeda.com/what-we-do/research-and-development/takeda-clinical-trial-transparency/.

Ethics Approval and Informed Consent

The PAIR study conforms to the Declaration of Helsinki aspects of ethical considerations (ethics committee/institutional review board approval and participants' informed consent). Before enrollment, the study protocol and informed consent form were reviewed and approved by the independent ethics committees/institutional review boards of all participating sites in accordance with local requirements (see Supplementary Table 6). Written consent before enrollment was obtained from all adult patients and caregivers of patients <18 years of age.

Author Contributions

Amy D. Shapiro, Kate Khair, and Jerome Teitel were principal investigators of the PAIR study and recruited and treated patients; they also contributed to the study concept and design. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

Amy D. Shapiro received grant/research support from Novo Nordisk, Bayer Healthcare, Bioverativ, Genentech, Prometic Life Sciences, Kedrion, Sangamo Biosciences, Bio Products Laboratory, Octapharma, OPKO, Daiichi Sankyo, Chugai Pharmaceutical, Glover Blood Therapeutics, and Shire (a Takeda company). Kate Khair received grant/research support from Baxalta/Shire (a Takeda company), CSL Behring, Novo Nordisk, Pfizer, Sobi, and uniQure; and is on the speaker bureau of Bayer, Novo Nordisk, Octapharma, Pfizer, Roche, Shire (a Takeda company), and Sobi. Jerome Teitel has served as a consultant and participated on advisory boards for Baxalta US Inc., Bayer, Octapharma, Biogen, CSL Behring, Roche, and Takeda. He is also the data monitoring committee for BioMarin. Jaco Botha is an employee of Takeda Pharmaceuticals International AG and Takeda stock owner. Alejandro Fernandez was an employee of Takeda Pharmaceuticals International AG at the time of this work. The authors report no other conflicts of interest in this work.

References

1. Kreuz W, Ettingshausen CE, Zyschka A, et al. Inhibitor development in previously untreated patients with hemophilia A: a prospective long-term follow-up comparing plasma-derived and recombinant products. *Semin Thromb Hemost*. 2002;28(3):285–290. doi:10.1055/s-2002-32664

2. Gouw SC, van den Berg HM, Fischer K, et al. Intensity of factor VIII treatment and inhibitor development in children with severe hemophilia A: the RODIN study. *Blood*. 2013;121(20):4046–4055. doi:10.1182/blood-2012-09-457036

3. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia*. 2003;9(4):418–435. doi:10.1046/j.1365-2516.2003.00780.x

4. Hay CR. Factor VIII inhibitors in mild and moderate-severity haemophilia A. *Haemophilia*. 1998;4(4):558–563. doi:10.1046/j.1365-2516.1998.440558.x

5. Scharrer I, Bray GL, Neutzling O. Incidence of inhibitors in haemophilia A patients – a review of recent studies of recombinant and plasma-derived factor VIII concentrates. *Haemophilia*. 1999;5(3):145–154. doi:10.1046/j.1365-2516.1999.00300.x

6. Lorenzo JI, López A, Altisent C, Aznar JA. Incidence of factor VIII inhibitors in severe haemophilia: the importance of patient age. *Br J Haematol*. 2001;113(3):600–603. doi:10.1046/j.1365-2141.2001.02828.x

7. Astermark J. Why do inhibitors develop? Principles of and factors influencing the risk for inhibitor development in haemophilia. *Haemophilia*. 2006;12(s3):52–60. doi:10.1111/j.1365-2516.2006.01261.x

8. Astermark J, Altisent C, Batorova A, et al. Non-genetic risk factors and the development of inhibitors in haemophilia: a comprehensive review and consensus report. *Haemophilia*. 2010;16(5):747–766. doi:10.1111/j.1365-2516.2010.02231.x

9. Scalone L, Mantovani LG, Mannucci PM, Gringeri A; The COCIS Study Investigators. Quality of life is associated to the orthopaedic status in haemophilic patients with inhibitors. *Haemophilia*. 2006;12(2):154–162. doi:10.1111/j.1365-2516.2006.01204.x

10. Morfini M, Haya S, Tagariello G, et al. European study on orthopaedic status of haemophilia patients with inhibitors. *Haemophilia*. 2007;13(5):606–612. doi:10.1111/j.1365-2516.2007.01518.x

11. Valentino LA, Kempton CL, Kruse-Jarres R, et al. US guidelines for immune tolerance induction in patients with haemophilia A and inhibitors. *Haemophilia*. 2015;21(5):559–567. doi:10.1111/hae.12730

12. Srivastava A, Santagostino E, Dougall A, et al. WFH guidelines for the management of hemophilia, 3rd edition. *Haemophilia*. 2020;26(Suppl 6):1–158. doi:10.1111/hae.14046

13. DiMichele DM, Hoots WK, Pipe SW, Rivard GE, Santagostino E. International workshop on immune tolerance induction: consensus recommendations. *Haemophilia*. 2007;13(s1):1–22. doi:10.1111/j.1365-2516.2007.01497.x

14. Hay CRM, DiMichele DM; International Immune Tolerance Study. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood*. 2012;119(6):1335–1344. doi:10.1182/blood-2011-08-369132

15. Nakar C, Manco-Johnson MJ, Lail A, et al. Prompt immune tolerance induction at inhibitor diagnosis regardless of titre may increase overall success in haemophilia A complicated by inhibitors: experience of two U.S. centres. *Haemophilia*. 2015;21(3):365–373. doi:10.1111/hae.12608

16. DiMichele DM, Kroner BL; The North American Immune Tolerance Study Group. The North American Immune Tolerance Registry: practices, outcomes, outcome predictors. *Thromb Haemost*. 2002;87(1):52–57. doi:10.1055/s-0037-1612943

17. Freiburghaus C, Berntorp E, Ekman M, Gunnarsson M, Kjellberg B, Nilsson IM. Tolerance induction using the Malmö treatment model 1982–1995. *Haemophilia*. 1999;5(1):32–39. doi:10.1046/j.1365-2516.1999.00195.x

18. Lenk H; ITT Study Group. The German registry of immune tolerance treatment in hemophilia—1999 update. *Haematologica*. 2000;85(10 Suppl):45–47.

19. Mariani G, Kroner B. International immune tolerance registry, 1997 update. *Vox Sang*. 1999;77(Suppl 1):25–27. doi:10.1159/000056710

20. Mauser-Bunschoten EP, Nieuwenhuis HK, Roosendaal G, van den Berg HM. Low-dose immune tolerance induction in hemophilia A patients with inhibitors. *Blood*. 1995;86(3):983–988. doi:10.1182/blood.V86.3.983.983

21. Oldenburg J, Schwaab R, Brackmann HH. Induction of immune tolerance in haemophilia A inhibitor patients by the 'Bonn Protocol': predictive parameter for therapy duration and outcome. *Vox Sang*. 1999;77(Suppl 1):49–54. doi:10.1159/000056717

22. Auerswald G, Thompson AA, Recht M, et al. Experience of Advate rAHF-PFM in previously untreated patients and minimally treated patients with haemophilia A. *Thromb Haemost*. 2012;107(6):1072–1082. doi:10.1160/TH11-09-0642

23. Valentino LA, Recht M, Dipaola J, et al. Experience with a third generation recombinant factor VIII concentrate (Advate®) for immune tolerance induction in patients with haemophilia A. *Haemophilia*. 2009;15(3):718–726. doi:10.1111/j.1365-2516.2008.01960.x

24. Rocino A, de Biasi R. Successful immune tolerance treatment with monoclonal or recombinant factor VIII concentrates in high responding inhibitor patients. *Vox Sang*. 1999;77(Suppl 1):65–69. doi:10.1159/000056720

25. Scheibel E, Ingerslev J, Dalsgaard-Nielsen J, Stenbjerg S, Knudsen JB. Continuous high-dose factor VIII for the induction of immune tolerance in haemophilia A patients with high responder state: a description of eleven patients treated. *Thromb Haemost*. 1987;58(4):1049–1052. doi:10.1055/s-0038-1646054

26. Dimichele DM, Hay CR. The international immune tolerance study: a multicenter prospective randomized trial in progress. *J Thromb Haemost*. 2006;4(10):2271–2273. doi:10.1111/j.1538-7836.2006.02127.x

27. Yoshioka A, Ishii E, Ueno T, et al. The international immune tolerance induction study and its follow-up study on Japanese hemophilia A patients with inhibitors. *Int J Hematol*. 2015;101(4):362–368. doi:10.1007/s12185-015-1734-z

28. Advate [antihemophilic factor (recombinant)] lyophilized powder for reconstitution, for intravenous injection [prescribing information]. Lexington, MA: Baxalta US Inc.; 2018. Available from: https://www.shirecontent.com/PI/PDFs/ADVATE_USA_ENG.pdf. Accessed November 15, 2021.

29. Iorio A, Marcucci M, Cheng J, et al. Patient data meta-analysis of Post-Authorization Safety Surveillance (PASS) studies of haemophilia A patients treated with rAHF-PFM. *Haemophilia*. 2014;20(6):777–783. doi:10.1111/hae.12480

30. Romanov V, Marcucci M, Cheng J, Thabane L, Iorio A. Evaluation of safety and effectiveness of factor VIII treatment in haemophilia A patients with low titre inhibitors or a personal history of inhibitor. Patient data meta-analysis of rAFH-PFM Post-Authorization Safety Studies. *Thromb Haemost*. 2015;114(1):56–64. doi:10.1160/TH14-10-0882

31. Wall C, Moore J, Thachil J. Catheter-related thrombosis: a practical approach. *J Intensive Care Soc*. 2016;17(2):160–167. doi:10.1177/1751143715618683

32. Spotts G, Abbuehl BE, Luu HD, et al. Safety and efficacy profile of rAHF-PFM for immune tolerance induction as assessed in 3 clinical trials (PUP ITI, PRE-PAIR, and PAIR). *Blood*. 2010;116(21):3670. doi:10.1182/blood.V116.21.3670.3670

33. Antun A, Monahan PE, Manco-Johnson MJ, et al. Inhibitor recurrence after immune tolerance induction: a multicenter retrospective cohort study. *J Thromb Haemost*. 2015;13(11):1980–1988. doi:10.1111/jth.13143

34. Coppola A, Margaglione M, Santagostino E, et al. Factor VIII gene (F8) mutations as predictors of outcome in immune tolerance induction of hemophilia A patients with high-responding inhibitors. *J Thromb Haemost*. 2009;7(11):1809–1815. doi:10.1111/j.1538-7836.2009.03615.x

35. Mariani G, Kroner B; Immune Tolerance Study G. Immune tolerance in hemophilia with factor VIII inhibitors: predictors of success. *Haematologica*. 2001;86(11):1186–1193.
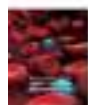
Taylor & Francis

The Impact of WhatsApp on the Blood Donation Process in Saudi Arabia

Qarmoosha Rasheed Al-Hajri, Asma Alfayez, Demah Alsalman, Fahad Alanezi, Hala Alhodaib, Saja A Al-Rayes, Afnan Aljaffary, Bashair AlThani, Heba AlNujaidi, Atheer K Al-Saif, Razaz Attar, Duaa Aljabri, Sama'a Al-Mubarak, Mona M Al-Juwair, Sumaiah Alrawiai & Turki M Alanzi

ORIGINAL RESEARCH

The Impact of WhatsApp on the Blood Donation Process in Saudi Arabia

Qarmoosha Rasheed Al-Hajri[1]
Asma Alfayez [1]
Demah Alsalman [1]
Fahad Alanezi [2]
Hala Alhodaib [3]
Saja A Al-Rayes [1]
Afnan Aljaffary [1]
Bashair AlThani [4]
Heba AlNujaidi [5]
Atheer K Al-Saif[5]
Razaz Attar[6]
Duaa Aljabri [1]
Sama'a Al-Mubarak [1]
Mona M Al-Juwair[1]
Sumaiah Alrawiai [1]
Turki M Alanzi [1]

[1]Health Information Management and Technology Department, College of Public Health, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [2]Community College, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [3]Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyad, Saudi Arabia; [4]College of Business Administration, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [5]College of Public Health, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [6]Princess Nourah Bint Abdul Rahman University, Riyad, Saudi Arabia

Correspondence: Turki M Alanzi
Health Information Management and Technology Department, College of Public Health, Imam Abdulrahman Bin Faisal University, 234, King Faisal Road, Dammam, 31441, Saudi Arabia
Tel +966 133331211
Email talanzi@iau.edu.sa

Background: WhatsApp was the most popular messenger app used in Saudi Arabia with 71% of the total population using it in 2020. WhatsApp is increasingly being used as a tool for mobile health (m-health) interventions; however, concerning blood donation, there is a lack of research studies on the topic.

Objective: This study aims to measure the general awareness levels of the blood donation process and assess blood donation history, the motivators and inhibitors to donating blood, and to assess the impact of WhatsApp on the blood donation process in Saudi Arabia.

Methods: In this research study, a descriptive quantitative cross-sectional analysis was adopted. A questionnaire survey was designed using Google Forms and distributed online through social media applications to collect data. All citizens aged above 18 years of age were eligible to participate in the survey. There were a total of 150 participants in the study.

Results: More than 90% of participants were aware of their blood group, blood donation requirements, and causes to be deferred from the donation. Furthermore, 27% of participants donated blood because their relatives or friends needed blood, 26% donated due to human solidarity, 18% did not donate blood because of their health condition and 14% did not because of the fear of needles. About 33% of participants relied on WhatsApp to search for blood donors, and all the requests were fulfilled with blood donors. In addition, 94% of participants strongly believed that the WhatsApp application had a significant role in bridging the gap for blood banks' need for blood donors.

Conclusion: Social media applications such as WhatsApp can bridge the gap between blood banks, blood donors and the patients in need of blood in Saudi Arabia, where there is a shortage of blood donors.

Keywords: WhatsApp, blood donation, Saudi Arabia

Introduction

Social media plays a significant role in rapidly transforming the healthcare needs in Saudi society. The global rise in social media amounts to 9.2% annually. WhatsApp is the most popular messenger app in Saudi Arabia with 71% of the total population using it in 2020.[1] WhatsApp is increasingly being used as a tool for mobile health (m-health) interventions; however, there is a lack of research concerning blood donation and the use of messenger apps.[2,3]

Globally, 120 million people donate blood every year. However, this is insufficient to reach the global need because many patients are in need of a transfusion. Regular blood donations are required to ensure there is always a supply for those in need.[4–6] According to King Faisal Specialist Hospital & Research Centre (KFSHRC), 205 donors were needed daily: 100 donors with O blood group, 50

donors with A blood group, 50 donors with B blood group, and five donors with AB blood group in Saudi Arabia.[7] In cooperation with the Ministry of Health, KFSHRC highlights the Wateen application as an initiative that seeks to reduce the communication gap between blood banks and donors so that donating blood becomes easier.[7] There are approximately 800,000 donors registered on the Wateen application in Saudi Arabia,[8] reflecting the importance of integrating mobile applications and social media in the blood donation process. The need for an effective and efficient blood donation and supply process was noticed during the COVID-19 pandemic when the blood banks were alerted due to a decrease in blood supply with a high demand for blood transfusion for patients. Many blood banks used the WhatsApp platform for blood donor mobilization campaigns to motivate donors to donate blood and meet the patients' demand for transfusion,[9] and for plasma donation, which proved to be effective for generating immunity among the COVID-19 patients.[10–12]

A recent study[13] identified four major potential challenges with respect to blood donation during the COVID-19 pandemic: blood/component shortage, donor/staff safety, consumable supply/logistics and catering to the convalescent plasma need. In addition, frequent lockdowns and curfews affected the blood collection process during the pandemic. However, various social media applications were effective in acquiring and retaining blood donors in these conditions. Social media applications such as Twitter were effective in raising blood donation requests and dissemination, reducing the gap between blood donors and the people in need.[14,15] Among the various social media applications, WhatsApp was effective in managing the blood donation process.[16] However, a similar study in Brazil identified there was no significant impact of WhatsApp in increasing the number of blood donors and the retention rates of existing donors. Further, the difference in the blood donors' return rates were identified in the study, suggesting specific interventions should be created according to the different stages of the donors' careers.[2] Similarly, another study highlighted the need for drawing, poetry, or singing contests about donating to keep active communication with donors.[17] However, blood transfusion in Saudi Arabia is essentially a hospital-based process, with the primary sources being from a combination of a growing number of voluntary non-remunerated donations and family members.[18] Although there are other sources, such as regional blood banks and the Saudi Red

Crescent, there is a lack of communication between the blood banks, blood donors, hospitals and people in need.[19] Due to the growing demand for blood donors, the WhatsApp platform being the most useful app among Saudi people, and the lack of research related to this area, this study aims to measure the impact of WhatsApp on the blood donation process in Saudi Arabia.

Methods
Study Settings and Participants
In this research study, a descriptive quantitative cross-sectional analysis method was adopted to investigate the general awareness about the blood donation process, motivators and inhibitors of donating blood, and to measure the impact of WhatsApp on the blood donation process in Saudi Arabia. A questionnaire-based survey was conducted. The participants were provided with information about the study's purpose at the beginning of the survey. Besides, voluntary participation in the questionnaire survey was mentioned. The Institutional Review Board at Imam Abdulrahman Bin Faisal University in Dammam, Saudi Arabia, approved the ethical protocol of this research and it was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from the participants.

Questionnaire Design
The research team modified the questionnaire exported from Alanzi and Alsaeed study to reach the research objectives.[16] The questionnaire survey included closed-ended questions with multiple choices from which the respondents were required to choose one of the available options. To ensure the participants could actively participate in the study, the questions were designed in a straightforward format to minimize errors in the findings. The questionnaire survey was translated forward and backward into the Arabic language and an academic expert reviewed it to check its clarity. The questionnaire survey was divided into three sections. The first section contained three questions about the participants' demographic data. (i) What is your gender? (male, female); (ii) What is your age group in years? (18–28, 29–39, 40–50, 51+); (iii) What is your nationality? (Saudi, Non-Saudi). The second section contained eight questions about a general awareness of blood donation, participants' history as blood donors, motivators and inhibitors to donating blood. (i) What is your blood type? (O+, O-, A+, A-, B+,

B-, AB+, AB-, I do not know); (ii) Did you know there are requirements to become a blood donor? (yes, no); (iii) Did you know there are causes to the donor being deferred or refused from blood donation? (yes, no); (iv) Did you know the donor can donate blood for only one component such as platelets? (yes, no); (v) How often did you donate blood? (never, first time, frequently); (vi) Did you donate blood before for only one blood component? (yes, no); (vii) What is your motivation to donate blood? (relative or a friend needs blood, human solidarity, personal satisfaction of helping others, beneficial to my health, seeing or receiving an advertising campaign, friends or relatives are blood donors and they encourage me, urgent call for blood, my blood type is rare and thus always necessary); (viii) What are your inhibitors to donate? (nothing inhibits me to donate, I can't donate due to my health condition, fear of needles, I have been deferred from donation before, fear of needing blood in future, fear of infectious disease transmission, the sight of blood is unpleasant, I get nothing in return). The third section contained seven questions to measure the impact of WhatsApp on the blood donation process in Saudi Arabia. (i) Have you ever looked for a blood donor for you/relatives by WhatsApp? (yes, no); (ii) Has your blood donation request ever been fulfilled with blood donors by WhatsApp? (yes, no); (iii) How frequently do you send broadcasts or campaigning requests for blood donation on WhatsApp? (never, sometimes, often, always); (iv) How frequently do you receive broadcasts or campaigning requests for blood donation on WhatsApp? (never, sometimes, often, always); (v) What are the sources of broadcasts from which you receive the requests for blood donation through WhatsApp? (families and friends, organizations, anonymous); (vi) What is your most likely response when you receive a blood donation request on WhatsApp? (nothing, donate blood only, share it with others only, donate blood and share it with others, I would have liked to donate but I could not); (vii) Do you think there is a role for WhatsApp in bridging the gap of blood banks' need for blood donors? (yes, no, maybe). The questionnaire survey is shown in Appendix A.

A pilot study was conducted with ten reviewers, one was an academic reviewer, and the others were the authors' colleagues at Imam Abdulrahman Bin Faisal University. The results were analyzed for validity and reliability using Cronbach's alpha for all items in the questionnaire, which were identified to be greater than 0.80; thus, the questionnaire was validated. Furthermore,

ethical approval was obtained from the Imam Abdulrahman Bin Faisal University.

The nine people were randomly selected.

Data Collection

The questionnaire survey was conducted online using the Google Forms platform. The participants were provided with information about the study and the measures taken, such as ensuring the anonymity, data protection, and so on. On the survey's first page, a check box was provided for participants to agree/provide their consent to participate in the survey. The questionnaire survey was distributed through social media channels including WhatsApp, Facebook, Instagram, etc. The study included citizens of Saudi Arabia, who have donated blood, and even those who have not. They were selected by using a non-probability sampling technique, specifically a convenience sample. Since the survey was sent to the public through online networks, formal sample size calculations were not possible.[20] Thus, everyone who completed the questionnaire was included, as there are no exclusion criteria in the data collection process. The study was conducted for a period of four weeks from 1st June 2021 to 29th June 2021. The study had a total of 150 participants.

Statistical Analysis

A basic descriptive statistical analysis in terms of frequencies and percentages was carried out in this study using the statistical package for social sciences (SPSS) version 23.

The participants' demographic information is shown in Table 1. As seen in the table, 67 (45%) males participated in the study, while 83 (55%) females did. In addition, the age of the majority of the participants (78%) was below 39 years old. Moreover, 93% of participants were Saudi.

Table 1 Participants' Demographic Information

Demographic Characteristics		Frequency	Percent
Gender	Female	83	55.3
	Male	67	44.7
Age group in years	18–28	58	38.7
	29–39	59	39.3
	40–50	23	15.3
	51 and above	10	6.7
Nationality	Non-Saudi	10	6.7
	Saudi	140	93.3

Table 2 Participants' General Awareness About the Blood Donation Process

General Awareness About the Blood Donation Process		Frequency	Percent
What is your blood type?	O+	79	52.7
	A+	36	24.0
	B+	10	6.7
	AB+	1	0.7
	O-	14	9.3
	A-	3	2.0
	I do not know	7	4.7
Did you know there are requirements to become a blood donor?	Yes	141	94.0
	No	9	6.0
Did you know there are causes to the donor being deferred or refused from blood donation?	Yes	141	94.0
	No	9	6.0
Did you know the donor can donate blood for only one component, such as platelets?	Yes	88	58.7
	No	62	41.3

Furthermore, Table 2 shows the participants' general awareness about the blood donation process. The majority of participants knew their blood group; half of them were O+ blood group (see Figure 1), while only 5% did not know their blood group. Also, 94% of participants were aware of the requirements to become a blood donor, and that there were causes for donors to be deferred or refused from blood donation. However, the awareness level dropped to 59% when the participants were asked if they could donate blood for only one component such as platelets.

Table 3 shows that 44.7% of the participants had previously donated blood, while 55.5% noted that they had never participated in a blood donation process. Of the participants, 13.3% had donated blood for only one blood component, such as platelets. With respect to the motivations
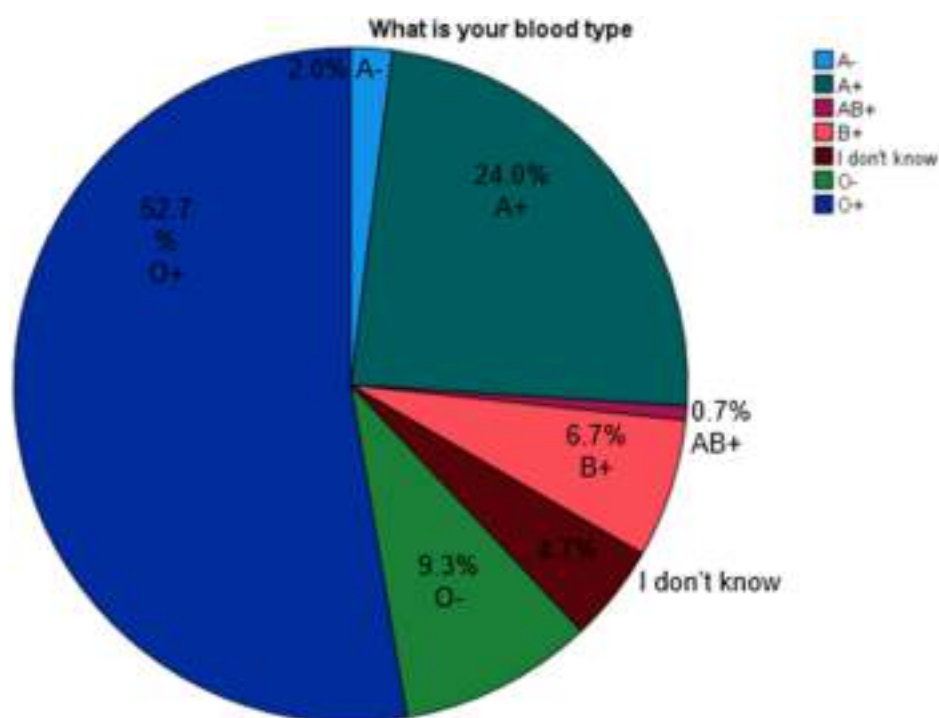
Figure 1 The variation of blood groups among the participants.

Table 3 Blood Donation History and Motivators and Inhibitors for Blood Donation

Blood Donation History and Motivators and Inhibitors for Blood Donation		Frequency	Percent
How frequent did you donate blood?	Never	83	55.3
	First time	49	32.7
	Frequently	18	12
Did you donate blood before for only one blood component?	No	130	86.7
	Yes	20	13.3
What is your motivation to donate blood?	Relative or a friend needs blood	41	27.3
	Human solidarity	39	26.0
	Personal satisfaction of helping others	34	22.7
	Beneficial to my health	23	15.3
	Seeing or receiving an advertising campaign	5	3.3
	Friends or relatives are blood donors and they encouraged me	4	2.7
	Urgent call for blood	4	2.7
What are your inhibitors to donate?	Nothing inhibits me to donate	68	45.3
	I can´t donate due to my health condition	27	18.0
	Fear of needles	21	14.0
	I have been deferred from donation before	13	8.7
	Fear of needing blood in the future	10	6.7
	Fear of infectious disease transmission	6	4.0
	The sight of blood is unpleasant	4	2.7
	I get nothing in return	1	0.7

Table 4 The Impact of the WhatsApp Platform on the Blood Donation Process

The Impact of the WhatsApp Platform in the Blood Donation Process		Frequency	Percent
Have you ever looked for a blood donor for you/relatives through WhatsApp?	No	100	66.7
	Yes	50	33.3
Has your blood donation request ever been fulfilled with blood donors by WhatsApp?	No	101	67.3
	Yes	49	32.7
How frequently do you send broadcasts or campaigning requests for blood donation on WhatsApp?	Never	108	72.0
	Sometimes	36	24.0
	Often	5	3.3
	Always	1	0.7
How frequently do you receive broadcasts or campaigning requests for blood donation on WhatsApp?	Never	48	32.0
	Sometimes	54	36.0
	Often	26	17.3
	Always	22	14.7
What are sources of broadcasts from which you receive the requests for blood donation through WhatsApp?	Families and Friends	107	71.3
	Organizations	22	14.7
	Anonymous	21	14.0
What is your most likely response when you receive the blood donation requests on WhatsApp?	I would like to donate but I cannot	40	26.7
	Share it with others only	37	24.7
	Donate blood only	13	8.7
	Donate blood and share it with others	31	20.7
	Nothing	29	19.3

behind donating blood, 27% of the subjects stated they donated blood due to their relatives or friends needing blood. This was followed by 26% of the participants who noted they only donated for a sense of human solidarity, and 23% indicated they donated blood to be satisfied when helping others. The remaining respondents donated blood for other reasons, as shown in Table 3. Related to the inhibitors to donating, 18% of the respondents stated they did not donate blood because of their health condition, and 1% pointed out they did not engage in the process because they did not get anything in return. Further, 14% noted they did not participate because of their fear of needles, while 3% found the sight of blood unpleasant.

Another relevant part of the investigation was to assess the impact of WhatsApp platform on the blood donation process. Table 4 shows that 33% of participants looked for blood donation opportunities by using WhatsApp, and all the requests for blood donation via WhatsApp were fulfilled with blood donors. Almost 28% of subjects, ranging from sometimes to always, participated in sending broadcasts or campaigning for requests for blood donation on WhatsApp, while 72% of participants responded they never participated in sending broadcasts or campaigning requests for blood donation on WhatsApp. Further, 32% of participants stated they never received broadcasts or campaigning requests for blood donation on WhatsApp, while others, ranging from sometimes to always, received broadcasts or campaigning requests for blood donation on WhatsApp. For the broadcasts of blood

donation requests that participants received through WhatsApp, 71% were from families and friends, while 15% of participants received broadcasts about blood donation requests from organizations and others received broadcasts about blood donation requests from anonymous sources. Participants' response to the broadcasts requesting blood donors was measured and 27% of them said they wanted to donate but couldn't, while 25% shared the broadcast with others only, 21% donated blood and shared broadcast, 9% donate blood only, and 19% of participant did nothing.

Finally, as shown in Figure 2, 94% of participants believed there was a role for WhatsApp in bridging the gap of blood banks' need for blood donors and only 6% thought there was no role of WhatsApp in bridging this gap.

Discussion

Based on the analyzed results, it is evident that WhatsApp plays a significant role in bridging the gap for blood banks' need for blood donors by using broadcasts, posts and campaigns in Saudi Arabia. This evidence is confirmed considering the information provided in Table 4, which shows that most of the participants in Saudi Arabia, who have sent broadcasts requesting blood donation messages through WhatsApp have had their requests fulfilled. This finding suggests that WhatsApp can promote blood donation programs to a high proportion of Saudi citizens. Moreover, this finding is supported by Alanzi and Alsaeed[16] and Waheed et al's[9] studies where they found WhatsApp was the most
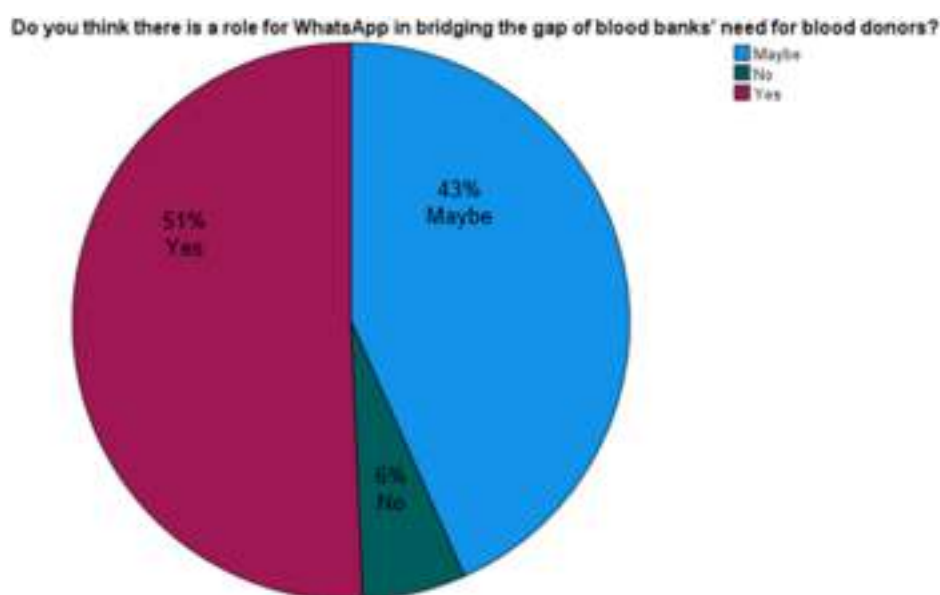

Figure 2 The impact of WhatsApp in bridging the gap between blood banks and blood donors in the participants' view.

useful social media tool in receiving the blood donation requests. Also, Table 4 shows that most of the sources of broadcasts and posts received through WhatsApp were from friends and family. Similar to what Alanzi and Alsaeed found in their study, friends and family were the primary source of broadcasts about blood donation.[16] In the same way, almost a half of the participants expressed they shared the broadcasts about the need for blood donors, while one-third of participants donated blood in response to the broadcasts received through the WhatsApp platform.

Furthermore, the participants expressed their opinion about their motivations and inhibitor factors from blood donation. In this sense, and according to Table 3, half of the participants considered that the motivation to donate blood was that a relative or friend needed blood, 26% felt human solidarity by donating, and 18% said they did not donate blood and were inhibited due to their health conditions.

As shown in Table 2, most participants were aware of the blood donation requirements and the causes for being deferred from donating. Nevertheless, around 60% of participants were not aware of the ability to donate only one component of blood. When the awareness was enhanced, the people who feared needing blood in the future donated blood for only one component, such as platelets, which have a life span of about eight days.[21]

Implementing an effective blood donation program in Saudi Arabia using WhatsApp platforms is possible. Alanzi and Alsaeed study supports these suggestions because their research outcome indicated that WhatsApp's popularity was essential and critical in promoting blood donation in Saudi Arabia.[16] Besides, the program would be more successful if it allowed the families and friends to share information about blood donation based on what is approached in our study, namely that families and friends were the major sources of broadcasts about blood donation. Moreover, the Saudi people's awareness of blood donation through social media, TV advertisements and campaigns should be increased.

This research's main limitations were limited resources, a small sample size, and short surveying time. Firstly, the sample identified in this study is low (150 participants); as a result, generalizing the results must be done with care. Secondly, while there are other popular social media applications, only WhatsApp was considered for the analysis. This study has both practical and theoretical implications. Firstly, there is a lack of research in Saudi Arabia on the use of social media applications in the blood donation process. Considering this gap, this study contributes to the existing literature. In addition, hospitals and healthcare administrative agencies can use this study's findings to understand the user's reliance on and behaviors surrounding using social media platforms for the blood donation process. Therefore, integrating these applications with hospitals, blood banks and donors could help improve the blood donation and management process.

Consequently, future studies will be addressed to avoid these limitations and evaluate, using statistical techniques, whether WhatsApp effectively improves the blood donation process in Saudi Arabia if the blood banks use it and promote voluntary non-remunerated blood donation, a family member's donation, or both contributions. It would also be interesting to compare a blood donor's behavioral intention before and after receiving messages on WhatsApp and between those who donate and those who do not.

Conclusion

This study's outcomes indicated that the Saudi people were aware of the blood donation process and half of them donated blood and were motivated to do so by a relative or a friend who needed blood and human solidarity, while those who did not donate blood were inhibited by their health condition. The WhatsApp platform announced the need for blood donors and the needs were fulfilled after announcements. Meaning, WhatsApp can bridge the communication gap between blood banks and the patients in need in Saudi Arabia, where there is a shortage of blood donors. Furthermore, as the number of users on WhatsApp is considerably high, social media applications can be integrated into eHealth platforms to streamline the resources and ensure the availability and accessibility of blood among the hospitals and the patients, which can improve operational efficiency. Overall, social media applications such as WhatsApp have huge potential to improve healthcare operations, which need to be explored more in the future research studies.

Acknowledgment

Disclosure

The authors report no conflicts of interest in this work.

Reference
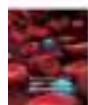
1. Saudi Arabia social media statistics 2020 (Infographics) - GMI Blog [Internet]; [cited December 31, 2020]. Available from: https://www.globalmediainsight.com/blog/saudi-arabia-social-media-statistics/. Accessed November 6, 2021.

2. Rodrigues Lucena TF, Queiroz Negri L, Marcon D, Yamaguchi MU. Is WhatsApp effective at increasing the return rate of blood donors? *Telemed e Health*. 2020;26(3):304–309. doi:10.1089/tmj.2019.0024

3. Healy J, Murphy M. Social marketing: the lifeblood of blood donation? In: *The Customer is NOT Always Right? Marketing Orientations in a Dynamic Business World*; 2017:811.

4. Blood transfusion safety [Internet]; [cited December 31, 2020]. Available from: https://www.who.int/westernpacific/health-topics/blood-transfusion-safety. Accessed November 6, 2021.

5. Ali R, Hafez T, Ali A, Abd-Alsabour N. Blood bag: a web application to manage all blood donation and transfusion processes. 2017 International Conference on Wireless Communications, Signal Processing and Networking (WiSPNET); 2017. doi:10.1109/WiSPNET.2017.8300136.

6. Asamoah-Akuoko L, Hassall O, Bates I, Ullum H. Blood donors' perceptions, motivators and deterrents in Sub-Saharan Africa - a scoping review of evidence. *Br J Haematol*. 2017;177 (6):864–877. doi:10.1111/bjh.14588

7. Blood donation [Internet]. King Faisal Specialist Hospital & Research Centre; [cited December 31, 2020]. Available from: https://www.kfshrc.edu.sa//en/home/giving/blood. Accessed November 6, 2021.

8. Wateen. Wateen app [Internet]; [cited December 31, 2020]. Available from: https://www.wateenapp.org/#download. Accessed November 6, 2021. Sindhi.

9. Waheed U, Wazeer A, Saba N, Qasim Z. Effectiveness of WhatsApp for blood donor mobilization campaigns during COVID-19 pandemic. *ISBT Sci Ser*. 2020;15:378–380. doi:10.1111/voxs.12572

10. Klassen S, Senefeld J, Johnson P, et al. The effect of convalescent plasma therapy on mortality among patients with COVID-19: systematic review and meta-analysis. *Mayo Clin Proc*. 2021;96 (5):1262–1275. doi:10.1016/j.mayocp.2021.02.008

11. Shi H, Zhou C, He P, et al. Successful treatment with plasma exchange followed by intravenous immunoglobulin in a critically ill patient with COVID-19. *Int J Antimicrob Agents*. 2020;56(2):105974. doi:10.1016/j.ijantimicag.2020.105974

12. Ye M, Fu D, Ren Y, et al. Treatment with convalescent plasma for COVID-19 patients in Wuhan, China. *J Med Virol*. 2020;92 (10):1890–1901. doi:10.1002/jmv.25882

13. Arcot PJ, Kumar K, Mukhopadhyay T, Subramanian A. Potential challenges faced by blood bank services during COVID-19 pandemic and their mitigative measures: the Indian scenario. *Transfus Apher Sci*. 2020;59(5):102877. doi:10.1016/j.transci.2020.102877

14. Abbasi R, Maqbool O, Mushtaq M, et al. Saving lives using social media: analysis of the role of twitter for personal blood donation requests and dissemination. *Telemat Inform*. 2018;35(4):892–912. doi:10.1016/j.tele.2017.01.010

15. Mathur P, Ayyar M, Chopra S, Shahid S, Mehnaz L, Shah RR. Identification of emergency blood donation request on Twitter. Proceedings of the 3rd Social Media Mining for Health Applications (SMM4H) Workshop & Shared Task. Brussels, Belgium; 2018:27–31.

16. Alanzi T, Alsaeed B. Use of social media in the blood donation process in Saudi Arabia. *J Blood Med*. 2019;10:417–423. doi:10.2147/JBM.S217950

17. Khomenko LM. Social marketing communications for blood donors retention. Socio-humanitarian aspects of modern society development: Proceedings of the eighth All-Ukrainian scientific conference of students, graduate students, teachers and staff. Sumy State University; 2020:312–315.

18. Elsafi S. Demographic pattern of blood donors and pre-donation deferral causes in Dhahran, Saudi Arabia. *J Blood Med*. 2020;11:243–249. doi:10.2147/JBM.S254168

19. Otifi H, Asiri M, Ahmad M, et al. Measuring public awareness about blood donation in Assir, South-Western Saudi Arabia. *Transfus Clin Biol*. 2020;27(3):122–127. doi:10.1016/j.tracli.2020.06.005

20. Al-rayes SA, Aldossary H, Aldoukhi E, Alahmedalyousif Z, Aldawood G, Alumran A. The awareness and utilization of 937-telephone health services in Saudi Arabia: cross-sectional survey study. *Inform Med Unlocked*. 2020;20:100393. doi:10.1016/j.imu.2020.100393

21. Thrombopoietin and platelet production in chronic immune thrombocytopenia- ClinicalKey [Internet]; [cited December 31, 2020]. Available from: https://www-clinicalkey-com.library.iau.edu.sa/#!/content/playContent/1-s2.0-S0889858809001725?returnurl=null&referrer=null. Accessed November 6, 2021.

Differences in Clinical Nature and Outcome Among Young Patients Suffering from an Acute Coronary Syndrome

Mohammad Saeed Al-Shahrani, Faisal Ahmad Katbi, Abdulaziz Mohammad Al-Sharydah, Saad Dhafer AlShahrani, Talal Mosfer Alghamdi & Mohammad Adnan Al-Sharidah

Published online: 02 Dec 2021.

Submit your article to this journal 🗗

Article views: 204

View related articles 🗗

View Crossmark data 🗗

Citing articles: 8 View citing articles 🗗

ORIGINAL RESEARCH

Differences in Clinical Nature and Outcome Among Young Patients Suffering from an Acute Coronary Syndrome

Mohammad Saeed Al-Shahrani [1,2]
Faisal Ahmad Katbi [1,2]
Abdulaziz Mohammad Al-Sharydah [3]
Saad Dhafer AlShahrani [4]
Talal Mosfer Alghamdi [5]
Mohammad Adnan Al-Sharidah [1]

[1]Department of Emergency, King Fahd Hospital of the University, Imam Abdulrahman Bin Faisal University, Khobar City, Eastern Province, Saudi Arabia; [2]Department of Critical Care, King Fahd Hospital of the University, Imam Abdulrahman Bin Faisal University, Khobar City, Eastern Province, Saudi Arabia; [3]Department of Diagnostic and Interventional Radiology, King Fahd Hospital of the University, Imam Abdulrahman Bin Faisal University, Khobar City, Eastern Province, Saudi Arabia; [4]Department of Emergency, Royal Commission Hospital, Jubail City, Eastern Province, Saudi Arabia; [5]Department of Anesthesia, King Fahd Hospital of the University, Imam Abdulrahman Bin Faisal University, Khobar City, Eastern Province, Saudi Arabia

Purpose: Acute coronary syndrome (ACS) is a life-threatening cardiac disease identified by acute, regional reductions in coronary blood flow, resulting in myocardial ischemia, or infarction, and manifesting as discomfort in the chest area, neck, or arms. Frequently, ACS is provoked by an atherosclerotic plaque; therefore, coronary atherosclerosis is converted into a chronic disease to an acute medical emergency. The purpose of this study was to explore the differences among these variables in patients less than 45 years of age suffering from this major health problem compared to older adults admitted with an ACS diagnosis, and to adopt an optimized temporary management.

Patients and Methods: A retrospective chart review study was conducted on a total of 652 ACS patients admitted at King Fahad Hospital of the University (KFHU) between 2015 and 2020. The patients' medical records were utilized for obtaining demographic data, presenting symptoms, risk factors, and clinical outcomes.

Results: Overall, 652 patients were enrolled. Of these, 109 patients (16.7%) were under 45, with a mean age of 38 ± 7. Younger patients showed a higher rate of palpitation (23.9% versus. 13.6%; P = 0.019). A positive smoking history and a family history of CAD were seen more often in younger patients (42.2% vs 27.3%, P < 0.001; 22.9% vs 9.4%, P < 0.001, respectively). Older patients had greater renal impairment with higher creatinine (median = 1.10 mg/dl (range, 0.3–13.0) vs 1.0 (0.3–19.0; p = 0. 001), BUN (median = 16.0 (mange, 0.9–141.0) vs 12.0 (0.9–49.0); P < 0.001)). Younger patients had higher levels of LDL and total cholesterol (median 138c. 115; p < 0.001) and cholesterol (median 209 vs 178.5; p < 0.001). Hospital mortality was 0.9% in younger patients versus 7.4% in older patients (P = 0.004).

Conclusion: Palpitations, smoking, family history, higher LDL levels, and total cholesterol levels were more prevalent in adults younger than 45 years old with ACS. Impaired renal function, hypertension, and diabetes were more in older patients with ACS.

Keywords: acute coronary syndrome, young adults, clinical features, in-hospital outcome

Introduction

Cardiovascular disease, which includes coronary heart disease and stroke, is the leading cause of death and disease burden globally.[1] Acute coronary syndrome (ACS) is characterized by acute local reduction in blood flow to the myocardium, also known as ischemia.[2] Ischemic heart disease may present with a variety of symptoms, eg, a symptom complex called acute coronary syndrome (ACS), which may lead to a diagnosis of one of unstable angina (UA), non-ST-segment elevation myocardial infarction (NSTEMI), or ST-segment elevation myocardial infarction

Correspondence: Mohammad Saeed Al-Shahrani
Email msshahrani@iau.edu.sa

1011

(STEMI). The former is a clinical diagnosis, whereas the latter two are diagnosed from the abnormalities seen on the EKG, possibly complemented by serum enzyme assays, eg, of troponin C.[3]

Atherosclerosis is a chronic inflammatory process of the arterial wall, and although it starts early in life, it takes years to be of serious pathological severity, so that young people do not present with ACS very often.[4,5] However, in recent years, coronary artery disease (CAD) has been affecting younger patients, which raises concern due to the accompanying results of premature morbidity and mortality.[5] Patients less than 45 years of age represent 6–10% of cardiac infarctions in the United States.[5–7] Smoking was found to be the prevailing risk factor and was frequently found along with coronary events in young patients.[8,9] ACS presents with similar clinical features irrespective of age 9 The main presenting symptom is chest pain in both the young as well as in the elderly.[10] It is also important to note that the severity and presentation of the diseases caused by atherosclerosis seem to differ between men and women in their different stages of life.[11] For example, men are more likely to develop advanced coronary artery disease (CAD) compared to pre-menopausal women.[12] However, post-menopausal women are more affected by an adverse prognosis than their male counterparts.[12]

Publications on ACS from Saudi Arabia are limited and there is a definite paucity of new information about the increasing prevalence among young patients less than 45 years of age.[13] We hypothesized that the clinical severity and outcomes of ACS patients are different for young people compared to older patients, owing to their different lifestyles and cultural differences. The aim of this present study was to explore the demographic characteristics, cardiovascular risk factors, short-term outcomes and complications in young adults below the age of 45 years compared to older adults who were admitted with a diagnosis of ACS at the King Fahd University Hospital between 2015 and 2020, in order to update the expertise on this major health problem. This study complements the scarce existing data on the acute coronary syndrome in youth and adults and is considered helpful for resource planning and short-term management optimization.

Materials and Methods
Setting
The study was conducted at King Fahad Hospital of the University (KFHU), a tertiary care university teaching hospital with 600 beds in the Eastern Province of Saudi Arabia. It is a retrospective study that was conducted using medical records of all ACS patients admitted between 2015 and 2020. Demographic data, presenting symptoms, risk factors, and clinical outcomes were obtained. The local institutional research body approved this study.

Study Design and Study Population
A descriptive retrospective study was conducted with the sample size determined by the number of patients in King Fahd Hospital of the University in Al-Khobar city, and this was a total of 652 patients. All methods were performed in accordance with STROBE guidelines and regulations of cohort studies.

Inclusion and Exclusion Criteria
We recruited all patients who were diagnosed with ACS based on the international classification of diseases version 10 ICD-10, including younger adult patients (from 18 to 45 years old) and older patients (above 45-year-old). We excluded patients labeled DNR (do not resuscitate), and incomplete patient files (48 patients).

Statistical Analysis
All categorical variables were represented by frequency as percentages, and were analyzed using chi-squared and Fisher's exact tests. Continuous variables were represented by median with range because the data did not follow the normal distribution. The Mann–Whitney U-test was used for the comparison between the two groups. All analyses were performed using SPSS (21 version). A P value of less than 0.05 was considered significant by Kruskal–Wallis test.

Ethical Approval
This study was performed in accordance with the Helsinki Declaration of 1975 (revised in 1983) and the Imam Abdulrahman Bin Faisal University Institutional Review Board considered the descriptive and retrospective nature of this study and granted approval for the study to be conducted at KFHU (IRB-UGS-2016-01-075). Anonymized data were collected, analyzed, and reported only in aggregate form, and no identifiable participant information (image, face, name, etc.) was revealed in the study.

Results
A total of 652 patients were enrolled in this study without significant difference in gender distribution (M:F, 1:1) (Figure 1). Of these, 109 (16.7%) patients were less than
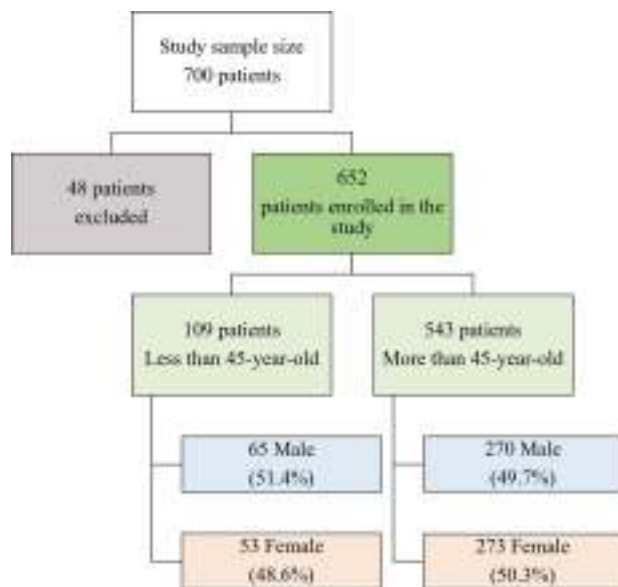
Figure 1 Flowchart illustrating the inclusion and exclusion criteria for the study and the size of the patient groups.

45 years of age. The mean age was 38 ± 7 vs 60 ± 11 years in the young and old groups, respectively ($P < 0.001$). Table 1 shows that younger patients had a higher frequency of palpitations (23.9% vs 13.6%; $P = 0.019$). Older patients had higher frequencies of hypertension, eg, a systolic blood pressure reading of >140 mmHg (68.7% vs 37.6%; $P < 0.001$), diabetes (61.1% vs 37.6%, $P < 0.001$), and history of CAD (52.3% vs 30.3%; $P < 0.001$), but less frequent history of smoking, and positive family history, which were higher among younger patients

Table 1 Clinical Presentations of ACS Upon Admission According to Age

Presentation	Less Than 45-Year-Old (n= 109)	More Than 45-Year-Old (n= 543)	P value*
Chest pain	97 (89%)	444 (81.8%)	0.071**
Epigastric pain	11 (10.1%)	60 (11%)	0.929
Syncope/ Pre-Syncope	10 (9.2%)	48 (8.8%)	0.834
Dyspnea/ SOB	49 (45%)	243 (44.8%)	0.286
Palpitation	26 (23.9%)	74 (13.6%)	0.019**
Sweating	38 (34.9%)	185 (34.1%)	0.604
Nausea and vomiting	29 (26.6%)	126 (23.2%)	0.446
Cardiac arrest	3 (2.8%)	38 (7%)	0.246

Notes: *Statistical significance was set at p-values <0.05. **Statistically significant by Kruskal–Wallis test.
Abbreviations: SOB, Shortness of breath; n, total number of patients.

Table 2 Risk Factors of ACS Patients According to Age

Risk Factor	Less Than 45-Year-Old (n= 109)	More Than 45-Year-Old (n= 543)	P value*
HTN	41 (37.6%)	373 (68.7%)	<0.001**
DM	41 (37.6%)	332 (61.1%)	<0.001**
Abnormal lipid profile	60 (55.0%)	227 (41.8%)	0.038
Smoking history	46 (42.2%)	148 (27.3%)	<0.001**
Family history	25 (22.9%)	51 (9.4%)	<0.001**
CAD history	33 (30.3%)	284 (52.3%)	<0.001**

Notes: *Statistical significance was set at p-values <0.05. **Statistically significant by Kruskal–Wallis test.
Abbreviations: HTN, Hypertension; DM, Diabetes Mellitus; n, total number of patients.

(42.2% vs 27.3%, $P < 0.001$, 22.9% vs 9.4%; $P < 0.001$) (Table 2).

Table 3 shows the laboratory outcomes; older patients had significantly higher creatinine levels (median = 1.10 mg/dl (range, 0.3–13.0) vs 1.0 (0.3–19.0); p = 0. 001), BUN (Median = 16.0 (Range, 0.9–141.0) vs 12.0 (0.9–49.0); P < 0.001), but less in Hb, LDL cholesterol and total cholesterol, which were high among younger patients (14.4 g/dl (8.4–17.6) vs 13.5 g/dl (0.11–158); P < 0.001, 138 mg/dl (43–224) vs 115 mg/dl (25–411); p < 0.001, 209 mg/dl (59–303) vs 178.5 mg/dl (18–485); p < 0.001) (Figure 2).

Table 4 shows the length of hospital admission and mortality, and there was a significant difference in mortality, that is, the in-hospital mortality was 7.4% among older patients and 0.9% among younger patients (P = 0.004).

Discussion

Acute coronary syndrome (ACS) in young adults is a rare entity, yet it occurs. Assessing the clinical features, risk factors, and outcomes, which may be due to either primary disease or secondary complications, would set several measures to prevent further episodes of ACS. In this study, our cutoff point to define young patients was 45 years. It was determined upon similar studies in Thailand, Singapore, Israel, and California.[14,15] While other studies from Japan, Poland, Germany, Australia, New Zealand, and the USA have defined young adults to be under 40 years.[15] Our findings are as follows: First, 109 ACS patients were younger than 45 years with a mean age of 38 ± 7. Domestic and international studies found that 5–10% of ACS manifest before the age of 46.[16,17]

Our estimated prevalence was slightly higher than the reported percentages. The authors attributed the increased

Table 3 Comparison of Laboratory Work Up Results in ACS Patients According to Age

Lab. Result	Less Than 45-Year-Old (n= 109) (Median, Average)	More Than 45-Year-Old (n= 543) (Median, Average)	P value*
Creatinine (mg/dL)	1 (0.3–19)	1.1 (0.3–13.0)	0.001**
BUN (mg/dL)	12 (0.9–49.0)	16 (0.9–141.0)	<0.001**
Hemoglobin (g/dL)	14.4 (8.4–17.6)	13.5 (0.11–158)	<0.001**
Troponin (ng/mL)	1.63 (0.04–450)	1.08 (0.01–165.5)	0.286
LDL (mg/dL)	138 (43–224)	115 (25–411)	<0.001**
HDL (mg/dL)	36 (10–56.0)	36 (9–172.0)	0.283
Triglyceride (mg/dL)	139 (20–1111)	126 (1.18–557.0)	0.103
Total Cholesterol (mg/dL)	209 (59–303)	178.5 (18–485)	<0.001**

Notes: *Statistical significance was set at p-values <0.05. **Statistically significant by Kruskal–Wallis test.
Abbreviations: n, total number of patients; lab., laboratory; ACS, acute chest syndrome; BUN, blood urea nitrogen; LDL, low-density lipoproteins; HDL, high-density lipoproteins; g, gram; mg, milligram; dL, deciliter; mL, milliliter.

ACS exposure to health system's transformation and adopting western lifestyle as a new way of living in Saudi Arabia as risk factors. Second, regarding risk factors, older patients had a higher frequency of hypertension, diabetes, and a history of CAD. Also, Systemic Mastocytosis (SM) is proven to be a major risk factor for developing acute coronary syndrome.[18] However, unlike younger patients, they had less frequent history of smoking and family history of ACS. Furthermore, a recent retrospective cohort study of two academic institutions suggests an increased likelihood of encountering ACS with a family history of hypercholesterolemia.[19] Previous studies reported that 82% of young patients who suffered from ACS were smokers which makes smoking a crucial risk factor.[20–22] Chua et al interestingly found that the rate of smoking patients aged ≤35 years who suffered from MI was significantly higher than in patients >65 years of age with the same condition.[21]

Aligning with the findings of other studies, it is suggested that both history of smoking and family cardiovascular disease are risk factors in young patients with ACS (Figure 2).[20–22] A study in Thailand reached the same conclusion, whereas it discovered that the most common risk factor of ACS in patients aged <45 years of age was

Figure 2 The patient is 42 years old, suffering from atypical chest pain. He's overweight, and his lab results came back positive for dyslipidemia. Cardiac CT scan performed to evaluate the presence of coronary heart disease. An ECG-gated cardiac CT acquisition was performed using an ECG-modulated radiation dose. A sublingual nitroglycerin tablet given three minutes prior to the procedure. (**A**) The left anterior descending artery presents a benign diffuse disease with moderate calcification and no apparent obstructive lesions (arrow). However, the presence of calcification impedes an accurate evaluation of stenosis. (**B**) The left ventricle is slightly dilated, the left ventricular volume at the end of diastolic is 211 mL, and the volume at the end of systole is 114 mL. The calculated ejector fraction is 46%. An organized small wall-mounted apical clot was displayed (arrow), which denotes an infarction of the old LAD territory with an organized old LV apical clot. There is an akinese of the medium and distal septum and the major part of the apex (not shown).

Table 4 In-Hospital Outcome for ACS Patients According to Age

Outcome	Less Than 45-Year-Old (n= 109) (Median, Average)	More Than 45-s (n= 543) (Median, Average)	P value*
In-hospital stay (days)	5 (0–97)	5 (0–26)	0.709
Mortality	1 (0.9%)	40 (7.4%)	0.004**

Notes: *Statistical significance was set at p-values <0.05. **Statistically significant by Kruskal–Wallis test.
Abbreviations: n, total number of patients; ACS, acute chest syndrome.

smoking.[14] A different study found that patients whose parents developed cardiovascular disease of early onset had an increased risk of cardiovascular disease.[23]

Both quantity and duration of smoking accelerate the development of atherogenic cardiovascular disease.[24,25] Furthermore, smoking also alters the defensive response of the immune system against vascular damage, which is characterized by an increase in oxidative influence on lipid peroxidation, endothelial cell dysfunction, and generation of foam cells in the tunica media.[25,26] It also leads to increased platelet aggregation, disrupts the metabolic activities of lipoproteins, and tends to reduce HDL cholesterol.[25,26] Cigarette smoking is linked to increased levels of inflammatory markers. During the acute phase of the inflammatory process, C-reactive protein is elevated, white blood cells and fibrinogen are increased, and serum albumin is decreased.[24–26] Smoking can also increase myocardial load due to stimulation by catecholamines and reduce consumption of O2 due to inhalation of carbon monoxide, which may cause tachycardia, vasoconstriction of blood vessels, and which may modify the permeability of the vessel wall.[25,26]

Primary and secondary prevention specifically are important in this subset of patients. In addition, one study has shown that diabetes mellitus was more prevalent in younger adults. Emphasized by several authors, ACS is clinically similar in both young patients and the elderly.[9,27–29] However, in our study, we found that palpitations occurred more frequently in younger adults.

In addition, some laboratory parameters including creatinine, and BUN, were higher in older patients, but HGB, LDL, and cholesterol levels were higher among younger patients. This result was similar to those of previous studies.

In our study, the length of stay was not significantly different between the two age groups, although other studies have shown that older patients with ACS had longer hospital stays.[30,31] Besides, in-hospital mortality was 7.4% among older patients and 0.9% among younger patients.

Patients who were discharged from the hospital were followed by general cardiologists in an outpatient setting after an average of one month and then every year. Our findings align with those of a recent cohort of Yang et al, which demonstrated equity among both age groups in terms of short- and long-term outcomes.[32]

This study confirmed a previously established correlation between the modifiable risk factors of ACS, and adoption of healthy dietary habits and exercise programs.[29,32] Compared to the western diet, changing nutritional behaviors and seeking weight reduction and reducing BMI strategies in patients with CAD and metabolic syndrome found to show significant improvement in patients' inflammatory and metabolic biomarkers.[33] This current study is newly conducted by licensed academic physicians from various medical specialties involved in the management of ACS including emergency medicine, vascular interventional radiology, anesthesiology, and intensive care medicine with adequate experience in ACS management, in a teaching hospital. Similar national and international studies have only examined the relationship of ACS to sex and age – young or old. In contrast, our study examined the influence of all these determinants and their contribution to the clinical severity and outcomes in patients with ACS. Potential limitations of our study include the fact that our selected design was retrospective. However, our sample size was reasonably large (652 cases), which in our opinion reduced information bias and therefore strengthened the results. We accept the limitation of not to imploy the types of ACS in this study which might be a trigger for future studies. The changes in waist circumference which were reported to be linked to increased risk of ACS especially with waist circumference ≥102 cm in males and ≥88 cm in females were not included in our data collection.[33]

Conclusion

Palpitations, positive smoking history, positive family history, and higher lipid profile were more prevalent in adults younger than 45 years old with ACS than in older ones.

Impaired renal function, hypertension, and diabetes were more common in older patients with ACS. Younger patients seem to have a better outcome in terms of mortality with no difference in length of hospital stay.

Abbreviations

ACS, acute coronary syndrome; CAD, coronary artery disease; NSTEMI, non-ST-segment elevation myocardial infarction; STEMI, ST-segment elevation myocardial infarction.

Data Sharing Statement

The datasets generated during and analysed during the current study are not publicly available due to the privacy of research participants but are available from the corresponding author on reasonable request.

Consent to Participate

Written informed consent was obtained from the patient. The Standing Committee for Research Ethics on Living Creatures (SCRELC) from the Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia approved this project by IRB number: IRB-UGS-2016-01-075. No patients were involved neither in the design, recruitment and conduct of this study, nor in the development of outcome measures.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Disclosure

The authors declare no conflicts of interest for this work.

References

1. Redfern J, Hyun K, Chew DP, et al. Prescription of secondary prevention medications, lifestyle advice, and referral to rehabilitation among acute coronary syndrome in patients: results from a large prospective audit in Australia and New Zealand. *Heart*. 2014;100(16):1281–1288. doi:10.1136/heartjnl-2013-305296

2. Kumar A, Kar S, Fay WP. Thrombosis, physical activity, and acute coronary syndrome. *J Appl Physiol*. 2011;111(2):599–605. doi:10.1152/japplphysiol.00017.2011

3. Aroney CN, Aylward P, Kelly AM, et al; Acute Coronary Syndrome Guidelines Working Group. Guidelines for the management of acute coronary syndromes. *Med J Aust*. 2006;184:S1–S32.

4. Jednacz E, Rutkowska-Sak L. Assessment of the body composition and parameters of the cardiovascular risk in juvenile idiopathic arthritis. *Biomed Res Int*. 2015;2015. doi:10.1155/2015/619023

5. Choudhury L, Marsh JD. Myocardial infarction in young patients. *Am J Med*. 1999;107(3):254–261. doi:10.1016/S0002-9343(99)00218-1

6. Egred M, Viswanathan G, Davis GK. Myocardial infarction in young adults. *Postgrad Med J*. 2005;81(962):741–745. doi:10.1136/pgmj.2004.027532

7. Chouhan L, Hajar HA, Pompisiello JC. Comparison of thrombolytic therapy for acute myocardial infarction in patients aged <35 and >55 years. *Am J Cardiol*. 1993;71:157–159. doi:10.1016/0002-9149(93)90731-q

8. Wolfe MW, Vacek JL. Myocardial infarction in the young. Angiographic features and risk factor analysis of patients with myocardial infarction at or before the age of 35 years. *Chest*. 1988;94(5):926–930. doi:10.1378/chest.94.5.926

9. Thomas D. Athérosclérose [Atherosclerosis]. In: *Cardiologie* [Cardiology]. Paris: Ellipses; 1994:135–151. French.

10. Joussein-Remacle S, Delarche N, Bader H, Bader H, Lasserre R, Estrade G. Facteurs de risque de l'infarctus du myocarde du sujet jeune: registre prospectif sur un an [Risk factors in a young population with acute myocardial infarction: one year prospective study]. *Ann Cardiol Angeiol*. 2006;55:204–209. French. doi:10.1016/j.ancard.2006.05.001

11. Capolaghi B, Charbonnier B, Dumonet M, et al. Recommandations sur la prescription, le dosage et l'interprétation des troponines cardiaques [Recommendations on the prescription, dosage and interpretation of cardiac troponins]. *Ann Biol Clin*. 2005;63:245–261. French.

12. Piro M, Della Bona R, Abbate A, Biasucci LM, Crea F. Sex-related differences in myocardial remodeling. *J Am Coll Cardiol*. 2010;55(11):1057–1065. doi:10.1016/j.jacc.2009.09.065

13. Al-khadra AH. Clinical profile of young patients with acute myocardial infarction in Saudi Arabia. *Int J Cardiol*. 2003;91(1):9–13. doi:10.1016/S0167-5273(02)00579-X

14. Nemetz PN, Roger VL, Ransom JE, Bailey KR, Edwards WD, Leibson CL. Recent trends in the prevalence of coronary disease: a population-based autopsy study of non-natural deaths. *Arch Intern Med*. 2008;168(3):264–270. doi:10.1001/archinternmed.2007.79

15. Tungsubutra W, Tresukosol D, Buddhari W, et al. Acute coronary syndrome in young adults: the Thai ACS registry. *J Med Assoc Thai*. 2007;90 Suppl 1(1):81–90.

16. Tuzcu EM, Kapadia SR, Tutar E, et al. High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults: evidence from intravascular ultrasound. *Circulation*. 2001;103(22):2705–2710. doi:10.1161/01.CIR.103.22.2705

17. Al-Nozha MM, Arafah MR, Al-Mazrou YY, et al. Coronary artery disease in Saudi Arabia. *Saudi Med J*. 2004;25(9):1165–1171.

18. Battisha A, Sawalha K, Madoukh B, et al. Acute myocardial infarction in systemic mastocytosis: case report with literature review on the role of inflammatory process in acute coronary syndrome. *Curr Cardiol Rev*. 2021;16(4):333–337. doi:10.2174/1573403X16666200331123242

19. Singh A, Gupta A, Collins BL, et al. Familial hypercholesterolemia among young adults with myocardial infarction. *J Am Coll Cardiol*. 2019;73(19):2439–2450. doi:10.1016/j.jacc.2019.02.059

20. Patel JV, Dwivedi S, Hughes EA, Gregory YH. Premature coronary artery disease: an inferred cardiovascular variant or a South Asian genetic disorder? *Thromb Haemost*. 2008;99:991–992. doi:10.1160/TH08-05-0286

21. Chua SK, Hung HF, Shyu KG, et al. Acute ST-elevation myocardial infarction in young patients: 15 years of experience in a single center. *Clin Cardiol*. 2010;33(3):140–148. doi:10.1002/clc.20718

22. Teo KK, Ounpuu S, Hawken S, et al. INTERHEART Study investigators tobacco use and risk of myocardial infarction in 52 countries in the INTERHEART study: a case-young non-MS patients study. *Lancet*. 2006;368(9536):647–658. doi:10.1016/S0140-6736(06)69249-0

23. Huang J, Qian HY, Li ZZ, Zhang JM. Comparison of clinical features and outcomes of patients with acute myocardial infarction younger than 35 years with those older than 65 years. *Am J Med Sci*. 2013;346(1):52–55. doi:10.1097/MAJ.0b013e318265e33e

24. Scheuner MT, Whitworth WC, McGruder H, Yoon PW, Khoury MJ. Familial risk assessment for early-onset coronary heart disease. *Gen Med*. 2006;8(8):525–531.

25. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease, an update. *J Am Coll Cardiol*. 2004;43 (10):1731–1737. doi:10.1016/j.jacc.2003.12.047

26. Bakhru A, Erlinger TP. Smoking cessation and cardiovascular disease risk factors: results from the third national health and nutrition examination survey. *PLoS Med*. 2005;2(6):528–535. doi:10.1371/journal.pmed.0020160

27. Leone A. Relationship between cigarette smoking and other coronary risk factors in atherosclerosis: risk of cardiovascular disease and preventive measures. Department of internal medicine, City Hospital Massa, Carrara, Italy. *Curr Pharm Des*. 2003;9 (29):2417–2423. doi:10.2174/1381612033453802

28. Benacerraf A, Castilloy-Fennoy A, Goffinet D, Krantz D. L'infarctus du myocarde avant 36 ans: à propos de 20 cas [Myocardial infarction before age 36: about 20 cases]. *Arch Mal Coeur*. 1978;77 (7):756–764. French.

29. Collet JP, Zeitouni M, Procopi N, et al. Long-term evolution of premature coronary artery disease. *J Am Coll Cardiol*. 2019;74 (15):1868–1878. doi:10.1016/j.jacc.2019.08.1002

30. Kanitz MG, Giovannucci SJ, Jones JS, Mott M. Myocardial infarction in young adults: risk factors and clinical features. *Emerg Med J*. 1996;14:139–145. doi:10.1016/0736-4679(95)02089-6

31. Fan G, Fu K, Jin C, et al. A medical costs study of older patients with acute myocardial infarction and metabolic syndrome in hospital. *Clin Interv Aging*. 2015;10:329–337. doi:10.2147/CIA.S70372

32. Yang J, Biery DW, Singh A, et al. Risk factors and outcomes of very young adults who experience myocardial infarction: the partners YOUNG-MI registry. *Am J Med*. 2020;133(5):605–612. doi:10.1016/j.amjmed.2019.10.020

33. Arbel Y, Havakuk O, Halkin A, et al. Relation of metabolic syndrome with long-term mortality in acute and stable coronary disease. *Am J Cardiol*. 2015;115(3):283–287. doi:10.1016/j.amjcard.2014.10.037

Prognostic Factors and Clinical Considerations for Iron Chelation Therapy in Myelodysplastic Syndrome Patients

Sarah Parisi & Carlo Finelli

Published online: 03 Dec 2021.

Submit your article to this journal ☑

View related articles ☑

View Crossmark data ☑

REVIEW

Prognostic Factors and Clinical Considerations for Iron Chelation Therapy in Myelodysplastic Syndrome Patients

Sarah Parisi[1]
Carlo Finelli[2]

[1]IRCCS Azienda Ospedaliero-Universitaria di Bologna, Istituto di Ematologia "Seràgnoli", Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale, Università di Bologna, Bologna, Italy; [2]IRCCS Azienda Ospedaliero-Universitaria di Bologna, Istituto di Ematologia "Seràgnoli", Bologna, Italy

Abstract: Iron chelation therapy (ICT) is an important tool in the treatment of transfusion-dependent lower-risk myelodysplastic syndrome (MDS) patients. ICT is effective in decreasing iron overload and consequently in limiting its detrimental effects on several organs, such as the heart, liver, and endocrine glands. Besides this effect, ICT also proved to be effective in improving peripheral cytopenia in a significant number of MDS patients, thus further increasing the clinical interest of this therapeutic tool. In the first part of the review, we will analyze the toxic effect of iron overload and its mechanism. Subsequently, we will revise the clinical role of ICT in various subsets of MDS patients (low, intermediate, and high risk MDS, patients who are candidates for allogeneic stem cell transplantation).

Keywords: iron overload, myelodysplastic syndrome, iron chelation therapy, hepcidin

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal, acquired diseases of the hematopoietic stem cell, characterized by a variable degree of peripheral cytopenias due to ineffective erythropoiesis and by the risk of progression to acute myeloid leukemia (AML).

The risk of progression to AML is predicted at diagnosis by several scores, which allow classification of MDS patients in low, intermediate, and high-risk and to choose the more suitable therapeutic approach.[1,2]

The International Prognostic Scoring System (IPSS) was designed in order to classify MDS patients in different risk groups and to predict the risk of AML transformation and overall survival (OS). IPSS is applicable at diagnosis and allows the classification of MDS patients into four risk groups (low, intermediate 1 and 2, and high risk), according to the percentage of bone marrow blasts, cytogenetic features, and number of cytopenias.

Subsequently, other risk scores were proposed, in order to better characterize patients; among the other risk scores, the revised IPSS (IPSS-R) divides MDS patients into five risk groups.[2]

Low-risk MDS are characterized by peripheral cytopenias and most patients become red blood cell (RBC) transfusion dependent during their disease history, thus determining iron overload and consequent organ damage.

MDS patients are frequently transfusion-dependent and iron overload can represent a significant complication due to iron toxicity on the liver, heart and the

Correspondence: Sarah Parisi
Email sarah.parisi2@unibo.it

1019

endocrine system. It was also demonstrated that iron over-load has a detrimental effect on the hematopoietic bone marrow, thus enhancing ineffective erythropoiesis and favoring the appearance of genomic abnormalities, with an increased risk of AML transformation.[3]

Several observations suggested that iron chelation therapy may have a positive effect on MDS patients outcome and on the basis of these observations a randomized trial was conducted in order to clarify the role of iron chelation therapy on low and intermediate-risk MDS patients.[4]

International guidelines also recommend consideration of iron chelation therapy in high-risk MDS patients who are candidates for allogeneic stem cell transplantation or in high risk patients who respond to disease-modifying therapies, such as hypomethylating agents,[5,6] in order to minimize the detrimental effect of iron overload.

Iron chelation therapy in MDS patients is indicated until the patient becomes transfusion independent and/or until normalization of parameters indicating iron overload.

Here we review data about iron overload and iron chelation therapy in transfusion-dependent low and intermediate-risk MDS patients. This work is mainly focused on the clinical use of ICT in low, intermediate, and high risk MDS patients according to the available data from literature.

Iron Overload Mechanisms in MDS Patients

Iron overload is a frequent finding in MDS patients[7] and several studies demonstrated its detrimental effect on overall and leukemia-free survival due to iron toxic effect on several organs, like the liver, heart and endocrine glands.[8] Chronic transfusion therapy is the most important risk factor for iron overload, even if it was noticed that some patients develop iron overload at an earlier stage of the disease, even before receiving transfusions, thus suggesting a dysregulation of iron homeostasis factors as a cause.[9] Chronic transfusion regimen and ineffective erythropoiesis are the main drivers of iron overload in MDS patients.

Several studies demonstrated that hepcidin, the small hepatic peptide hormone able to regulate iron absorption by binding to its receptor ferroportin, is downregulated in hematologic disorders characterized by ineffective erythropoiesis, first of all in transfusion-dependent beta-thalassemia. A similar pathway is supposed to be involved in iron overload in MDS patients.

Hepcidin action is mediated by its binding to ferroportin, that is highly expressed on duodenal enterocytes

and macrophages and that is internalized and degraded after hepcidin binding, thus blocking iron absorption. Hepcidin production by the liver is enhanced by increased plasma and hepatic iron levels and by inflammation, while it is downregulated in the case of ineffective erythropoiesis. It has been postulated that hepcidin downregulation has a key role in the establishment of iron overload in diseases characterized by enhanced ineffective erythropoiesis, such as MDS. Santini et al analyzed serum hepcidin levels of 113 MDS patients and demonstrated the lowest levels in refractory anemia with ring sideroblasts (RARS) and the highest in refractory anemia with excess blasts (RAEB) and in chronic myelomonocytic anemia (CMML).[10] Notably, RARS patients are known to have the highest levels of toxic non-transferrin-bound iron.

When hepcidin is downregulated, intestinal iron absorption and iron release by macrophages is enhanced, resulting in iron overload.[11–21] The excess of absorbed iron is in part utilized for erythropoiesis, but the increased number of erythroid precursors typical of MDS is not able to fully utilize iron, which accumulates as non-transferrin-bound iron (NTBI) and causes organ damage. Ultimately, iron overload is maintained by hepcidin inhibition mediated by enhanced bone marrow proliferation and iron is inadequately used due to ineffective erythropoiesis and defective red blood cell (RBC) maturation.

As stated by Santini et al, a diverse degree of hepcidin downregulation has been recently described in some MDS subtypes, in particular the lowest serum levels of hepcidin were observed in patients with refractory anemia with ring sideroblasts (MDS-RS) and refractory cytopenia with multilineage dysplasia and ring sideroblasts (MDS-MLD-RS). Clinically, patients affected by MDS-MLD-RS are known to develop iron overload even before being transfusion-dependent and to have a pretransfusional hepcidin/ferritin ratio significantly decreased, with hepcidin levels inappropriately low for the degree of iron loading (evaluated by serum ferritin levels).[10,22] Moreover, MDS-MLD-RS subtype (according to the WHO 2008 classification), hallmarked by the high prevalence of *SF3B1* mutation and bone marrow erythroid precursors with iron deposits in their mitochondria (ring sideroblasts), are characterized by a particularly high degree of ineffective erythropoiesis, thus enhancing hepcidin inhibition.[23,24] Iron incorporation within mitochondria prevents heme production, with consequent hypoxia and enhances ineffective erythropoiesis and hepcidin downregulation.[25]

Some genetic alterations have been associated with an increased risk of developing iron overload in MDS patients.[26]

SF3B1 mutation is typical of MDS-RS and MDS-MLD-RS and it is known to inhibit erythropoiesis by the dysregulation of RNA splicing of the transcription factors *TAL1* and *GATA1*.[27] *SF3B1* mutation has a direct effect on iron accumulation, as it is associated with splicing alterations of some genes that regulate iron metabolism.[28,29] Some patients with MDS show *TET2* mutation, which has been correlated with the dysregulation of several genes involved in iron metabolism.[30]

The potential role of hemochromatosis gene mutations on iron regulation have been investigated in MDS patients, but no association with clinical outcomes could be established.[31]

Besides ineffective erythropoiesis and hepcidin regulation, transfusion therapy is the most important risk factor for iron overload in MDS patients, but it is at the same time a hallmark of supportive care in low and intermediate-1 risk MDS patients. Every unit of transfused blood contains about 200 mg of iron, so patients who require four RBC units per month will receive about 10 g iron per year, whereas natural iron losses are only 1 to 2 mg daily and the normal amount of body iron is 4 g. Therefore, given that iron overload can frequently develop in MDS patients, iron chelation therapy can be a useful tool to counteract this event and its harmful consequences.

Toxic Effects of Iron Overload

Iron overload may cause several toxic effects on different organs.

Heart disease, in particular arrhythmias and heart failure, is one of the more common causes of nonhematologic morbidity and mortality in MDS patients[32,33] who are, per se, more at risk of heart failure because of age and anemia.

Pascal et al demonstrated that 17–27% of transfusion-dependent MDS patients develop heart disease with T2* magnetic resonance imaging-documented iron overload.[34]

Furthermore, there is some evidence that clonal hematopoiesis and the consequent inflammatory state,[35–37] as well as vascular impairment induced by NTBI and ROS production predispose to atherosclerotic cardiovascular disease.[38–42]

The role of iron chelation therapy in delaying cardiac morbidity has been suggested by retrospective studies.[43]

The results of the only prospective study by Angelucci et al confirmed that iron chelation therapy with deferasirox is able to prolong significantly event free survival in patients with low- to intermediate-1-risk MDS compared to placebo.[4]

Iron overload first becomes evident in the liver, that is an iron storage organ, when hepatic stored iron is tenfold increased compared to normal concentration (15–20 mg/g dry weight vs normal: 2 mg/g dry weight).[44,45] Liver iron overload is present in about 80% of transfusion-dependent MDS patients and correlates to fibrosis and liver dysfunction, which lead to decreased overall survival and dismal prognosis. Iron chelation therapy can reduce the rate of hepatic-related deaths in MDS patients[46] and probably can prevent cirrhosis.[47]

MDS patients have an increased risk of infections compared to the general population because of cytopenias, in particular neutropenia and impairment of the immune function. Some studies show an increased incidence of bacterial, viral and fungal infections and infection-related mortality in transfusion-dependent MDS patients with signs of iron overlaod,[48] suggesting a role of iron overload, but the exact mechanism is not fully understood (Figure 1). Two main mechanisms are thought to be related to increased risk of infections and iron overload: free unbound iron (NTBI) can be used by pathogens for their growth and excess iron is able to impair immune function.[49]

Iron can impair the function of cellular immunity, in particular it has a detrimental effect on macrophages, neutrophils and lymphocytes and decreases the production of cytokines and nitric oxide involved in the regulation of the immune response.

There is some evidence that iron chelation therapy is able to delay the occurrence of infections and to decrease the incidence of infection-related mortality, but further studies are needed to clarify this point.[50–55]

Iron Chelation Therapy: Indications in Low-risk MDS Patients

As previously discussed, iron overload can rapidly develop in MDS patients and it is recognized to be toxic for several organs and even for the bone marrow niche, thus favoring ineffective erythropoiesis, accumulation of genomic alterations and increased risk of AML transformation.[3]

So, iron chelation therapy appears to be an important tool in MDS patients, in order to prevent or minimize the detrimental effects of iron overload.
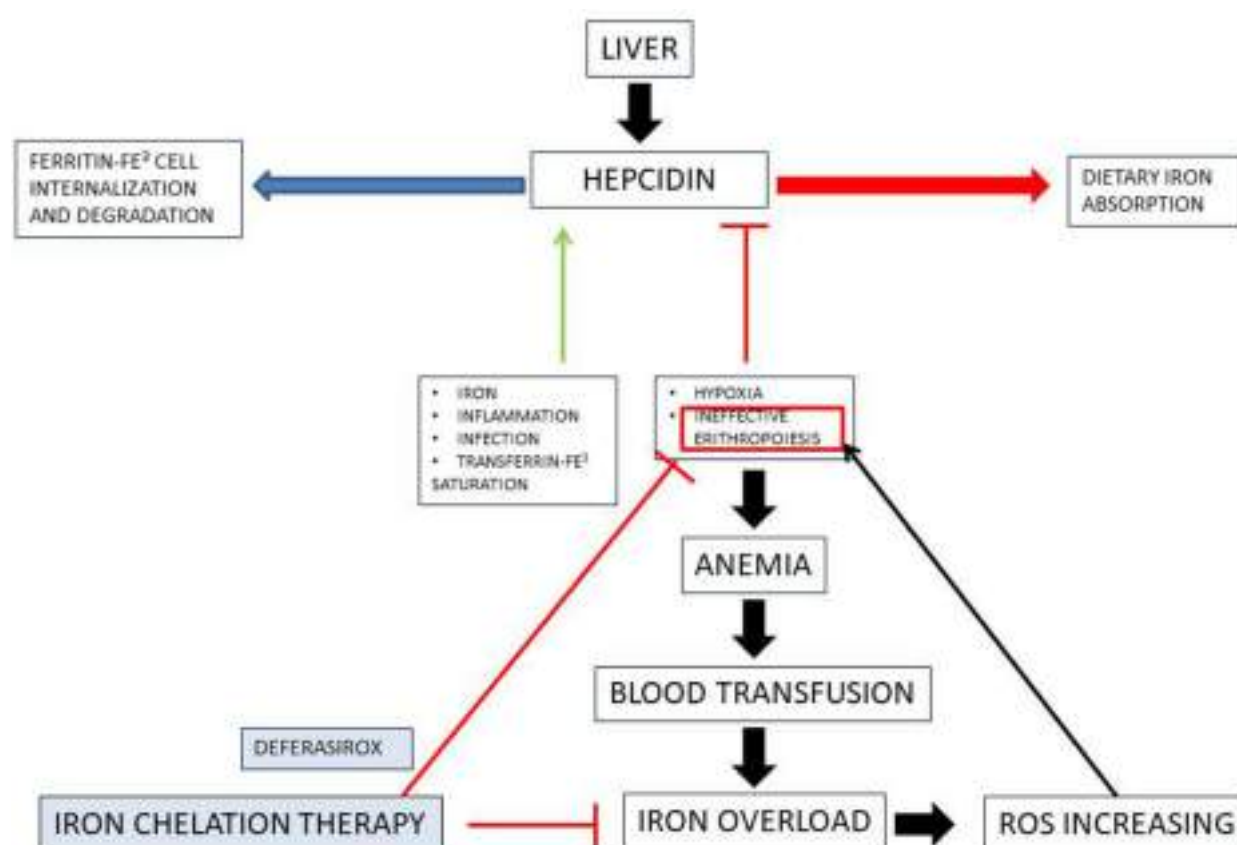
Figure 1 Iron overload and its effects in myelodysplastic syndrome.

Patients who could benefit from iron chelation therapy would be studied in order to demonstrate and evaluate the degree of iron overload and organ damage prior to therapy onset and during treatment. The diagnostic work-up includes several analyses, in particular it is important to establish the exact amount of the transfusion regimen and to calculate the consequent iron intake.

Serum ferritin and transferrin saturation represent important tools to evaluate iron overload and to monitor its trend during therapy. Even in the absence of validated data, a serum ferritin level above 1000 ng/mL is considered to be suggestive for iron overload, but it is recommended to evaluate ferritin together with transferrin saturation, as ferritin levels can be inappropriately high in inflammatory states and in the presence of hepatocellular necrosis.[56] Transferrin saturation levels above 60–70% are correlated with free iron in plasma as non-transferrin-bound iron (NTBI) and labile plasma iron (LPI), a subcomponent of NTBI, that is a potent redox-active form which causes the increase of intracellular radical oxygen species (ROS) with a consequent intracellular

oxidative stress.[57] Currently, standard tests to evaluate NTBI and LPI are not available for clinical practice.

The prospective EPIC study (evaluation of patients' iron chelation with exjade) was conducted in order to evaluate the possibility of using plasma markers other than serum ferritin (SF) to assess iron overload in transfusion-dependent thalassemia, sickle cell disease, and MDS. The study concluded that transferrin saturation (TfSat) is not a good marker of iron overload during chelation therapy while transferrin levels or total iron binding capacity (TIBC) or LPI could represent a valid tool for monitoring patients, even if further studies are needed for conclusive data.[58]

The assessment of organ damage is possible by performing magnetic resonance studies of the liver, heart and pancreas and can be repeated during therapy to evaluate its efficacy.

Based on these considerations, international guidelines recommend to start iron chelation therapy in low-risk MDS patients when one or more of these conditions are present:[5,59,60]

- Transfusion dependency and at least 20 RBC units transfused (currently, it is under discussion whether to start chelators before the 20 transfused units based on the observation that oxidative stress may be present even earlier)[61]

- Serum ferritin levels above 1000 ng/dL

- Life expectancy longer than 12 months

About discontinuation of chelators, current guidelines recommend to go on with therapy as long as the transfusional need is present.[62]

Currently, two different iron chelators are available for the treatment of iron overload in MDS patients: deferoxamine and deferasirox. These agents differ in route and timing of administration as well as side-effect profiles. We will explain these drugs in more detail in the paragraph "Iron chelation: options".

Iron Chelation Therapy: Indications in High-risk MDS Patients

High-risk MDS patients, according to IPSS, R-IPSS and WPSS prognostic scores, account for about 20–30% of the entire MDS population. After the introduction of disease-modifying therapies such as hypomethylating drugs the outcome among this cohort of patients improved significantly[63] and the impact of iron overload and iron toxicity became evident, with a significant number of deaths due to infectious complications and cardiac diseases.[64]

An increasing number of patients can be considered as candidates for iron chelation therapy, with the same indications regarding starting and discontinuation as low-risk MDS, even if some patients will not receive chelation therapy because of a short life expectancy or renal and/or hepatic impairment.

Rose et al published a retrospective study on 51 intermediate to very high risk, transfusion dependent, MDS patients (according to R-IPSS prognostic score) treated with deferasirox in association (71%) or not (29%) with azacitidine. Patients who received deferasirox had a significant decrease in serum ferritin and an improvement in liver function and in one case a hematological improvement was observed.[65] The authors concluded that the results of the study were comparable, in terms of safety and efficacy, with those observed in lower-risk MDS.

Several studies showed that deferasirox treatment can reduce infection incidence among high-risk MDS patients[6,66] and, more intriguingly, there is some evidence of a synergistic effect of deferasirox and hypomethylating agents, since it was observed that iron overload and

consequent oxidative stress is able to enhance hypermethylation of important tumor suppressor genes.[67]

On the basis of the abovementioned data, it could be appropriate to propose iron chelation therapy to intermediate and high-risk MDS patients if they have the following characteristics:

- Good performance status

- Age ≤65 years

- No significant comorbidities and adequate liver and renal function

- Patients candidates to receive disease-modifying therapies (hypomethylating agents and/or allogeneic stem cell transplantation).

Iron Chelation Therapy: Indications in MDS Patients Candidate for Allogeneic Stem Cell Transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative therapy for MDS patients, but only a minority of fit patients can be considered for this therapeutic option because of high risk of transplant-related mortality and morbidity due to the advanced age of most MDS patients.

Several studies confirmed that patients who have iron overload show an inferior outcome post HSCT[68,69] and a prospective, multicenter study (ALLIVE) demonstrated a significant correlation between high levels of NTBI at baseline and the incidence of non-relapse mortality and between NTBI positivity and inferior overall survival (OS).[70] It has been observed that conditioning regimen itself can increase NTBI levels and that NTBI/LPI levels drop after engraftment, when iron can be utilized again by restored erythropoiesis.

Given the detrimental effect of high levels of NTBI on transplant outcome, in terms of higher incidence of infections and poor engraftment, patients who are candidates for HSCT should receive iron chelation therapy pre and peritransplantation.[71,72]

In 2019 Cremers et al showed that the reduction of iron overload within six months after HSCT, and not prior to HSCT, can improve relapse-free survival (90% in chelated patients vs 56% in untreated patients).[73]

Iron Chelation Therapy: Options

Deferoxamine, the first approved iron chelator, is characterized by limited use in MDS patients because it can be administered only by intravenous, intramuscular, or

subcutaneous injection and it has a very short half-life. Therefore, to be effective it requires a prolonged daily subcutaneous or intravenous infusion of 8–12 h. For this reason the use of deferoxamine in MDS patients has been limited and there are no randomized studies, that were conducted only in beta-thalassemia patients.

Side effects are injection reactions and ocular and otic toxicity.[74]

Another iron chelator is deferiprone, which has a very limited use in MDS patients because it can be responsible for neutropenia as a major side effect.

The most widely used iron chelator in MDS patients is deferasirox (DFX). DFX is an N-substituted bis-hydroxyphenyl-triazole, a class of tridentate iron chelators and it is able to bind ferric ions forming a soluble complex with high binding affinity for iron in its trivalent form.[75–80] Preclinical and clinical studies demonstrated that DFX is two to five times more effective than deferoxamine in binding and excreting iron and that it is able to reduce liver and cardiac iron burden.[81–83]

Angelucci et al published the results of the only randomized, placebo-controlled, study—the TELESTO study—on the use of deferasirox in low and intermediate-1-risk MDS patients.[4]

The authors demonstrated that patients receiving DFX had a significantly longer median event free survival (EFS) than patients receiving placebo (approximately one year), even if they were unable to explain this result with a specific improvement in one of the main organs affected by iron overload.

Serum ferritin level monitoring seemed to be the most suitable way to evaluate clinical benefit of DFX. The study did not provide a conclusive answer about the observation that DFX can induce hematological improvement.[84] On the basis of the 2006 IWG criteria, hematological improvement was observed in 27 of 121 (22.3%) deferasirox recipients vs 14 of 68 (20.6%) placebo recipients; this analysis was performed excluding patients receiving concomitant, potential erythroid-modifying treatments.

The study also confirmed the safety of DFX, increased levels of serum creatinine being the most frequent adverse event.

Some concerns about the TELESTO study have to be reported. Even if the TELESTO trial had been designed as a Phase 3 randomized study, slow enrollment and high dropout forced investigators to downgrade it to Phase 2 trial providing only exploratory value of evidence regarding outcomes. Also the issue of EFS benefit was put in question,[85] since significant separation of EFS curves occurred only after three years of therapy when the study was profoundly affected by dropout and crossover between the two groups.

Several studies demonstrated that DFX can induce additional effects other than the decrease of serum ferritin levels and tissue deposition of iron. These additional effects are about overall survival, the risk of leukemia transformation, and hematological improvement.

The effect of iron chelation therapy in MDS patients on survival was shown by a number of retrospective studies and by a meta-analysis that reported the results of eight different studies involving 1562 patients: this meta-analysis demonstrated a significant advantage in terms of overall survival in chelated MDS patients vs nonchelated patients, with a mean difference in median OS of 61.2 months.[62] The results coming from a meta-analysis can be controversial because authors could only utilize observational studies (no randomized trials were available) and because some selection bias could have conditioned the results (better prognosis in patients receiving ICT, different drugs for chelation).

Leitch et al published a prospective trial which showed a better survival in MDS patients receiving ICT independently from age, R-IPSS score, comorbidities, and disease-modifying therapies.[86]

The IRON2 retrospective trial reported an increase in OS (133 vs 105 months; $p=0.009$) and an increase in cardiac event free survival (137 vs 90 months; $p=0.004$) in MDS patients receiving ICT (mostly DFX, 72%) than patients not treated with ICT.[87]

The results of the previously mentioned TELESTO study, further supported the significant effect of DFX on EFS, reporting a median EFS of 1440 days in the chelated patients vs 1091 days in patients not treated with DFX ($p=0.015$).[4]

Preclinical studies suggested that ICT can reduce the risk of leukemic evolution by decreasing the ROS production and consequent genetic instability.[88] A prospective study published by Lyons et al confirmed the positive effect of ICT, mostly with DFX, in reducing leukemic evolution and increasing leukemia free survival (LFS) in transfusion-dependent MDS patients.[89] In a cohort of 263 patients who received ICT the LFS was 40.6 months, compared to 27.3 months in nonchelated patients.

Several studies showed that a significant percentage of MDS patients treated only with DFX obtained an improvement of peripheral cytopenia that could be defined as

a hematological improvement according to the international working group response criteria.[90]

In the EPIC study (evaluation of patients' iron chelation with exjade) 10 to 20% of patients receiving DFX obtained a significant erythroid response.[91] Angelucci et al published the results of the study conducted by the Gruppo Italiano Malattie Ematologiche dell'adulto (GIMEMA) about the use of deferasirox in transfusion-dependent, lower-risk MDS patients: besides the reduction in serum ferritin of more than 36% following 12 months of therapy, a significant proportion of patients (11%) obtained transfusion independence.[92]

The multicenter trial by Nolte et al showed an hematological improvement in 18% of lower risk MDS patients receiving deferasirox, platelet response being the more frequent one (30%) and erythroid response the less frequent (6%).[93]

In the study by List et al 51 (28%) of 173 patients obtained hematological improvement according to international working group 2006 criteria.[94] Similar results came from two Italian registry data, the Roman Myelodysplasia Group (GROM) and the Basilicata Registry: among 118 transfusion-dependent MDS patients receiving DFX, 17.6% obtained an hematological improvement in the erythroid serie, 5.9% in platelets and 7.1 in neutrophils.[95]

Breccia et al published their data about 40 MDS patients who received deferasirox outside of clinical trials: efficacy in serum ferritin reduction and safety were confirmed even in association with other therapies (such as azacitidine); moreover, four patients had a reduction of transfusion requirement and a mean increase in Hb levels of 2 g/dL.[96]

The abovementioned data were consistent with results from Extend and EXjange studies, published by Gattermann et al. Treatment with deferasirox proved to be effective in serum ferritin levels reduction in chelation-naïve (p=0.0002) and prechelated patients (p=0.06).[97]

The main studies on the use of deferasirox in low-risk MDS patients are summarized in Table 1.

The biologic reason for this result is controversial, but the two main mechanisms that can explain the possibility of obtaining a hematological improvement with ICT are the reduction in ROS production and NFk-B pathway inhibition.

ICT protects from iron overload and from its detrimental effects, first of all oxidative stress that may damage nucleic acids, proteins and lipids. Rachmilewitz et al showed a decrease of ROS in red blood cells (RBC) after three months of chelation therapy with deferasirox in a cohort of 15 low-risk MDS patients.[98] Chan et al established a correlation between serum ferritin levels and ROS in $CD34^+$ cells in MDS patients.[99] These observations suggest a role of ICT in reducing oxidative stress by decreasing ferritin levels and a possible positive effect on hematopoiesis.

In the US03 trial 9.4% of MDS patients treated with deferasirox obtained an hematological improvement and in this cohort of patients a normalization of LPI values was seen after chelation therapy, this further suggesting a link between oxidative stress and peripheral cytopenias.[100]

The second pathway that could explain the role of ICT in the improvement of cytopenias is the reduction of NF-kB activity. NF-kB is a transcription factor involved in myelopoiesis and its role in the pathogenesis of MDS is fully described[101,102] as well as the efficacy of NF-kB inhibitors on leukemic cells. NF-kB pathway is activated by ROS, which are increased in transfusion-dependent MDS patients with iron overload.

Messa et al demonstrated that deferasirox, but not the other commercially available iron chelators, acts as a selective inhibitor of NF-kB in leukemic cells in vitro. The study suggests that deferasirox is able to inhibit NF-kB action independently from iron sequestering, but the exact cellular target is still unknown.[103]

These data pave the way to the use of iron chelation therapy in MDS patients not only for the treatment of secondary hemosiderosis, but also as a target therapy against malignant clones, in association with other therapies.

Conclusions

In the last few years multiple reviews have been published on the topic, so it is worth giving a perspective about the latest findings in the current review.

It is well known that the prognosis of patients with MDS is related both to the biological characteristics of the disease and, especially for lower-risk MDS, to age and comorbidities. It is also important to note that prognosis can be negatively affected by several factors, such as the severity of cytopenias and iron overload induced by red blood cell transfusions. The excess of iron not only is responsible for organ damage, but it is able to induce genomic instability and to modify the hematopoietic niche, favoring progression to acute leukemia.

Iron overload can be effectively treated with iron chelation therapy and, in particular with deferasirox, the use

Table 1 Principal Studies on Deferasirox in Low-risk MDS Patients

Author (Year)	Study Type	Patients N	Dosage (mg/kg/Day)	Ferritin at Baseline ng/mL (Median)	Ferritin at 12th Month ng/mL (Median)	p	Adverse Events	Erythroid Response (%)
Gattermann (2010) EPIC[91]	Phase IIIb prospective multicenter openlabel single-arm	341	10–30	2730	1904	0.002	GI, skin rash	22.6
Angelucci (2014) GIMEMA MDS0306[92]	Prospective, open-label, single-arm, multicenter trial	152	10–20	1966	1475	<0.0001	GI, renal	15.5
Nolte (2013)[93]	Open-label, single arm, multicenter trial	50	20–30	2447	1685	0.01	GI, renal, skin rash	11.0
List (2009)-US03[100]	Phase II, open-label	176	20	2771	2210	<0.001	GI, renal	15.0
Maurillo (2015)[95]	Retrospective	118	10–20	1773	1300	<0.001	GI, renal	19.0
Breccia (2012)[96]	Single-institution experience	40	10–30	2878	1400	<0.001	GI, skin rash	10.0
Gattermann (2012)-EXTEND[97]	Prospective, 1-year, noninterventional, observational, multicentre	123	10–20	2679	2000	0.0002	GI, renal, skin rash	NR
Gattermann (2012)-EXJANGE[97]	Prospective, 1-year, non-interventional, observational, multicenter	44	10–30	2442	2077	0.06	GI, renal, skin rash	NR

of which is recommended by international guidelines in lower-risk MDS patients, after the demonstration that it can significantly prolong event free survival and most likely also overall survival.

The observation that a number of MDS patients can obtain an improvement of peripheral cytopenias during therapy with deferasirox put increasing interest on this treatment and probably paved the way to new studies, in order to confirm this data. Further studies are also needed to evaluate and clarify the biological basis of hematological improvement induced by ICT in MDS patients.

Disclosure

Sarah Parisi: no conflict of interest to declare.

Carlo Finelli: Celgene BMS: research funding, advisory committees, speaker fees; Novartis: advisory committees, speaker fees; Takeda: consultancy.

References

1. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes [erratum appears in Blood 91:1100, 1998]. *Blood*. 1997;89:2079–2088. doi:10.1182/blood.V89.6.2079
2. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood*. 2012;120(12):2454–2456. doi:10.1182/blood-2012-03-420489
3. Leitch HA, Buckstein R, Zhu N, et al. Iron overload in myelodysplastic syndromes: evidence based guidelines from the Canadian consortium on MDS. *Leuk Res*. 2018;74:21–41. doi:10.1016/j.leukres.2018.09.005
4. Angelucci E, Li J, Greenberg P, et al. Iron chelation in transfusion-dependent patients with low- to intermediate-1–risk myelodysplastic syndromes. *Ann Intern Med*. 2020;172(8):513–522. doi:10.7326/M19-0916
5. Santini V, Alessandrino PE, Angelucci E, et al. Clinical management of myelodysplastic syndromes: update of SIE, SIES, GITMO practice guidelines. *Leuk Res*. 2010;34:1576–1588. doi:10.1016/j.leukres.2010.01.018
6. Musto P, Maurillo L, Simeon V, et al. Iron-chelating therapy with deferasirox in transfusion-dependent, higher risk myelodysplastic syndromes: a retrospective, multicentre study. *Br J Haematol*. 2017;177:741–750. doi:10.1111/bjh.14621

7. Fenaux P, Rose C. Impact of iron overload in myelodysplastic syndromes. *Blood Rev*. 2009;23(Suppl 1):S15–19. doi:10.1016/S0268-960X(09)70005-0

8. Malcovati L, Porta MG, Pascutto C, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. *J Clin Oncol*. 2005;23:7594–7603. doi:10.1200/JCO.2005.01.7038

9. Cortelezzi A, Cattaneo C, Cristiani S, et al. Nontransferrin-bound iron in myelodysplastic syndromes: a marker of ineffective erythropoiesis? *Hematol J*. 2000;1:153–158. doi:10.1038/sj.thj.6200028

10. Santini V, Girelli D, Sanna A, et al. Hepcidin levels and their determinants in different types of myelodysplastic syndromes. *PLoS One*. 2011;6:e23109. doi:10.1371/journal.pone.0023109

11. Ganz T, Nemeth E. Hepcidin and disorders of iron metabolism. *Annu Rev Med*. 2011;62:347–360. doi:10.1146/annurev-med-050109-142444

12. Pullarkat V. Objectives of iron chelation therapy in myelodysplastic syndromes: more than meets the eye? *Blood*. 2009;114:5251–5255. doi:10.1182/blood-2009-07-234062

13. Donovan A, Lima CA, Pinkus JL, et al. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab*. 2005;1:191–200. doi:10.1016/j.cmet.2005.01.003

14. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*. 2004;306:2090–2093. doi:10.1126/science.1104742

15. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell*. 2010;142:24–38. doi:10.1016/j.cell.2010.06.028

16. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med*. 2007;13:1096–1101. doi:10.1038/nm1629

17. Tanno T, Noel P, Miller JL. Growth differentiation factor 15 in erythroid health and disease. *Curr Opin Hematol*. 2010;17:184–190. doi:10.1097/MOH.0b013e328337b52f

18. Kroot JJ, Kemna EH, Bansal SS, et al. Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization. *Haematologica*. 2009;94:1748–1752. doi:10.3324/haematol.2009.010322

19. Castagna A, Campostrini N, Zaninotto F, Girelli D. Hepcidin assay in serum by SELDI-TOF-MS and other approaches. *J Proteomics*. 2010;73:527–536. doi:10.1016/j.jprot.2009.08.003

20. Winder A, Lefkowitz R, Ghoti H, et al. Urinary hepcidin excretion in patients with myelodysplastic syndrome and myelofibrosis. *Br J Haematol*. 2008;142:669–671. doi:10.1111/j.1365-2141.2008.07225.x

21. Murphy PT, Mitra S, Gleeson M, Desmond R, Swinkels DW. Urinary hepcidin excretion in patients with low grade myelodysplastic syndrome. *Br J Haematol*. 2009;144:451–452. doi:10.1111/j.1365-2141.2008.07455.x

22. Cui R, Gale RP, Zhu G, et al. Serum iron metabolism and erythropoiesis in patients with myelodysplastic syndrome not receiving RBC transfusions. *Leuk Res*. 2014;38:545–550. doi:10.1016/j.leukres.2014.01.016

23. Bondu S, Alary A-S, Lefèvre C, et al. A variant erythroferrone disrupts iron homeostasis in SF3B1-mutated myelodysplastic syndrome. *Sci Transl Med*. 2019;11:eaav5467. doi:10.1126/scitranslmed.aav5467

24. Zhu Y, Li X, Chang C, et al. SF3B1-mutated myelodysplastic syndrome with ring sideroblasts harbors more severe iron overload and corresponding over-erythropoiesis. *Leuk Res*. 2016;44:8–16. doi:10.1016/j.leukres.2016.02.011

25. Shenoy N, Vallumsetla N, Rachmilewitz E, et al. Impact of iron overload and potential benefit from iron chelation in low-risk myelodysplastic syndrome. *Blood*. 2014;124:873–881. doi:10.1182/blood-2014-03-563221

26. Vinchi F, Hell S, Platzbecker U. Controversies on the consequences of iron overload and chelation in MDS. *Hemasphere*. 2020;4(3):e357. doi:10.1097/HS9000000000000357

27. Jin S, Su H, Tran NT, et al. Splicing factor SF3B1K700E mutant dysregulates erythroid differentiation via aberrant alternative splicing of transcription factor TAL1. *PLoS One*. 2017;12:e0175523. doi:10.1371/journal.pone.0175523

28. Dolatshad H, Pellagatti A, Fernandez-Mercado M, et al. Disruption of SF3B1 results in deregulated expression and splicing of key genes and pathways in myelodysplastic syndrome hematopoietic stem and progenitor cells. *Leukemia*. 2015;29:1092–1103. doi:10.1038/leu.2014.331

29. Visconte V, Avishai N, Mahfouz R, et al. Distinct iron architecture in SF3B1-mutant myelodysplastic syndrome patients is linked to an SLC25A37 splice variant with a retained intron. *Leukemia*. 2015;29:188–195. doi:10.1038/leu.2014.170

30. Inokura K, Fujiwara T, Saito K, et al. Impact of TET2 deficiency on iron metabolism in erythroblasts. *Exp Hematol*. 2017;49:56–67 e5. doi:10.1016/j.exphem.2017.01.002

31. Fabiani E, Valentini L, Cilloni D, Voso MT. Could haemochromatosis (HFE) gene mutations affect response to iron chelation in myelodysplastic syndrome? - Response to Lucijanic and Kusec. *Br J Haematol*. 2019;186(4):639–640. doi:10.1111/bjh.15938

32. Della Porta MG, Kuendgen A, Malcovati L, et al. Myelodysplastic syndrome (MDS)-specific comorbidity index for predicting the impact of extra-hematological comorbidities on survival of patients with MDS. *Blood*. 2008;112:11. doi:10.1182/blood-2008-02-078170

33. Brunner AM, Blonquist TM, Hobbs GS, et al. Risk and timing of cardiovascular death among patients with myelodysplastic syndromes. *Blood Adv*. 2017;1:2032–2040. doi:10.1182/bloodadvances.2017010165

34. Pascal L, Beyne-Rauzy O, Brechignac S, et al. Cardiac iron overload assessed by T2* magnetic resonance imaging and cardiac function in regularly transfused myelodysplastic syndrome patients. *Br J Haematol*. 2013;162:413–415. doi:10.1111/bjh.12368

35. Fuster JJ, MacLauchlan S, Zuriaga MA, et al. Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science*. 2017;355:842–847. doi:10.1126/science.aag1381

36. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498. doi:10.1056/NEJMoa1408617

37. Swirski FK. From clonal haematopoiesis to the CANTOS trial. *Nat Rev Cardiol*. 2018;15:79–80. doi:10.1038/nrcardio.2017.208

38. Vinchi F, Porto G, Simmelbauer A, et al. Atherosclerosis is aggravated by iron overload and ameliorated by dietary and pharmacological iron restriction. *Eur Heart J*. 2019:1–16. doi:10.1093/eurheartj/ehy883

39. Xu S. Iron and atherosclerosis: the link revisited. *Trends Mol Med*. 2019;25:659–661. doi:10.1016/j.molmed.2019.05.012

40. Vinchi F, Muckenthaler MU, Da Silva MC, et al. Atherogenesis and iron: from epidemiology to cellular level. *Front Pharmacol*. 2014;5:1–20.3. doi:10.3389/fphar.2014.00094

41. Vinchi F, Simmelbauer A, Altamura S, et al. Iron accelerates cardiovascular disease: the importance of maintaining iron balance. *Atherosclerosis*. 2016;252:E241–E242. doi:10.1016/j.atherosclerosis.2016.07.030

42. Vinchi F. Non-transferrin-bound iron in the spotlight: novel mechanistic insights into the vasculotoxic and atherosclerotic effect of iron. *Antioxid Redox Signal*. 2021;35(6):387–414. doi:10.1089/ars.2020.8167

43. Wong CAC, Leitch HA. Delayed time from RBC transfusion dependence to first cardiac event in lower IPSS risk MDS patients receiving iron chelation therapy. *Leuk Res*. 2019;83:106170. doi:10.1016/j.leukres.2019.106170

44. Telfer PT, Prestcott E, Holden S, et al. Hepatic iron concentration combined with long-term monitoring of serum ferritin to predict complications of iron overload in thalassaemia major. *Br J Haematol*. 2000;110:971–977. doi:10.1046/j.1365-2141.2000.02298.x

45. Milic S, Mikolasevic I, Orlic L, et al. The role of iron and iron overload in chronic liver disease. *Med Sci Monit*. 2016;22:2144–2151. doi:10.12659/MSM.896494

46. Neukirchen J, Fox F, Kündgen A, et al. Improved survival in MDS patients receiving iron chelation therapy - a matched pair analysis of 188 patients from the Düsseldorf MDS registry. *Leuk Res*. 2012;36:1067–1070. doi:10.1016/j.leukres.2012.04.006

47. Metzgeroth G, Dinter D, Schultheis B, et al. Deferasirox in MDS patients with transfusion-caused iron overload—a phase-II study. *Ann Hematol*. 2009;88:301–310. doi:10.1007/s00277-008-0588-3

48. Girmenia C, Candoni A, Delia M, et al. Infection control in patients with myelodysplastic syndromes who are candidates for active treatment: expert panel consensus-based recommendations. *Blood Rev*. 2019;34:16–25. doi:10.1016/j.blre.2018.10.002

49. Ganz T. Iron and infection. *Int J Hematol*. 2018;107:7–15. doi:10.1007/s12185-017-2366-2

50. Wong CAC, Wong SAY, Leitch HA. Iron overload in lower international prognostic scoring system risk patients with myelodysplastic syndrome receiving red blood cell transfusions: relation to infections and possible benefit of iron chelation therapy. *Leuk Res*. 2018;67:75–81. doi:10.1016/j.leukres.2018.02.005

51. Kontoghiorghes GJ, Kolnagou A, Skiada A, et al. The role of iron and chelators on infections in iron overload and non iron loaded conditions: prospects for the design of new antimicrobial therapies. *Hemoglobin*. 2010;34:227–239. doi:10.3109/03630269.2010.483662

52. Thompson MG, Corey BW, Si Y, et al. Antibacterial activities of iron chelators against common nosocomial pathogens. *Antimicrob Agents Chemother*. 2012;56:5419–5421. doi:10.1128/AAC.01197-12

53. Neupane GP, Kim DM. In vitro time-kill activities of ciprofloxacinalone and in combination with the iron chelator deferasirox against Vibrio vulnificus. *Eur J Clin Microbiol Infect Dis*. 2010;29:407–410. doi:10.1007/s10096-010-0875-5

54. Lyons RM, Marek BJ, Paley C, et al. Relation between chelation and clinical outcomes in lower-risk patients with myelodysplastic syndromes: registry analysis at 5 years. *Leuk Res*. 2017;56:88–95. doi:10.1016/j.leukres.2017.01.033

55. Leitch HA. Optimizing therapy for iron overload in the myelodysplastic syndromes. *Drugs*. 2011;71:155–177. doi:10.2165/11585280-000000000-00000

56. Angelucci E, Pilo F. Iron chelation therapy in MDS – the final answer. *Clin Lymphoma Myeloma*. 2019;19(S1):S75–S76. doi:10.1016/j.clml.2019.07.425

57. Pilo F, Angelucci E. A storm in the niche: iron, oxidative stress and haemopoiesis. *Blood Rev*. 2018;32:29–35. doi:10.1016/j.blre.2017.08.005

58. Porter JB, El-Alfy M, Viprakasit V, et al. Utility of labile plasma iron and transferrin saturation in addition to serum ferritin as iron overload markers different underlying anemias before and after deferasirox treatment. *Eur J Haematol*. 2016;96(1):19–26. doi:10.1111/ejh.12540

59. Greenberg PL, Stone RM, Al-Kali A, et al. Myelodysplastic syndromes, version 2.2017, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2017;15:60–87. doi:10.6004/jnccn.2017.0007

60. Malcovati L, Hellstrom-Lindberg E, Bowen D, et al. Diagnosis and treatment of primary myelodysplastic syndromes in adults: recommendations from the European LeukemiaNet. *Blood*. 2013;122:2943–2964. doi:10.1182/blood-2013-03-492884

61. Bennett JM; Working Group on Transfusional Iron Overload. Consensus statement on iron overload in myelodysplastic syndromes. *Am J Hematol*. 2008;83:858–861. doi:10.1002/ajh.21269

62. Mainous AG, Tanner RJ, Hulihan MM, et al. The impact of chelation therapy on survival in transfusional iron overload: a meta-analysis of myelodysplastic syndrome. *Br J Haematol*. 2014;167:720–723. doi:10.1111/bjh.13053

63. Zeidan AM, Griffiths EA. To chelate or not to chelate in MDS: that is the question! *Blood Rev*. 2018;32:368–377. doi:10.1016/j.blre.2018.03.002

64. Goldberg SL, Chen E, Corral M, et al. Incidence and clinical complications of myelodysplastic syndromes among United States Medicare beneficiaries. *J Clin Oncol*. 2010;28:2847–2852. doi:10.1200/JCO.2009.25.2395

65. Rose C, Brechignac S, Vassilief D, et al. Does iron chelation therapy improve survival in regularly transfused lower risk MDS patients? A multicenter study by the GFM (Groupe Francophone des Myelodysplasies). *Leuk Res*. 2010;34:864–870. doi:10.1016/j.leukres.2009.12.004

66. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, Phase III study. *Lancet Oncol*. 2009;10:223–232. doi:10.1016/S1470-2045(09)70003-8

67. Goncalves AC, Cortesao E, Oliveiros B, et al. Oxidative stress levels are correlated with P15 and P16 gene promoter methylation in myelodysplastic syndrome patients. *Clin Exp Med*. 2016;16:333–343. doi:10.1007/s10238-015-0357-2

68. Platzbecker U, Bornhäuser M, Germing U, et al. Red blood cell transfusion-dependence and outcome after allogeneic peripheral blood stem cell transplantation in patients with de novo myelodysplastic syndromes (MDS). *Biol Blood Marrow Transplant*. 2008;14:1217–1225. doi:10.1016/j.bbmt.2008.08.006

69. Alessandrino EP, Della Porta MG, Bacigalupo A, et al. Prognostic impact of pre-transplantation transfusion history and secondary iron overload in patients with myelodysplastic syndrome undergoing. allogeneic stem cell transplantation: a GITMO study. *Haematologica*. 2010;95:476–484. doi:10.3324/haematol.2009.011429

70. Wermke M, Eckoldt J, Götze KS, et al. Enhanced labile plasma iron and outcome in acute myeloid leukaemia and myelodysplastic syndrome after allogeneic haemopoietic cell transplantation (ALLIVE): a prospective, multicentre, observational trial. *Lancet Haematol*. 2018;5:e201–e210. doi:10.1016/S2352-3026(18)30036-X

71. Petzer V, Wermke M, Tymoszuk P, et al. Enhanced labile plasma iron in hematopoietic stem cell transplanted patients promotes *Aspergillus* outgrowth. *Blood Adv*. 2019;3(11):1695–1700. doi:10.1182/bloodadvances.2019000043

72. Isidori A, Loscocco F, Visani G, et al. Iron toxicity and chelation therapy in hematopoietic stem cell transplant. *Transpl Cell Ther*. 2021;27:371379. doi:10.1016/j.jtct.2020.11.007

73. Cremers EMP, de Witte T, de Wreede L, et al. A prospective non interventional study on the impact of transfusion burden and related iron toxicity on outcome in myelodysplastic syndromes undergoing allogeneic hematopoietic cell transplantation. *Leuk Lymphoma*. 2019;60:2404–2414. doi:10.1080/10428194.2019.1594215

74. Mahesh S, Yelena Ginzburg AV. Iron overload in myelodysplastic syndromes. *Leuk Lymphoma*. 2008;49:427–438. doi:10.1080/10428190701843221

75. Stumpf JL. Deferasirox. *Am J Health Syst Pharm*. 2007;64(6):606–616. doi:10.2146/ajhp060405

76. Vanorden HE, Hagemann TM. Deferasirox – an oral agent for chronic iron overload. *Ann Pharmacother*. 2006;40(6):1110–1117. doi:10.1345/aph.1G566

77. Shashaty G, Frankewich R, Chakraborti T, et al. Deferasirox for the treatment of chronic iron overload in transfusional hemosiderosis. *Oncology*. 2006;20(14):1799–1806.

78. Barton JC. Chelation therapy for iron overload. *Curr Gastroenterol Rep*. 2007;9(1):74–82. doi:10.1007/s11894-008-0024-9

79. Piga A, Galanello R, Forni GL, et al. Randomized Phase II trial of deferasirox (Exjade, ICL670), a once-daily, orally-administered iron chelator, in comparison to deferoxamine in thalassemia patients with transfusional iron overload. *Haematologica*. 2006;91(7):873–880.

80. Nisbet-Brown E, Olivieri NF, Giardina PJ, et al. Effectiveness and safety of ICL670 in iron-loaded patients with thalassaemia: a randomised, double-blind, placebo-controlled, dose-escalation trial. *Lancet*. 2003;361(9369):1597–1602. doi:10.1016/S0140-6736(03)13309-0

81. Cappellini MD, Cohen A, Piga A, et al. A Phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with β-thalassemia. *Blood*. 2006;107(9):3455–3462. doi:10.1182/blood-2005-08-3430

82. Pennell DJ, Porter JB, Cappellini MD, et al. Deferasirox for up to 3 years leads to continued improvement of myocardial T2* in patients with β-thalassemia major. *Haematologica*. 2012;97 (6):842–848. doi:10.3324/haematol.2011.049957

83. Porter JB. Deferasirox: an effective once-daily orally active iron chelator. *Drugs Today*. 2006;42(10):623–637. doi:10.1358/dot.2006.42.10.1009901

84. Killick SB. Iron chelation therapy in low risk myelodysplastic syndrome. *Br J Haematol*. 2017;177:375–387. PMID: 28300275. doi:10.1111/bjh.14602

85. Lucijanic M, Lovrinov M, Skelin M. Iron chelation in transfusion-dependent patients with low- to intermediate-1-risk myelodysplastic syndromes. *Ann Intern Med*. 2020;173(7):595. doi:10.7326/L20-1055

86. Leitch HA, Parmar A, Wells RA, et al. Overall survival in lower IPSS risk MDS by receipt of iron chelation therapy, adjusting for patient-related factors and measuring from time of first red blood cell transfusion dependence: an MDS-CAN analysis. *Br J Haematol*. 2017;179:83–97. doi:10.1111/bjh.14825

87. Remacha AF, Arrizabalaga B, Villegas A, et al. Evolution of iron overload in patients with low-risk myelodysplastic syndrome: iron chelation therapy and organ complications. *Ann Hematol*. 2015;94:779–787. doi:10.1007/s00277-014-2274-y

88. Malcovati L, Della Porta MG, Cazzola M. Predicting survival and leukemic evolution in patients with myelodysplastic syndrome. *Haematologica*. 2006;91:1588–1590.

89. Lyons RM, Marek BJ, Paley C, et al. Comparison of 24-month outcomes in chelated and non-chelated lower-risk patients with myelodysplastic syndromes in a prospective registry. *Leuk Res*. 2014;38:149–154. doi:10.1016/j.leukres.2013.11.004

90. Cheson BD, Greenberg PL, Bennett JM, et al. Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. *Blood*. 2006;108(2):419–425.

91. Gattermann N, Finelli C, Porta MD, et al. Deferasirox in iron overloaded patients with transfusion-dependent myelodysplastic syndromes: results from the large 1-year EPIC study. *Leuk Res*. 2010;34:1143–1150. doi:10.1016/j.leukres.2010.03.009

92. Angelucci E, Santini V, Di Tucci AA, et al. Deferasirox for transfusion-dependent patients with myelodysplastic syndromes: safety, efficacy, and beyond (GIMEMA MDS0306 Trial). *Eur J Haematol*. 2014;92:527–536. doi:10.1111/ejh.12300

93. Nolte F, Hochsmann B, Giagounidis A, et al. Results from a 1-year, open-label, single arm, multi-center trial evaluating the efficacy and safety of oral Deferasirox in patients diagnosed with low and int-1 risk myelodysplastic syndrome (MDS) and transfusion-dependent iron overload. *Ann Hematol*. 2013;92:191–198. doi:10.1007/s00277-012-1594-z

94. List AF, Baer MR, Steensma DP, et al. Deferasirox reduces serum ferritin and labile plasma iron in RBC transfusion-dependent patients with myelodysplastic syndrome. *J Clin Oncol*. 2012;30:2134–2139. doi:10.1200/JCO.2010.34.1222

95. Maurillo L, Breccia M, Buccisano F, et al. Deferasirox chelation therapy in patients with transfusion-dependent MDS: a 'realworld' report from two regional Italian registries: gruppo Romano Mielodisplasie and Registro Basilicata. *Eur J Haematol*. 2015;95:52–56. doi:10.1111/ejh.12476

96. Breccia M, Fisinger P, Loglisci G, et al. Deferasirox treatment for myelodysplastic syndromes: "real-life" efficacy and safety in a single-institution patient population. *Ann Haematol*. 2012;91:1345–1349. doi:10.1007/s00277-012-1481-7

97. Gattermann N, Jarish A, Shlag R, et al. Deferasirox treatment of iron-overloaded chelation-naïve and prechelated patients with myelodysplastic syndromes in medical practice: results from the observational studies Extend and EXJjange. *Eur J Haematol*. 2012;88(3):260–268. doi:10.1111/j.1600-0609.2011.01726.x

98. Rachmilewitz E, Merkel D, Ghoti H, et al. Improvement of oxidative stress parameters in MDS patients with iron overload treated with deferasirox. *Blood*. 2008;112(11):A924–A925. doi:10.1182/blood.V112.11.2675.2675

99. Chan LSA, Buckstein R, Reis MD, et al. Iron overload and haematopoiesis in MDS: does blood transfusion promote progression to AML? *Blood*. 2008;112(11):928a. doi:10.1182/blood.V112.11.2685.2685

100. List AF, Baer MR, Steensma D, et al. Deferasirox (ICL670); Exjade) reduces serum ferritin (SF) and labile plasma iron (LPI) in patients with myelodysplastic syndromes (MDS). *Blood*. 2008;112(11):523a.

101. Guzman M, Neering SJ, Upchurch D, et al. Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood*. 2001;98 (8):2301–2307. doi:10.1182/blood.V98.8.2301

102. Carvalho G, Coquelle A, Vozenin MC, et al. NF-kappaB constitutes a potential therapeutic target in high-risk myelodysplastic syndrome. *Blood*. 2006;107(3):1156–1165. doi:10.1182/blood-2005-05-1989

103. Messa E, Carturan S, Maffè C, et al. Deferasirox is a powerful NF-κB inhibitor in myelodysplastic cells and in leukemia cell lines acting independently from cell iron deprivation by chelation and reactive oxygen species scavenging. *Haematologica*. 2010;95 (8):1308–1316. doi:10.3324/haematol.2009.016824
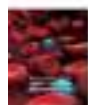
Clinical Utility of Subcutaneous Factor VIII Replacement Therapies in Hemophilia A: A Review of the Evidence

Yesim Dargaud & Maissa Janbain

Published online: 07 Dec 2021.

Dovepress

REVIEW

Clinical Utility of Subcutaneous Factor VIII Replacement Therapies in Hemophilia A: A Review of the Evidence

Yesim Dargaud [1,2]
Maissa Janbain [3]

[1]UR4609 Hemostase et Thrombose, Université Claude Bernard Lyon 1, Faculté de Médecine Lyon Est, Lyon, France; [2]Unité d'Hémostase Clinique, Hôpital Cardiologique Louis Pradel, Hospices Civils de Lyon, Lyon, France; [3]Hematology Department, Tulane School of Medicine, New Orleans, LA, USA

Abstract: Hemophilia therapies have tremendously improved over the last decades with the development of prolonged half-life factor VIII (FVIII) and FIX concentrates, non-factor therapies, such as emicizumab, anti-TFPI antibodies or siRNA antithrombin and gene therapy. All of these new molecules significantly reduced the burden of the disease and improved the quality of life of patients with severe hemophilia. Emicizumab, a non-factor therapy, is currently the only subcutaneous molecule available for prophylactic treatment of severe hemophilia A. Because of the subcutaneous route of delivery and similar efficacy to FVIII replacement therapy, emicizumab has been rapidly adopted by patients and their families. This clinical observation emphasizes the relevance and need for the development of subcutaneous FVIII concentrates. Here, we report evidence-based advantages and interest in the subcutaneous route of administration for the treatment of hemophilia A and review the stages of development of the different subcutaneous FVIII molecules.

Keywords: hemophilia A, factor VIII, recombinant von Willebrand factor fragment, prophylaxis, subcutaneous injection

Therapeutic Progress in Hemophilia A

Hemophilia A (HA) is an X-linked inherited bleeding disorder resulting from the partial or total deficiency of coagulation factor VIII (FVIII). Severe HA, characterized by a complete plasma FVIII deficiency, is a rare disease with a prevalence of 6 per 100,000 males.[1] Patients with severe HA have a lifelong spontaneous bleeding tendency with frequent joint bleeds, muscle hematomas, intracranial hemorrhages, and hematuria occurring even in the absence of any noticeable trauma. Repeated joint bleeds lead to chronic arthropathy responsible for limitation of movements, pain, disability and decrease in patients' quality of life.

Early prophylaxis is currently the standard of care for severe HA because it significantly reduces the risk of life-threatening bleeding, hemarthroses and joint damage, compared to on-demand therapy, which consists of treating bleeding episodes.[2,3] The need for frequent intravenous injections, due to the short half-life of FVIII molecule, ie 10–14 hours, has been one of the major barriers to the widespread use of prophylaxis, associated with difficult vein access and affordability. Thus, in the last decades extended half-life (EHL) coagulation factors have been developed to reduce the number of intravenous injections. Conjugation with

Correspondence: Yesim Dargaud
UR4609 Hemostase&Thrombose, Université Claude Bernard Lyon 1, Faculte de Medecine Lyon Est, 7, rue Guillaume Paradin, Lyon, 69008, France
Tel +3378778797
Email ydargaud@univ-lyon1.fr

polyethylene glycol (PEG) and fusion with albumin or Fc fragment of IgG1 have been used to generate several EHL products.[4] Pharmacokinetic (PK) improvements obtained with EHL-FVIII products were 1.3 to 1.8 fold-increased terminal half-life, mean trough FVIII levels between 1 and 3 IU/dL with infusions every 3–5 days and 30% reduction in the number of intravenous injections.[5] These characteristics improved patient adherence to prophylaxis, quality of life of patients and their families and opened new perspectives for more personalized prophylaxis regimens.[6] Despite the advantages of EHL-FVIII, it is to be noted that the improvements of PK for EHL-FVIII were modest compared to EHL-factor IX products produced with similar technologies and having 3 to 5 times-prolonged half-life. This difference is explained by the fact that EHL-FVIII molecules can still interact with endogenous von Willebrand factor (VWF), which has a relatively short half-life (~15 hours) and are cleared as part of the FVIII-VWF complex.[7] The experience with the first EHL molecules allowed a better understanding of the mechanisms responsible for the prolonged half-life and led to the development of new molecules. Thus, a novel recombinant FVIII molecule BIVV001 was recently bioengineered to overcome this limited half-life extension issue imposed by VWF.[8] BIVV001 is an EHL-FVIII fused to Fc fragment coupled with the FVIII binding D'D3 domain of VWF and two XTEN linkers aiming to reduce degradation and clearance.[9] A recent Phase 1–2a open-label clinical trial reported a significantly prolonged half-life of BIVV01 that was up to four times the half-life associated with standard recombinant FVIII (rFVIII), allowing an FVIII prophylaxis with a weekly treatment interval.[10]

Despite the improvements achieved, the exclusive intravenous route of administration of EHL-FVIII products remains a substantial source of burden for patients and caregivers. To date, only one non-factor therapy, emicizumab, has been administered subcutaneously. Emicizumab is a bispecific humanized monoclonal antibody mimicking activated FVIII activity into the tenase complex.[11] The molecule, designed for prophylaxis with once a week to once a month regimens and delivered subcutaneously, showed similar effectiveness to reference molecules in patients with and without inhibitors.[12] The transition from factor concentrates administered intravenously to a subcutaneous route of administration with emicizumab has been a strong argument in favor of the new drug for patients and their families.

Novel Routes for the Administration of Coagulation Factors

For a medication to be effective it must be administered appropriately. The route of administration of a drug affects its bioavailability, which determines both the onset and the duration of the pharmacological effect. The vast majority of treatments available for hemophilia are usually delivered intravenously. However, some subcutaneous drugs are used in certain situations, and research is very active for the development of new molecules with more convenient routes of administration.

The hemostatic drug desmopressin (DDAVP) is commonly and effectively used in most cases of mild or moderate bleedings of patients with mild HA. DDAVP can be administered intravenously, subcutaneously and as a nasal spray.[13] The nasal spray and subcutaneous DDAVP are very convenient for home treatment. Knowledge accumulated through research on EHL-factors and their interaction with neonatal Fc receptor (FcRn) might open new perspectives for nasal delivery of coagulation factors to treat hemophilia. It is known that the prolonged half-life of these molecules is related to the FcRn-mediated recycling of the modified FVIII/FIX molecules. FcRn is expressed in the nasal airway and the olfactory epithelium, and we may speculate that FcRn may play a role in Fc-mediated transport of IgG or albumin fused to FVIII/FIX across the nasal epithelium and might have significant potential for intranasal delivery.

Recently, Nichols et al[14] reported the first experience of orally delivered FVIII in a HA dog model. The authors evaluated safety and efficacy of delivering FVIII via orally ingestible robotic pills. Results were compared to a similar dose of FVIII 150 IU/kg administered intravenously or intraperitoneally. This early preclinical study showed that orally delivered FVIII restored hemostasis in HA dog. While waiting for the maturation of this work clinically, the progress achieved in adopting the subcutaneous route remains appealing.

Subcutaneous Coagulation Factors for the Treatment of Hemophilia A with or without Inhibitors

Subcutaneous deliveries of biotherapeutics for hemophilia, such as emicizumab or fitusiran (small interfering RNA that knocks down the synthesis of antithrombin), are convenient alternatives to intravenously delivered factor VIII. The first subcutaneous monoclonal antibodies were

developed and labeled in oncology. These drugs became rapidly preferred by patients as they reduced the burden of intravenous administrations. Subcutaneously delivered drugs have been proven effective, safe and are associated with lower healthcare costs,[15] even though they have different pharmacokinetic profiles compared to their peers administered intravenously. Maximum serum concentrations (C max) of these molecules are usually delayed because of a slow absorption rate from the subcutaneous tissue. In addition, a linear relationship was reported between molecular weight (MW) of biotherapeutics and the rate of lymphatic absorption. Molecules with MW greater than 16,000 Da are first absorbed by lymphatic vessels and pass through the thoracic duct, whilst smaller molecules cross directly through the capillary wall into the blood stream.[16] Human FVIII is a large molecule with an MW of 330,000 Da; B-domain deleted rFVIII is a metal ion linked 80,000- and 90,000- Da heterodimer.[17]

The first attempt of subcutaneous FVIII administration was reported in the late 1990s by Spira et al.[18] However, subcutaneous delivery of standard FVIII resulted in limited bioavailability: 5% to 10% in several animal models. In a mice model, Shi et al[19] confirmed this observation. The authors investigated whether subcutaneously administered FVIII could be transferred from extra-vascular space into the vascular space. They did not detect FVIII in the plasma following subcutaneous injection.

Fatouros et al studied the mechanisms underlying the low bioavailability of subcutaneous FVIII.[20] They concluded that subcutaneous rFVIII was inactivated in the subcutaneous tissue by proteolytic degradation, after binding to phospholipids. Von Willebrand (VWF) factor and FVIII circulate in blood as a non-covalently linked complex. VWF protects FVIII from proteolytic degradation by phospholipid-binding proteases like activated protein C.[21] This protection is due to the binding of D3 domain of the VWF molecule to the C1 domain of FVIII.

In the light of this knowledge, small recombinant VWF fragments were bioengineered to protect FVIII from phospholipid binding. Among the bioengineered VWF fragments, the best bioavailability results were obtained with vWF-12 construct. In a recent preclinical study, FVIII was co-administered with this "protector" recombinant VWF fragment to mini-pigs[22] and to factor VIII knock-out mice.[23] Solecka-Witulska et al[22] expressed the vWF-12 fragment as a dimer of the VWF-D'D3 region containing amino acids 1 to 1268 of the VWF sequence fused to a highly O-glycosylated 31 amino acids sequence repeated

twice at the C terminus. This VWF fragment has binding capacity to FVIII with high affinity (in the nM range) and inhibits the interaction between FVIII and phospholipids, which supports subcutaneous uptake of FVIII. Aachener mini-pigs received 100 IU/kg rFVIII co-delivered with VWF-12 fragment. Blood was taken prior, and 11 blood samples were taken at different time points up to 120 h after drug administration, and FVIII antigen concentrations were measured in each sample. The authors showed that the bioavailability of rFVIII alone was 2% whilst the co-administration of rFVIII with VWF-12 fragment increased the bioavailability of rFVIII to 41%.

Recently, Vollack-Hesse et al[23] compared pharmacokinetic (PK) profiles after a single dose of rFVIII administered intravenously (200 IU/kg) or subcutaneously (1000 IU/kg) in the presence or absence of the VWF-12 construct in a HA mice model. They confirmed enhanced bioavailability (up to 18.5%) of subcutaneous rFVIII co-administered with VWF-12. As expected, PK profiles of intravenous and subcutaneous rFVIII were very different. Subcutaneous FVIII had a slow absorption leading to a plasma Cmax 6 hours after drug administration and had 2.5-fold longer half-life compared to intravenous rFVIII. Pharmacodynamics of the co-administered rFVIII with vWF-12 fragment was evaluated with tail-clip assay that showed an efficient protection against bleeding during 24 hours after a single subcutaneous injection. These promising results, obtained using VWF-12 fragment as a chaperone to FVIII for subcutaneous administration, open new perspectives for the development of novel subcutaneous rFVIII molecules and an opportunity for patients with severe HA who may have replacement therapy with a subcutaneous rFVIII.

Subcutaneous delivery of coagulation factors was always feared for concerns about immunogenicity, particularly for FVIII. The mice data reports that the formation of anti-FVIII IgG antibodies did not increase after subcutaneous administration compared to intravenous route, which plays in favor of this approach.

Preclinical studies with glycopegylated rFVIII N8-GP in HA mice and cynomolgus monkeys showed improved bioavailability of the molecule compared to standard half-life FVIII.[24] Using this EHL-rFVIII molecule, Klamroth et al[25] reported the results of the first human subcutaneous rFVIII trial. This phase 1, double-blinded, multicenter trial had two parts: in the first part, patients received a single subcutaneous (SQ) dose (12.5–25-50 or 100 IU/kg) of turoctocog alfa pegol (N8-GP). The second part was

a multiple dose study and patients received 2000 or 4000 IU SQ. N8-GP daily during 3 months. Administered dose was dependent on the patient's body weight (below or ≥60 kg respectively). The authors studied pharmacokinetics, safety and preliminary efficacy of SQ N8-GP in previously treated patients with severe HA. The multiple dose study reported FVIII activity levels of 11.9% (9.0 to 15.6) at week 1 and 9.6% (7.3 to 12.7) at week 13 of the study. Five of the 26 patients (19.2%) developed non-neutralizing anti-N8-GP binding antibodies and 1 patient developed inhibitors to FVIII.

In parallel, a bioengineered subcutaneous factor VIIa has also been developed (activated marzeptacog alfa).[26] Activated recombinant factor VII (rFVIIa), administered intravenously, is commonly used for the treatment of hemophilia with inhibitors. The molecule has a very short half-life of 2.3 hours (range 1.7–2.7). Activated marzeptacog alfa (MarzAA) is a variant of rFVIIa with 4 amino acids substitutions. Two of the substitutions (Q286R and M298Q in the heavy chain) increase the catalytic activity of the molecule for factor X activation and two others (T128N and P129A, in the light chain) provide extended duration of biological activity. Recently, the results of a Phase 2 study investigating the prophylactic efficacy of subcutaneous MarzAA in hemophiliacs with inhibitors were published.[27] All patients had a baseline annualized bleeding rate (ABR) ≥12 events/year, as determined during the 6-months pre-treatment period. MarzAA was administered subcutaneously at a daily dose of 30μg/kg for 50 days. The volume of subcutaneous injection was low (0.6 mL). Subcutaneous MarzAA prophylaxis reduced mean ABR from 19.8 (range 12.2–26.7) to 1.6 (p = 0.009).

Strengths and Weaknesses of Subcutaneous FVIII Molecules

The rationale behind the design of subcutaneous FVIII molecules is to overcome the difficulties of intravenous delivery. While peripheral venipuncture is the first choice for venous access, central venous access devices are frequently used to facilitate repeated administration of clotting factor concentrates, particularly in very young children and adults with poor IV access. Subcutaneous route can also reduce the need for central venous access devices and their common complications, such as infection and thrombosis.[28] The development of subcutaneous FVIII concentrates may improve prophylaxis feasibility and patient's adherence.

Each intravenous injection of prophylaxis that cannot be performed increases the risk of breakthrough bleeds.

In addition, there are several advantages of subcutaneous FVIII concentrates over non-factor therapies that are delivered subcutaneously. These benefits include:

- The possibility of laboratory monitoring with usual, wildly available, easy to perform and cheap routine FVIII assays. The utility of laboratory monitoring is obvious in certain situations such as surgical settings and to individualize therapy and tailor it to patient's needs and lifestyle.

- No need to administer other procoagulant molecules in case of acute bleeding or surgery. This need persists with nonfactor therapy in some circumstances. Adding to the complexity of this scenario, where monitoring poses a major problem and concerns about interference between the two drugs persist, patients used to subcutaneous injections may lose with time the expertise and autonomy of intravascular administration. Subcutaneous FVIII molecules may help to overcome this issue.

- Non-factor therapies do not cover any potential non-hemostatic role of FVIII, and the long-term impact of nonfactor replacement therapy in patients with no inhibitors is still unclear. In addition to the concerns about the maintenance of immunological tolerability, the effect of such therapies on joint health remains the focus of ongoing research. Indeed, several clinical studies have described osteopenia in patients with HA. Twenty-seven per cent of patients with severe HA experience osteoporosis, responsible for a significant risk of fracture. It has been demonstrated that FVIII replacement partly prevents the loss of bone mineral density in patients with severe HA. Some authors suggest that the effect of FVIII on bone might be associated with the regulation of RANKL–OPG, which can influence the synthesis of osteoclasts and therefore bone resorption.[29] Thus, if these preliminary data are confirmed in well-designed clinical studies, FVIII replacement therapy may have a relevant advantage over nonfactor therapies.

In addition, the main advantages of subcutaneous FVIII concentrates over intravenous FVIII concentrates are:

- better quality of life and comfort for patients and their families
- no need for central venous access devices, allowing to start conveniently prophylaxis at very early age
- better adherence of patients to treatment and reduced breakthrough bleeds

1034

Journal of Blood Medicine 2021:12

- highest autonomy of patients with their treatment and lower costs
- potential lower immunogenicity as suggested by pre-clinical studies: decreased immunogenicity observed in animal models with co-delivery of rFVIII and VWF-12 fragment is promising. Some previous studies reported that VWF protects FVIII against endo-cytosis by dendritic cells, and reduces the presentation of FVIII to immune cells. Co-administration of FVIII with a particular VWF fragment may potentially reduce the recognition of FVIII by the immune system. If confirmed in clinical trials, this will allow to study whether SQ FVIII with VWF can be optimal to establish immune tolerance to FVIII in previously untreated patients, especially those at high risk of inhibitor development.

However, subcutaneous route of administration may also have some disadvantages. Large volumes of injection may be an issue in young children and may require multiple SQ injections, which is a clear source of discomfort for patients.

Conclusion

In conclusion, the development of subcutaneous FVIII molecules is of great clinical interest, reducing tremendously the burden of prophylaxis in severe HA patients, and therefore improving treatment adherence with subsequent reduction in breakthrough bleeds and better joint protection. However, even though a subcutaneous injection is easier to perform than an intravenous injection, it remains a painful procedure. Other administration routes that are less painful in nature and more practical for a busy school or professional schedule, such as the oral or intranasal route, may in the future expand the recent improvements offered by the subcutaneous route.

Disclosure

References

1. Iorio A, Stonebracker JS, Chambost H, et al. Establishing the prevalence and prevalence at birth of hemophilia in males: a meta-analytic approach using national registries. *Ann Intern Med*. 2019;171:540–546. doi:10.7326/M19-1208
2. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357:535–544. doi:10.1056/NEJMoa067659
3. Gringeri A, Lundin B, von Mackensen S, Mantovani L, Mannucci PM; ESPRIT Study Group. A randomized clinical trial of prophylaxis in children with hemophilia A (the ESPRIT Study). *J Thromb Haemost*. 2011;9:700–710. doi:10.1111/j.1538-7836.2011.04214.x
4. Mannucci PM. Hemophilia therapy: the future has begun. *Haematologica*. 2020;105:545–553. doi:10.3324/haematol.2019.232132
5. Mancuso ME, Mahlangu JN, Pipe SW. The changing treatment landscape in haemophilia: from standard half-life clotting factor concentrates to gene editing. *Lancet*. 2021;397:630–640. doi:10.1016/S0140-6736(20)32722-7
6. Oldenburg J, Hay CRM, Jiménez-Yuste V, et al. Design of a prospective observational study on the effectiveness and real-world usage of recombinant factor VIII Fc (rFVIIIFc) compared with conventional products in haemophilia A: the A-SURE study. *BMJ Open*. 2019;9(5):e028012. doi:10.1136/bmjopen-2018-028012
7. Pipe SW, Montgomery RR, Pratt KP, Lenting PJ, Lillicrap D. Life in the shadow of a dominant partner: the FVIII-VWF association and its clinical implications for hemophilia A. *Blood*. 2016;128(16):2007–2016. doi:10.1182/blood-2016-04-713289
8. Chhabra ES, Liu T, Kulman J, et al. BIVV001, a new class of factor VIII replacement for hemophilia A that is independent of von Willebrand factor in primates and mice. *Blood*. 2020;135:1484–1496. doi:10.1182/blood.2019001292
9. Fuller JR, Knockenhauer KE, Leksa NC, Peters RT, Batchelor JD. Molecular determinants of the factor VIII/von Willebrand factor complex revealed by BIVV001 cryo-electron microscopy. *Blood*. 2021;137:2970–2980. doi:10.1182/blood.2020009197
10. Konkle B, Shapiro AD, Quon DV, et al. BIVV001 fusion protein as factor VIII replacement therapy for hemophilia A. *N Engl J Med*. 2020;383:1018–1027. doi:10.1056/NEJMoa2002699
11. Oldenburg J, Mahlangu JN, Kim B, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377:809–818. doi:10.1056/NEJMoa1703068
12. Rodriguez-Merchan EC, Valentino AL. Emicizumab: review of the literature and critical appraisal. *Haemophilia*. 2019;25:11–20. doi:10.1111/hae.13641
13. Noye J, Rowell JA, Zeissink B, Connolly A, Mason JA. A fixed 'single vial' dose of subcutaneous desmopressin (DDAVP) produces adequate biologic responses for persons with mild haemophilia A. *Haemophilia*. 2021;27(4):e540–e542. doi:10.1111/hae.14287
14. Hashim MA, Yamaguchi A, Vo AT, et al. Orally delivered factor VIII restores hemostasis in a hemophilia A dog. ISTH 2021 Congress. Abstract number: OC 48.4.
15. Bittner B, Richter W, Schmidt J. Subcutaneous administration of biotherapeutics: an overview of current challenges and opportunities. *BioDrugs*. 2018;32:425–440. doi:10.1007/s40259-018-0295-0
16. Supersaxo A, Steffen WRH, Steffen H. Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. *Pharm Res*. 1990;7:167–169. doi:10.1023/A:1015880819328
17. Lind P, Larsson K, Spira J, et al. Novel forms of B-domain deleted recombinant factor VIII molecules. Construction and biochemical characterization. *Eur J Biochem*. 1995;232:19–27. doi:10.1111/j.1432-1033.1995.tb20776.x

18. Spira J, Widlund L, Österberg T, et al. Pharmaceutical formulation for subcutaneous, intramuscular or intradermal administration of factor VIII of factor IX. International patent application: US patent US5925739A, European patent EP0772452. International filing. 1995 March 30.

19. Shi Q, Kuether EL, Schroeder JA, Fahs SA, Montgomery RR. Intravascular recovery of VWF and FVIII following intraperitoneal injection and differences from intravenous and subcutaneous injection in mice. *Haemophilia*. 2012;18:639–646. doi:10.1111/j.1365-2516.2011.02735.x

20. Fatouros A, Liden Y, Sjostrom B. Recombinant factor VIII SQ-stability of FVIII:C in homogenates from porcine, monkey and human subcutaneous tissue. *J Pharm Pharmacol*. 2000;52:797–805. doi:10.1211/0022357001774651

21. Sadler JE. New concepts in von Willebrand disease. *Annu Rev Med*. 2005;56:173–191. doi:10.1146/annurev.med.56.082103.104713

22. Solecka-Wituska BA, Wuschko S, Kannicht C. Pharmacokinetics of subcutaneously administered simoctocog alfa with von Willebrand factor fragment candidates ina minipig model. *Blood*. 2017;130(9):1073. doi:10.1182/blood-2017-07-793356

23. Vollack-Hesse N, Olshko O, Werwitzke S, Solecka-Witulska B, Kannicht C, Tiede A. Recombinant VWF fragments improve bioavailability of subcutaneous factor VIII in hemophilia A mice. *Blood*. 2021;137:1072–1081. doi:10.1182/blood.2020006468

24. Rode F, Almholt K, Petersen M, et al. Preclinical pharmacokinetics and biodistribution of subcutaneously administered glycoPEGylated recombinant factor VIII (N8-GP) and development of a human pharmacokinetic prediction model. *J Thromb Haemost*. 2018;16:1141–1152. doi:10.1111/jth.14013

25. Klamroth R, Feistritzer C, Friedrich U, et al. Immunogenicity, safety, pharmacokinetics and preliminary efficacy results from the first human subcutaneous factor VIII trial (subcutaneous turoctocog alfa pegol, SC N8-GP) in previously treated patients with hemophilia A (Alleviate 1). *J Thromb Haemost*. 2020;18:341–351. doi:10.1111/jth.14660

26. Pittman D, Weston S, Shields K, et al. A novel FVIIa variant with increased potency and duration of effect compared to wildtype FVIIa. A study in a dog model of hemophilia A. *Blood*. 2011;118:2252. doi:10.1182/blood.V118.21.2252.2252

27. Mahlangu J, Levy H, Kosinova MV, et al. Subcutaneous engineered factor VIIa marzeptacog alfa (activated) in hemophilia with inhibitors: phase 2 trial of pharmacokinetics, pharmacodynamics, efficacy, and safety. *Res Pract Thromb Haemost*. 2021;5:e12576. doi:10.1002/rth2.12576

28. Titapiwatanakun R, Moir C, Pruthi RK, Stavlo PL, Schmidt KA, Rodriguez V. Central venous access devices for paediatric patients with haemophilia: a single-institution experience. *Haemophilia*. 2009;15:168–174. doi:10.1111/j.1365-2516.2008.01906.x

29. Cadé M, Muñoz-Garcia J, Babuty A, et al. FVIII at the crossroad of coagulation, bone and immune biology: emerging evidence of biological activities beyond hemostasis. *Drug Discov Today*. 2021. doi:10.1016/j.drudis.2021.07.015

Macrophage Activation Led Acute Heart Failure Managed Successfully with Immunosuppression

Karan Seegobin, Muhamad Alhaj Moustafa, Umair Majeed, Jordan C Ray, Marwan Shaikh, Liuyan Jiang & Han W Tun

CASE SERIES

Macrophage Activation Led Acute Heart Failure Managed Successfully with Immunosuppression

Karan Seegobin [iD][1]
Muhamad Alhaj Moustafa [iD][1]
Umair Majeed[1]
Jordan C Ray[2]
Marwan Shaikh[1]
Liuyan Jiang [iD][3]
Han W Tun [iD][1]

[1]Department of Hematology and Medical Oncology, Mayo Clinic, Jacksonville, FL, 32224, USA; [2]Departmentof Cardio-Oncology, Mayo Clinic, Jacksonville, FL, 32224, USA; [3]Department of Pathology, Mayo Clinic, Jacksonville, FL, 32224, USA

Abstract: Macrophage activation leading to multi-organ dysfunction/failure has been described in various hematologic disorders like hemophagocytic lympho-histiocytosis (HLH), also known as macrophage activation syndrome (MAS) and macrophage activation like syndrome (MALS). Congestive heart failure (CHF) appears to be an uncommon manifestation of macrophage activation. This novel entity of macrophage activation-associated cytokine-mediated CHF has not been well reported in the medical literature. We report two young female patients with acute CHF secondary to macrophage activation-associated cytokine storm. An extensive diagnostic workup was negative for other etiologies, such as ischemia, myocarditis, or infections. Their clinical, laboratory, and pathologic findings did not meet the diagnostic criteria for hemophagocytic syndrome (HPS)/MAS. However, both had laboratory and pathologic findings which were consistent with macrophage activation and cytokine storm. One patient met criteria for MALS. Therapeutically, our patients were promptly treated with steroids with or without anti-cytokine therapy with rapid restoration of cardiac function. Macrophage activation-induced disease may not always fulfil the diagnostic criteria for the currently known macrophage activation disorders. We suggest that markers of macrophage activation and cytokine levels should be part of the diagnostic workup in patients with otherwise unexplained acute CHF. Additional research is warranted to further elucidate the underlying mechanism of this disorder.

Keywords: macrophage activation syndrome, MAS, macrophage activation like syndrome, MALS, cytokine release storm, CRS, reversible systolic heart failure, immunosuppression

Plain Language Summary

Macrophages are cells that surround and kill microorganisms, remove dead cells, and stimulate the action of other immune system cells. These macrophages are found in many organs and produce cytokines which is a chemical that can drive inflammation. In excess these cytokines can affect other organs such as the heart and result in heart failure. Some laboratory tests that can indicate macrophage activation include elevated ferritin, triglyceride, and CRP. Steroids and other anti-inflammatory medications can suppress this inflammation and improve symptoms and organ function in these patients with macrophage activation and organ dysfunction. We report two cases with unexplained heart failure who were found to have features of macrophage activation after further workup. They both responded promptly to immunosuppressive treatment. We suggest that markers of macrophage activation should be evaluated in patients with unexplained CHF. In some proven cases of heart failure, secondary to macrophage activation, prompt treatment with immunosuppressants could lead to clinical improvement, and such treatment should be sought only after other causes of heart failure have been clearly ruled out and must be done by physicians with expertise in managing heart failure.

Correspondence: Han W Tun
Department of Hematology and Medical Oncology, Mayo Clinic Hospital, Jacksonville, FL, 32224, USA
Email Tun.Han@mayo.edu

1037

Introduction

Macrophage activation leading to multi-organ dysfunction/failure has been described in various hematologic disorders including hemophagocytic lympho-histiocytosis (HLH)/macrophage activation syndrome (MAS), and macrophage activation like syndrome (MALS).[1,2] Congestive heart failure (CHF) appears to be an uncommon manifestation of macrophage activation.[3] We found seven case reports of HLH presenting with systolic heart failure in the medical literature (Table 1), six of whom had complete recovery of cardiac function with immunosuppressive therapy; post treatment cardiac function was not reported in one case. Here we report two cases of acute CHF associated with laboratory and pathologic evidence of macrophage activation and cytokine storm without meeting the diagnostic criteria for MAS/HLH. One patient met the criteria for MALS. Both cases had increased M2 macrophages in the bone marrow. Both cases had a dramatic response to immunosuppressive therapy with full recovery of cardiac function.

Case 1

A 37-year-old female without any prior co-morbidity was hospitalized with acute onset of severe shortness of breath and fatigue. The initial examination showed blood pressure 102/96 mmHg, pulse 121 bpm, temperature 36.8 °C, respiratory rate 35 bpm and SpO2 94% on 2LO2. Bilateral inspiratory crackles were present on chest auscultation with normal heart sounds, S1 and S2, with added S3. Initial workup (Table 2) showed hemoglobin 9.3 g/dL (11.6–15), hematocrit 54% (35.5–44.9), platelet 76×10^9 (157–371), white cell count 2.4×10^9 (3.4–9.6), absolute neutrophil count 0.11×10^9 (1.56–6.45), absolute monocyte 3.0×10^9 (0.26–0.81), NT proBNP 17,280 pg/mL (<140), troponin T 11 (<10 ng/L), triglyceride 556 mg/dL (H), fibrinogen 312 mg/dL (200–393), ferritin 568 (11–307 mcg/L), CRP 92.4 (<8.0 mg/L), LDH 261 (122–222 U/L), presence of heterozygous prothrombin G20210A gene mutation, and interleukin-6, 8.6 pg/mL (<1.8), creatine kinase 80 U/L (26–192). Serial troponin T levels were 10 ng/L (6 hours after), and 8 ng/L (on day 2). Her HScore was 98 points based on the above results. Other lab workup including renal function panel, liver function panel, and autoimmune screen were unremarkable. Blood smear showed absolute neutropenia with left-shifted neutrophils and markedly increased immature monocytes. Electrocardiogram (EKG) showed sinus tachycardia with T inversion in the inferior leads. CT chest showed moderate bilateral pleural effusions, interstitial edema and central perivascular ground-glass opacities. Echocardiogram showed ejection fraction of 25%, with severe generalized left ventricular hypokinesis; normal left ventricular chamber size and thickness; normal right ventricular chamber size with reduced systolic function; right ventricle pressure 23 mmHg; right and left atrium was of normal size; mild

Table 1 Literature Review of Prior Cases with HLH Systolic Heart Failure

Case	Age	Association	Takotsubo Features	Treatment	Complete Recovery of Cardiac Function on Echo
Zhou JY, et al[14]	45-year-old female	Lamotrigine	Yes	Etoposide, dexamethasone	Yes
Otillio JK, et al[15]	6-year-old female	Infection	Yes	Etoposide, cyclosporine, and dexamethasone	Yes
Takeoka Y, et al[16]	73-year-old (A) and 56- year-old female (B)	EBV (A), T cell lymphoma (B)	Yes (A and B)	Etoposide, cyclosporine, and dexamethasone (A); carboplatin, cytarabine, dexamethasone (B)	Yes (A and B)
Prakash PB, et al[3]	1-year old female	Infection	No	(anakinra), methylprednisolone and tacrolimus	Yes
Abdin A[17]	59-year-old male	Unclear etiology	Yes	High-dose steroids	Yes
Ullah W, et al[18]	32-year-old male	Infection (HIV)	Yes	Dexamethasone, etoposide	Yes
Chizinga M, et al[9]	32-year-old male	Systemic juvenile idiopathic arthritis (SJIA)	No (right sided heart failure)	Dexamethasone, Anakinra.	Not reported, patient clinically improved

Table 2 Laboratory, Bone Marrow and ECHO Findings of Case 1 and 2

	Case 1	Case 2
Laboratory		
Hb (11.6–15)	9.3 g/dL	7.9 g/dL
WCC (3.4–9.6) ×10^9	2.4 /L	1.1 /L
Platelet (157–371)	76 /L	33 /L
Triglyceride	556 mg/dL	970 mg/dL
Ferritin (11–307)	731 mcg/L	5446 mcg/L
LDH (122–222)	261 U/L	534 U/L
IL 2 receptor (CD25) (≤1033)	——	1599 pg/mL
CRP (<8.0)	92.4 mg/L	——
IL-6 (<1.8)	8.6 pg/mL	10.2 pg/mL
Bone Marrow	Diffuse infiltration by CD68+ macrophages	Increased CD68 + macrophages
ECHO	EF 25%, severe left ventricular hypokinesis	EF 21%, global decrease in systolic function

mitral valve regurgitation, mild tricuspid valve regurgitation; normal aortic and pulmonary valve; small pericardial effusion (video 1). Abdominal ultrasound showed normal spleen and liver size. Initially she was started on broad-spectrum antibiotics (vancomycin, piperacillin and tazobactam) and diuretics. Her oxygen requirements increased within 24 hours of admission necessitating non-invasive positive-pressure ventilation with BIPAP at 50% FiO2, and ICU monitoring. She remained hemodynamically stable in the ICU and did not require inotropic support. After 72 hours, extensive workup for underlying infection including bacterial, viral, fungal and mycobacterial organisms were all negative; and despite antibiotics, interleukin-6 and ferritin continued to increase to 11.4, and 731 respectively, with increasing liver enzymes. Bone marrow biopsy showed hypercellularity with diffuse infiltration by CD68+ macrophages and atypical megakaryocytes; the macrophages are predominantly type 2 macrophages since they are negative for pSTAT1 and positive for CD163 (Figure 1). Chromosome analysis, acute myeloid leukemia (AML) FISH panel, next generation sequencing (NGS) for myeloid neoplasms (11 gene panel), and myeloproliferative neoplasm (MPN) panel (JAK2, CALR,

MPL) were all negative. Macrophage activation associated cytokine-mediated disease process was suspected in the absence of evidence for ischemia, myocarditis, autoimmune disorders, and infections. She was treated with dexamethasone 40 mg daily and had significant clinical improvement, no longer requiring oxygen after 5 days of steroids. Furthermore, echocardiogram after 5 days of steroids showed significant improvement in EF to 55% with normal left ventricular chamber size, wall thickness and regional wall motion; normal left ventricular diastolic function; normal right ventricular chamber size and function; estimated right ventricular systolic pressure 23 mmHg; mild mitral valve regurgitation and mild tricuspid valve regurgitation; normal pulmonary valve systolic velocity and small circumferential pericardial effusion (video 2). There was also resolution of hematologic abnormalities.

She was discharged on a 6-week steroid taper. One month later, ejection fraction was 62% and at 2-month follow-up she was clinically doing well. Unfortunately, several months later she was found to have avascular necrosis of the bilateral distal femur and proximal tibia and is currently followed by orthopedics for further care. She was started on Eliquis 10 mg twice daily for 7 days then 5 mg twice daily for 21 days, then 2.5 mg twice daily for 6 months in light of the possible association of her prothrombin gene mutation and development of avascular necrosis. Her pain symptom subjectively improved with the initiation of anticoagulation.

Case 2

A 41-year-old female was hospitalized with subjective fever, nausea, vomiting, and abdominal pain which were present for 3 weeks. Initial examination showed blood pressure 66/39 mmHg, pulse 135 bpm, temperature 36.6 °C, respiratory rate 35 bpm and SpO2 99%. Normal heart sounds and breath sounds were heard on auscultation. Initial workup (Table 2) showed hemoglobin 7.9 g/dL (11.6–15), hematocrit 24.7% (35.5–44.9), platelet 33×10^9 (157–371), white cell count 1.1×10^9 (3.4–9.6), absolute neutrophil count 0.5×10^9 (1.56–6.45), troponin T 17 (<10 ng/L), triglyceride 970 mg/dL, fibrinogen 601 mg/dL (200–393), ferritin 5446 (11–307 mcg/L), LDH 534 (122–222 U/L), interleukin 2 receptor (CD25) soluble 1599 pg/mL (≤1033), IL-6 10.2 pg/mL, prothrombin time 14.4 (9.4–12.5 sec), INR 1.3 (0.9–1.1), activated partial prothrombin time 35 (25–37 sec) and elevation of liver enzymes (total bilirubin 3.1 mg/dL (<1.2), direct bilirubin
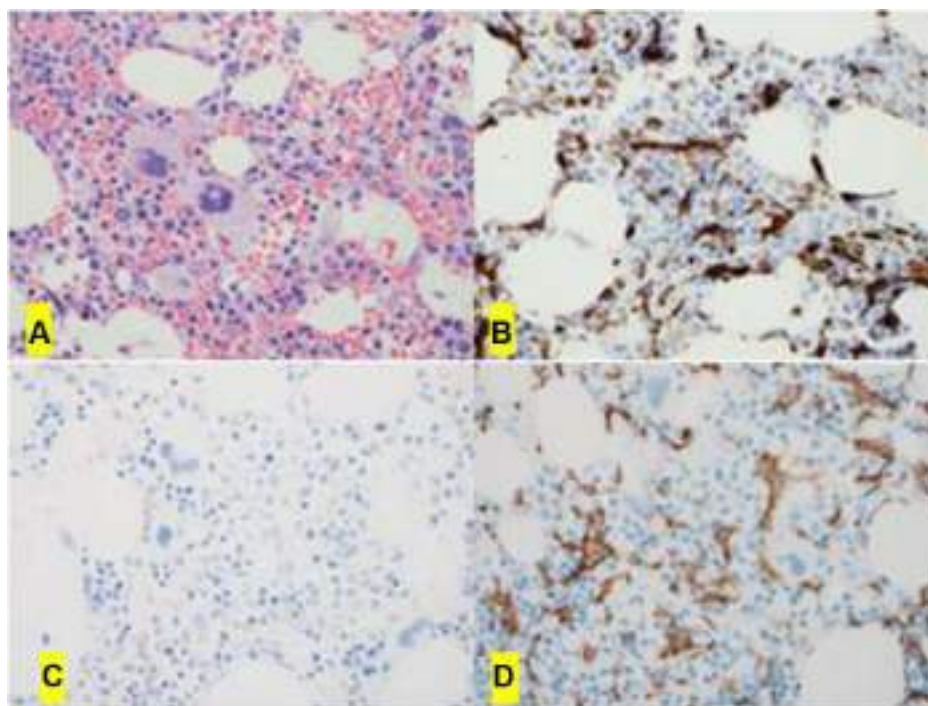
Figure 1 The bone marrow biopsy shows hypercellularity with increased megakaryocytes (**A**, H&E x40). The immunohistochemical studies show increased interstitial macrophages by CD68 (PGM1) (**B**, x40); and they are type 2 macrophages negative for pSTAT1 (**C**, x 0) but positive for CD163 (**D**, x40).

2.7 mg/dL (0–0.3), alanine aminotransferase 81 U/L (7–45), aspartate aminotransferase 241 U/L (8–43), alkaline phosphatase 117 U/L (35–104). Serial troponin T levels were 13 ng/L (day 2) and 18 ng/L D3 (day 3). Other laboratory workup included renal function panel and autoimmune screen were unremarkable. Her HScore was 152 based on the above results. Blood smear showed absolute leukopenia and lymphopenia. EKG showed sinus tachycardia with T inversions in V1.

Echocardiogram showed left ventricular ejection fraction 21% with a normal size and thickness left ventricle with multiple regional wall motion abnormalities in non−coronary distribution and associated global decrease in systolic function; grade 3/4 left ventricular diastolic dysfunction, consistent with severely elevated left ventricular filling pressure; normal right ventricular chamber size with mild global decrease in systolic function, right ventricular systolic pressure 42 mmHg, tricuspid annular plane systolic excursion (TAPSE) was 11 mm (20–22); mitral valve was normal with moderate mitral valve regurgitation and severe tricuspid valve regurgitation. Aortic and pulmonary valves were normal. No pericardial effusion. Normal size of both right and left atrium (video 3). Abdominal ultrasound did not show splenomegaly/hepatomegaly. She was

started on broad spectrum antibiotics (vancomycin, piperacillin and tazobactam) and admitted to ICU as she required inotropic support with milrinone starting at 0.375 mcg/kg/min and then titrated as needed to maintain blood pressure. Extensive workup for underlying infection including bacterial, viral, fungal and mycobacterial organisms were all negative. Bone marrow biopsy showed hypocellularity with left-shifted myelopoiesis, increased clusters of immature myeloid precursors, and increased CD68+ macrophages, which were predominantly type 2 macrophages negative for pSTAT1 and positive CD163 (Figure 2). Chromosome analysis, AML FISH, NGS for myeloid neoplasms (11 gene panel), MPN panel (JAK2, CALR, and MPL), immunoglobulin gene rearrangement, and T-cell receptor gene rearrangements were all unremarkable. Macrophage activation associated cytokine-mediated systemic disease process was suspected and treated with anakinra (anti-IL1) 100 mg subcutaneous for 7 days, and dexamethasone 10 mg once daily for 7 days followed by a 6-week steroid taper. She clinically improved rapidly and was completely off inotropic support after 6 days of immunosuppression treatment. Repeat echocardiogram after 7 days of immunosuppressive treatment showed significantly improved left ventricular
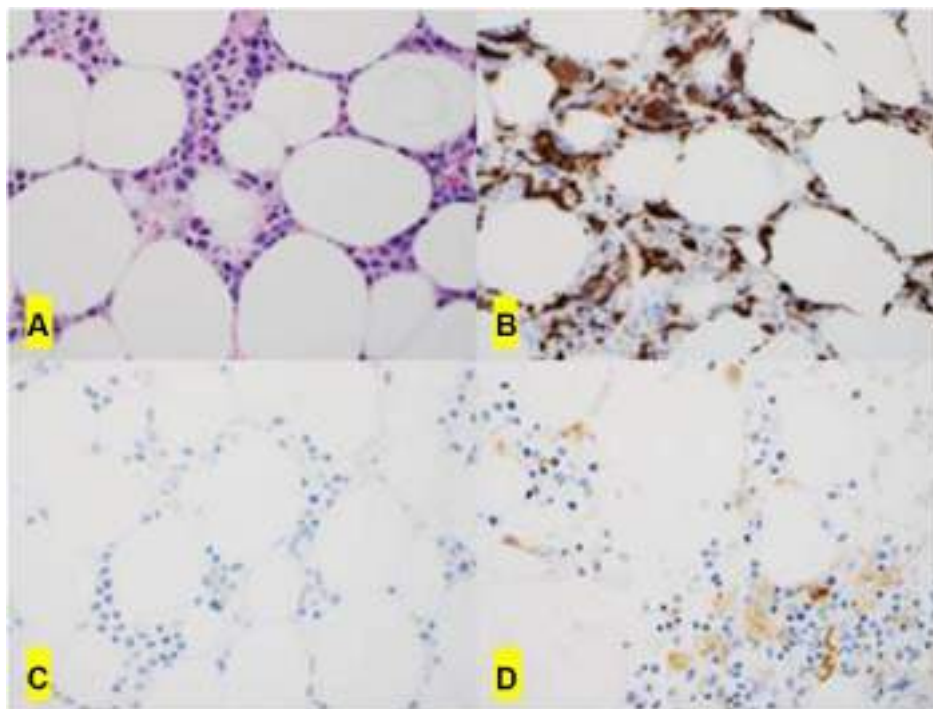
Figure 2 The bone marrow biopsy shows hypocellularity with left-shifted myelopoiesis (**A**, H&E x 0). Immunohistochemical studies shows increased interstitial macrophages by CD68 (PGM1) (**B**, x40); they are type 2 macrophages (M2) negative for pSTAT1 (**C**, x 0) and positive for CD163 (**D**, x40).

function with ejection fraction 70%; normal left ventricular chamber size and wall thickness with hyperdynamic systolic function; normal right ventricular chamber size and function; no regional wall motion abnormalities; no pericardial effusion (video 4). Complete resolution of cytopenias and normalization of liver function was also seen. At 8 months follow up she has no clinical features of heart failure.

Discussion

Our two young patients represent unique cases of acute CHF secondary to macrophage activation-associated cytokine storm with one patient meeting criteria for MALS. It is interesting that they did not meet the diagnostic criteria for known macrophage activation disorders such as HLH, also known as MAS.[2,4,5] They clearly had laboratory evidence of macrophage activation which includes elevated ferritin, triglyceride, CRP, IL-6, and CD25.[2] The other prominent finding is infiltration of the bone marrow by CD68+ type 2 macrophages, associated with pancytopenia which can be seen in HLH/MAS.[2] It appears that activated macrophages are located in the bone marrow compromising hematopoiesis and mediating CHF via cytokine storms. In our cases the underlying mechanism for

macrophage activation is not clear, negative extensive genetic and molecular testing together with rapid response to immune suppression would indicate a reactive process inciting macrophage activation. They both had increased M2 macrophages in the bone marrow. M2 macrophages which can be seen in MAS, are heterogeneous in comparison to M1 macrophages.[6] In these cells the production of proinflammatory mediators are downregulated.[6] While they are mostly described as anti-inflammatory, there is some data to suggest they may act as an innate immune sensor for bacteria and trigger production of pro-inflammatory cytokines.[6,7]

HLH/MAS is diagnosed if at least five of the eight criteria of the International Histiocyte Society (2004-HLH criteria), published in 2007, are met: (a) fever, (b) splenomegaly, (c) cytopenia of at least two lineages; (d) fasting triglycerides ≥265 mg/dl and fibrinogen ≤150 mg/dl; (e) hemophagocytosis in the bone marrow; (f) low or absent NK-cell activity; (g) ferritin ≥500 ng/mL; and soluble CD25 ≥2400 units/mL.[2] Presence of bone marrow hemophagocytosis is one of the criteria taken into consideration for the diagnosis of MAS.[8] MALS, which is often described in the setting of sepsis, came about due to the difficulty of performing a bone marrow biopsy in every

critically ill patient and is defined as an HS score more than 151 or patients with both hepatobiliary dysfunction (HBD) and disseminated intravascular coagulation (DIC). Both cases did not meet the criteria for HLH/MAS (they both had two out of the five criteria for HLH/MAS). We did not have NK cell activity or soluble CD 25 levels available in both cases. An HS score of case 2 met the criteria for MALS, further supporting the presence of macrophage activation. Our second case had more evidence of multiorgan dysfunction characterized by liver dysfunction, heart failure and lower degrees of cytopenias (with respect to case 1) and overall was more critically ill and required inotropic support. It is possible that she may have been on the more severe end of the disease spectrum of macrophage activating disorders; while our first patient who did not have liver dysfunction, less severe cytopenias and not requiring inotropic support may have been on a milder end of the spectrum characterizing this novel entity of macrophage activation-associated cytokine-mediated CHF. Bone marrow features of macrophage activation were present in both cases. One hypothesis is that macrophage activation in the bone marrow may be the first sign of the disease and if not diagnosed early may propagate from macrophage associated cytokine storm to the more severe end of the spectrum (manifesting as MALS or HLH) with HLH being the most severe of these entities.

The other unique aspect of our two patients is CHF as a prominent organ dysfunction. In our medical literature search, we identified only seven case reports of HLH with systolic heart failure (Table 1). Of note right ventricle failure without involvement of the left ventricle can also be involved.[9] It appears that CHF is uncommon in macrophage activation disorders.[3] In those cases and our two patients, immunosuppressive therapy worked very well with rapid restoration of cardiac function. The ECHO findings in both cases and non-rising troponin levels helped rule out other conditions such as takotsubo cardiomyopathy, myocarditis and acute myocardial infarction. Cardiac catheterization was not pursued considering their quick recovery with immunosuppressive medications.

We used steroid monotherapy in our first patient and steroid with anakinra (Anti-IL-1 monoclonal antibody) in our second patient with excellent therapeutic outcomes. Dexamethasone is a standard steroid used in management of HLH.[10] We chose to use anakinra in our second patient as her clinical status was rapidly deteriorating. IL-1 is an important cytokine which can activate an innate immune response with activation of macrophages.[11] In both

patients, IL-6 was elevated. It would have also been reasonable to try anti-IL-6 monoclonal antibody therapy. In some cases while patients respond to immunosuppressive medications additional supportive care, such as veno-arterial extracorporeal membrane oxygenation (VA-ECMO), may be needed and can be lifesaving.[9]

Unfortunately, our first patient developed avascular necrosis several months after she recovered from her episode of heart failure. There are many short-term and long-term side effects of high dose steroids, avascular necrosis is one such reported side effect. We felt other factors may have contributed to her avascular necrosis, such as the presence of her prothrombin G20210A gene mutation. Hypercoagulable states have been reported to be associated with avascular necrosis and anticoagulation has been reported to help in some cases. This was our rationale using Eliquis in this patient.[12,13] It is unclear if macrophage activation would have had a role in the pathogenesis of her avascular necrosis. Monitoring for adverse effects of steroids during the short- and long-term follow up of these patients is also important.

In conclusion, we report a novel macrophage activation-induced disease entity which does not fulfil the diagnostic criteria for HLH/MAS and is clinically characterized prominently by acute CHF and pancytopenia. We suggest that macrophage activation should be entertained as a differential diagnosis in patients with acute, otherwise unexplained, CHF. Markers of macrophage activation and hematologic parameters including complete blood counts and bone marrow biopsy should be evaluated to diagnose this entity. Therapeutically, patients with this disorder should be promptly treated with steroids and anti-cytokine therapy after considering the risk of the therapy vs benefit and only patients with proven cases should be treated with immunosuppressants. The clinical outcome could be better if the disease is recognized early in the course and appropriate therapy, such as immunosuppressants, started early with consideration of advanced therapies such as ECMO in refractory cases. Additional research is warranted to further elucidate the underlying disease mechanism.

Ethics Approval
Institutional approval was not required to publish the case details.

Consent
Written and informed consent was obtained from both patients for publication of this manuscript.

Acknowledgments

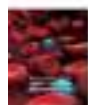## Author Contributions

Funding

Disclosure

References

1. Bracaglia C, Prencipe G, De Benedetti F. Macrophage activation syndrome: different mechanisms leading to a one clinical syndrome. *Pediatr Rheumatol Online J*. 2017;15(1):5. doi:10.1186/s12969-016-0130-4
2. Karakike E, Giamarellos-Bourboulis EJ. Macrophage activation-like syndrome: a distinct entity leading to early death in sepsis. *Front Immunol*. 2019;10:55. doi:10.3389/fimmu.2019.00055
3. Prakash PB, Raj S. Cytokine induced myocardial dysfunction in a patient with hemophagocytic lymphohistiocytosis. *J Am Coll Cardiol*. 2016;67(13_Supplement):1061. doi:10.1016/S0735-1097(16)31062-2
4. Ravelli A, Minoia F, Davi S, et al. Classification criteria for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a European League Against Rheumatism/American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. *Ann Rheum Dis*. 2016;75 (3):481–489. doi:10.1136/annrheumdis-2015-208982
5. Henter JI, Horne A, Arico M, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48(2):124–131. doi:10.1002/pbc.21039
6. Grom AA, Mellins ED. Macrophage activation syndrome: advances towards understanding pathogenesis. *Curr Opin Rheumatol*. 2010;22 (5):561–566. doi:10.1097/01.bor.0000381996.69261.71
7. Rőszer T. Understanding the mysterious m2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm*. 2015;2015:816460. doi:10.1155/2015/816460
8. Kyriazopoulou E, Leventogiannis K, Norrby-Teglund A, et al. Macrophage activation-like syndrome: an immunological entity associated with rapid progression to death in sepsis. *BMC Med*. 2017;15 (1):172. doi:10.1186/s12916-017-0930-5
9. Chizinga M, Kalra SS, Innabi A, Rackauskas M, Ataya A, Emtiazjoo A. Macrophage activating syndrome causing decompensated right heart failure. *Respir Med Case Rep*. 2021;33:101409. doi:10.1016/j.rmcr.2021.101409
10. Lerkvaleekul B, Vilaiyuk S. Macrophage activation syndrome: early diagnosis is key. *Open Access Rheumatol*. 2018;10:117–128. doi:10.2147/OARRR.S151013
11. Mehta P, Cron RQ, Hartwell J, Manson JJ, Tattersall RS. Silencing the cytokine storm: the use of intravenous anakinra in haemophagocytic lymphohistiocytosis or macrophage activation syndrome. *Lancet Rheumatol*. 2020;2(6):e358–e67. doi:10.1016/S2665-9913(20)30096-5
12. Björkman A, Burtscher IM, Svensson PJ, Hillarp A, Besjakov J, Benoni G. Factor V Leiden and the prothrombin 20210A gene mutation and osteonecrosis of the knee. *Arch Orthop Trauma Surg*. 2005;125(1):51–55. doi:10.1007/s00402-004-0760-8
13. Björkman A, Svensson PJ, Hillarp A, Burtscher IM, Rünow A, Benoni G. Factor V Leiden and prothrombin gene mutation: risk factors for osteonecrosis of the femoral head in adults. *Clin Orthop Relat Res*. 2004;425:168–172. doi:10.1097/00003086-200408000-00023
14. Zhou JY, Martinez JA, Shen JP. Lamotrigine-induced hemophagocytic lymphohistiocytosis with Takotsubo cardiomyopathy: a case report. *Journal of Medical Case Reports*. 2019;13(1):345.
15. Otillio JK, Harris JK, Tuuri R. A 6-year-old girl with undiagnosed hemophagocytic lymphohistiocytosis and takotsubo cardiomyopathy: a case report and review of the literature. *Pediatr Emerg Care*. 2014;30(8):561–565.
16. Takeoka Y, Nakamae M, Nakamae H, et al. Two cases of ampulla (takotsubo-shaped) cardiomyopathy associated with hemophagocytic lymphohistiocytosis. *Acta Haematol*. 2007;117(4):205–210.
17. Abdin A. Takotsubo Cardiomyopathy Due to Hemophagocytic Lymphohistiocytosis. *CHEST*. 2016;150(4):435A.
18. Ullah W, Hamid M, Grover H, Figueredo VM, Inayat F. Takotsubo cardiomyopathy secondary to haemophagocytic lymphohistiocytosis in HIV patients: a comprehensive review. *BMJ Case Rep*. 2019;12(4).

Novel Management and Screening Approaches for Haematological Complications of Gaucher's Disease

Pilar Giraldo & Marcio Andrade-Campos

Submit your article to this journal

View related articles

View Crossmark data

REVIEW

Novel Management and Screening Approaches for Haematological Complications of Gaucher's Disease

Pilar Giraldo[1,2]
Marcio Andrade-Campos[2,3]

[1]Haematology, Hospital Quironsalud, Zaragoza, Spain; [2]Foundation FEETEG, Zaragoza, Spain; [3]Haematology Department, Hospital del Mar, Barcelona, Spain

Purpose: Gaucher disease (GD) is the most common lysosomal storage disorder. The principal manifestations for its diagnosis and further monitoring include haematological manifestations such as anaemia, thrombocytopaenia, spleen enlargement, and bleeding disorders, among others. This review aims to summarise and update the role of haematological complications in GD diagnosis and follow-up, describe their management strategies, and to use these indicators as part of the diagnostic approach.

Materials and Methods: A systematic review following the recommendations of PRISMA-P 2020 was carried out. Publications indexed in the databases PubMed, Embase, Science Open, Mendeley, and Web of Science were electronically searched by three independent reviewers, and publications up to June 2021 were accessed. A total of 246 publications were initially listed, of which 129 were included for further review and analysis. Case reports were considered if they were representative of a relevant hematologic complication.

Results: From the first review dated in 1974 to the latest publication in 2021, including different populations confirmed that the haematological manifestations such as thrombocytopaenia and splenomegaly at diagnosis of GD type 1 are the most frequent features of the disease. The incorporation of haematological parameters to diagnosis strategies increases their cost-effectiveness. Hematologic parameters are part of the scoring system for disease assessment and the evaluation of therapeutic outcomes, providing reliable and accessible data to improve the management of GD. However, cytopaenia, underlying coagulation disorders, and platelet dysfunction need to be addressed, especially during pregnancy or surgery. Long-term haematological complications include the risk of neoplasia and immune impairment, an area of unmet need that is currently under research.

Conclusion: Haematological features are key for GD suspicion, diagnosis, and management. Normalization of hematological parameters is achieved with the treatment; however, there are unmet needs such as the underlying inflammatory status and the long-term risk of hematologic neoplasia.

Keywords: Gaucher disease, long-term complications, bleeding disorders, haematologic manifestations

Introduction

Gaucher disease (GD, OMIM230800) is the most prevalent of the lysosomal storage diseases (LSD). It affects approximately 1 in 40,000–100,000 inhabitants of the general population, but its frequency in Ashkenazi Jews is as high as ~1 in 850.[1–5] GD is classically described as three types according to neurological involvement. Type 1 GD (non-neuronopathic) is the most common form of presentation.

Correspondence: Pilar Giraldo
Email giraldocastellano@gmail.com

Type 3 is characterised in turn by three sub-types with different neurological manifestations (epilepsy, ataxia, saccadic eye movements, seizures) and other non-neurological features like heart valve infiltration and kyphosis. Type 2 GD is the most severe presentation with acute/subacute neurological impairment early in life (newborns to 1 year old); GD2 patients have a very short lifespan, usually around 2 years of age.[3–6]

GD is considered the prototype of the LSD, and was the first LSD to have a specific therapy. Among its multisystemic impairments, it is the LSD with the most implications compromising the haematopoietic system in terms of manifestations at diagnosis, follow-up, and development of complications.[1–3]

Haematological manifestations are key features for disease suspicion, as cytopaenia and spleen enlargement are almost universal in untreated patients. Among the most frequent manifestations at diagnosis, according to International Collaborative Gaucher Group (ICGG) and other registries, are anaemia (29%), thrombocytopaenia (62%), splenomegaly (91%), bleeding (20.6%), and bone pain (57.9%).[7,8] In our local experience, almost 80% of GD1 cases undergo a bone marrow exam as part of their patient journey for diagnosis, although a bone marrow aspirate is not necessary to make a diagnosis of GD.[8]

The target cell in this lysosomal disease is a blood cell, the monocyte-macrophage. Monocyte-macrophages originate from the common myeloid progenitor in the bone marrow; they express acid phosphatase, CD68, CD14, and HLA class II. The classical Gaucher cells are macrophages engorged with a PAS+ substrate and a crinkled paper morphology; they can be found in different tissues, but especially in the bone marrow and spleen. Gaucher cells also express CD163, CCL18, and the interleukin-1 receptor antagonist, characteristic of alternatively activated or M2 macrophages. The altered morphology of Gaucher cells is secondary to the defect in the enzymatic activity of the lysosomal hydrolase β-glucocerebrosidase; these cells and mainly all phagocytic cells show an impairment in their multiple functions that leads to multi-systemic repercussions.[9,10]

The hematopoietic system of an adult person produces more than 400 billion cells daily. With this high turnover, cellular components, especially membrane remnants, are constantly destroyed and phagocytosed by macrophages to be broken down and reused in the manufacture of new cells.[9] The accumulation of undegraded phagocytosed material in the cytoplasm of the macrophage due to impaired enzyme function leads to a thickening of the cells, which transform into Gaucher cells and displace the normal cellular network, increasing the size of the spleen and liver (the organs with the highest macrophage content), which also contributes to blood cell sequestration and increased anaemia and/or thrombocytopaenia. In the bone marrow, the displacement of haematopoiesis by Gaucher cell accumulation causes anaemia and thrombocytopaenia, which often leads to the suspicion of a haematological malignancy. It should be noted that, in several haematological malignancies, macrophages engorged with excess cellular material in the process of destruction are visualised in the bone marrow with a similar appearance to Gaucher cells (they are called pseudo Gaucher cells).[11] For that reason, Gaucher cells are not a pathognomonic finding of GD, but usually a finding that leads the diagnostic suspicion.

Other accompanying haematological changes are hyperferritinemia,[12,13] polyclonal or monoclonal gammopathy,[14] and other immune abnormalities as a decrease in the overall B-cells and an increase in NK and NK/T-cell population in the peripheral blood[15] are found with great frequency and contribute to identifying the underlying inflammatory component derived from monocyte-macrophage dysfunction.

Several haemostatic abnormalities have been described in GD, including prolonged prothrombin time (PT) and activated partial thromboplastin time (APPT), indicative of deficiencies in factor X, factor V, and factor XI (more frequent in Ashkenazi Jews). Also have been reported, but with little incidence deficiencies secondary to liver failure and rarely associated with von Willebrand disease.[16] Functional platelet defects such as abnormal platelet aggregation or adhesion defects could contribute to bleeding diathesis.[17]

In this review, a search was performed in the literature, focused on the haematological manifestations and complications with the aim of updating their incidence, describing novel management strategies, and highlighting these strategies as a part of the diagnostic work-up.

Materials and Methods
Systematic Literature Review Objectives
In this study, a systematic review was carried out in accordance with the Preferred Reporting Items for Systematic Review and Meta Analyses (PRISMA) guidelines.[18]

Eligibility Criteria

The inclusion criteria used for the selection of articles were all published original articles or review articles or registry data and guidelines about the diagnosis and follow-up of GD. Reports of single-case studies were considered if they were representative of a relevant hematologic complication.

Search Strategy

PubMed, Embase, Science Open, Mendeley, and Web of Science databases were electronically searched for relevant papers published up to June 2021, without language limitations. English descriptors were adapted according to the database. The following search strategy was entered into the database: ("haematological manifestations" OR "haematological complications" OR "haematological data" OR "screening haematological diagnosis") AND ("Gaucher disease").

A secondary manual search of the reference lists of the relevant articles was also carried out. In addition to these database searches, numerous permutations of our search terms (keywords: bone marrow, spleen, cytopaenia, bleeding, B-cell malignancy, gammopathy) were entered into Google Scholar and thoroughly searched for any additional articles not found in the database searches.

Two independent reviewers developed an Excel database in order to record the studies that met the inclusion criteria, including the abstract, title, date of publication, authors, and journal in order to facilitate the review and selection process.

Results

We reviewed and selected a total of 246 articles, including two online books updated annually. In the first review, the selection was reduced to 213, as shown in the flow chart in Figure 1. We excluded articles that referred only to basic research results without translatability, treatment results referring exclusively to bone disease or analysis of results that did not include haematological alterations or complications, to finally select 129 works that were included in this review.

Haematological Manifestations and Screening Programmes

The reviewed articles, from the first review from 1974 to the latest publication in 2021, included analyses of different populations, all of them with haematological manifestations such as thrombocytopaenia and splenomegaly at diagnosis. GD type 1 was the most frequent manifestation of the disease. Anaemia and hepatomegaly were also a constant in the clinical picture. The intensity and variability of these manifestations was very wide due to the
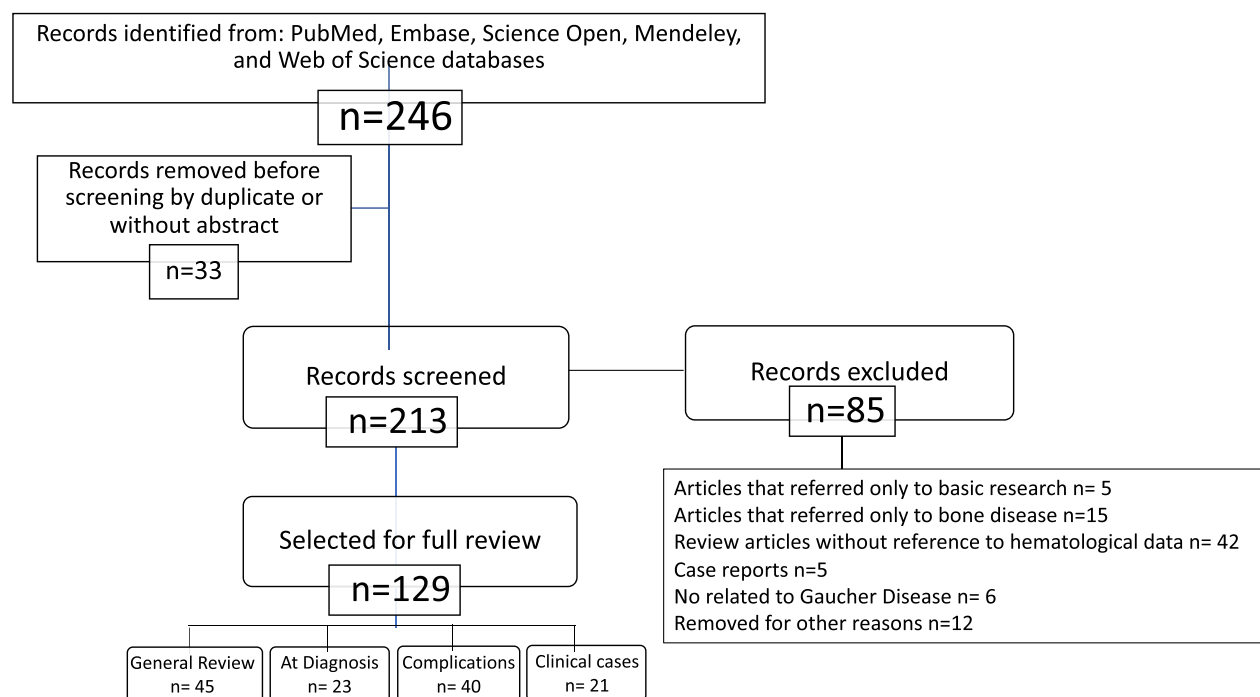

Figure 1 Flow chart. Identification of studies.

heterogeneity of the disease. Haematological manifestations may present as early as in the first months of life or go unnoticed for decades. Table 1 shows the selected publications in which data on haematological variables are referred to diagnosis in different populations; the majority of the selected articles were reviews.[6,19–38]

In the last decade, several screening programmes for lysosomal diseases have been developed in different populations using dried blood spots for early detection before symptoms appear. In a New York study, 15 GD cases were diagnosed,[39] while none were found in a Mexican study,[40] 2 GD cases in an Italian study,[41] and 5 GD cases in an Illinois study.[42] These studies were performed using both enzymatic analysis and multiplexed tandem mass spectrometry.[43] In China, using a fluorometric assay, 1 GD case was diagnosed.[44] In Taiwan, using mass spectrometry, no cases were diagnosed,[45] and in Brazil, using microfluidics, 2 GD cases were identified.[46] There is much controversy over the detection of false positives.[47] This approach is controversial because the incidence of cases in general population is low (~1/100,000 live births) in different populations and its application is only promoted in those communities with a high incidence of cases, such as in the Ashkenazi Jewish population, where the incidence is 1/850 births. Recently, the use of genetic analysis panels has been explored.[48] Active search for patients with subclinical disease by screening for cases with mild to moderate thrombocytopaenia and splenomegaly has provided variable results with generally low identification rates, ie, 4/73,[49] 55/787,[50] 7/196,[51] and 2/1000.[52] Another different approach is the family study, the current recommendation for low-incidence populations includes familial studies after an index case has been identified. In our practice, more than 10% of the GD cases included in the Spanish Registry were diagnosed following this recommendation.[53]

GD Biomarkers and Haematological Features

Biomarkers are useful tools to help in the disease diagnosis and follow-up. GD is the lysosomal disease that has the most validated biomarkers, which offer objective information about the situation of the disease to detect early complications and measure the response to therapy.

Chitotriosidase is an enzyme produced by activated macrophages; its activity correlates with the burden of the disease and is modified with treatment. Therefore, it is a useful tool to monitor the treatment response; however, other systemic inflammatory statuses or acute conditions such as systemic infections or acute GD complications can increase chitotriosidase activity. In addition, about 6% of the Caucasian population has a 24 base pair duplication in homozygosity in the *CHIT1* gene encoding the enzyme and lacks chitotriosidase activity.[54] The quantification in plasma of the substrate sphingolipid has rapidly become an important biomarker for diagnosis; glucosylsphingosine (Lyso-Gb1) is increased in GD, with rapid reduction after enzymatic therapy because it specifically reflects substrate accumulation in the body. Therefore, some screening studies have already incorporated these two determinations in combination with a minimum clinical haematological feature for suspicion to identify patients affected by GD. A study carried out by Fuller et al confirmed 9 GD patients by increased chitotriosidase activity among 1415 samples.[55] Recently, a study by Tang et al determined glucosylsphingosine in dried blood spot (DBS) samples of 142 high-risk patients with splenomegaly and/or thrombocytopaenia and

Table 1 Studies That Refer to Haematological Manifestations at Diagnosis

Author Year (Reference)	No Cases	Anaemia (%)	Thrombocytopaenia (%)	Hepatomegaly (%)	Splenomegaly (%)
Medoff and Bayrd 1954[20]	29		83.0	79.0	100
Giraldo P et al 2000[27]	155	46.0	83.5	61.2	71.7
Thomas et al 2012[31]	45	20.0	59.0	44.0	82.0
Essabar et al 2015[23]	11	56.0		100	100
Mistry et al 2017[30]	212	46.6	81.0	68.0	81.0
Weinreb et al 2021[28]	310	40.6	100	40.0	90.0

identified 52 GD patients.[56] Other strategies using algorithms have been tried.[57–59]

Screening programmes based on haematological indicators or biomarker determinations are shown in Table 2.

Coagulation Disorders

Bleeding is a frequent manifestation that occurs at different stages of the disease; at diagnosis, it is a symptom usually secondary to thrombocytopaenia, but a variety of coagulation factor abnormalities (fibrinogen, factor II, VII, VIII, X, XII), acquired coagulation factor deficiencies including von Willebrand factor (vWF) deficiency and specific inherited coagulation factor deficiencies (factor XI deficiency among

Ashkenazi Jews) have been described. In addition, abnormal platelet aggregation and adhesion can be present at diagnosis and may contribute to bleeding diathesis.[3,16,17,60–69] Table 3 shows the most relevant studies.

It is important to carry out a haemostatic evaluation including platelet count and basic haemostatic tests such as fibrinogen, prothrombin time, activated partial thromboplastin time as well as platelet function tests, especially if the patient is to undergo surgery to compensate for deficiencies.

In special situations, such as pregnancy, these abnormalities are of special importance, requiring the monitoring of haemostatic function and cellular counts to minimise the bleeding risk.[70–72]

Table 2 Screening Programmes for Lysosomal Diseases Based on Haematological Data or Biomarkers

Author Year (Reference)	Period Study	No Cases Screened	No Cases Identified	Positive Predictive Value (%)	General Prevalence of GD
Fuller et al 2011[55]	2003–2007	1415	9		Australia (retrospective)
Motta et al 2015[51]	2010–2013	196	7	18.4	Italy 1/100,000
Huang et al 2020[50]	2016–2019	786	55	37.4	China 1/80,000
Miyamoto et al 2021[52]	2016–2018	994	12		Japan 1/330,000
Tang et al 2021[56]		142	52		China

Abbreviation: GD, Gaucher disease.

Table 3 Haemostatic Abnormalities

Author Year (Reference)	No Cases	Decreased Factors	Platelets Dysfunction	Prolongation PT/APPT	Increased D Dimer or Reduction PC/PS
Billett et al 1996[60]	9	II, V, VIII, XI	–	Yes	–
Hollak et al 1997[61]	30	II; V; VII; VIII; IX, X, XI, XII	Yes	Yes	Yes
Katz et al 1999[62]	22	V, VIII, IX, XI, XII	–	Yes	–
Giona et al 2006[63]	15	II, V, VII, VIII, IX, X, XI, XII, vWF	Yes	Yes	–
Deghady et al 2006[64]	10	II, V, VII, VIII, IX, X, XI, XII	–	Yes	–
Spectre et al 2011[17]	48	–	Yes	–	–
Givol et al 2012[66]	7	XI	Yes	No	–
Mitrovic et al 2012[65]	31	II, V, VII, VIII, IX, X, XI, XII, vW	Yes	Yes	–
Komninaka et al 2020[67]	29	VIII, vWF, ADAMTS13	Yes	Yes	Yes

Abbreviations: PT, prothrombin time; APPT, activated partial thromboplastin time; PC, C protein; PS, S protein; vWF, von Willebrand factor.

Treatment Effect in Haematological Parameters

The goals of treatment focus on three main areas: recovery of hematologic parameters and a reduction in visceral volumes and bone marrow infiltration. Reversal of haematological alterations is one of the main goals of treatment and is achieved in more than 90% of cases with both enzyme replacement therapy and substrate reduction therapy. However, the underlying inflammatory process is not fully reversible with available treatments, and there is speculation about the importance of this situation in the functioning of the immune system and the development of long-term complications of the disease.[22] The application of scoring systems and monitoring guides facilitates the traceability of the response and monitoring indicators. Haematological parameters are easily measurable by any specialist.[29,73–79] However, there are other haematological alterations that can be observed in the follow-up of patients, such as megaloblastic features of the red blood cells, accompanied or not by folate or vitamin B12 deficiency.[80]

All the current available therapies have firmly demonstrated an improvement in haematological parameters, and improvement is rapid during the first 6 months, but continues progressively with the majority of patients normalising blood counts in 2–5 years.

Inflammation and Haematological Alterations

The methods to assess the inflammatory status and immunological alterations are not well defined in GD, although there are some determinations that may help to predict immune status and detect haematological complications that may arise in the long term. Hyperferritinaemia and immunologic abnormalities such as polyclonal and monoclonal gammopathy are detected very often both in children and adults.[12–15,81] Persistent immune alterations such as changes in T/B-cell populations, polyclonal gammopathy, and the cytokine profile, especially in splenectomised patients, have been related to long-term complications.[37,82–84] Also, a general increased incidence of haematological malignancies has been reported in GD patients since the early 1990s.[85–87]

Hyperferritinaemia and gammopathies also play a role in GD diagnosis, as they may be the reason for consultation with haematology in previously undiagnosed cases.[12,13] Isolated cholestasis[88] or elevated transaminases and hepatomegaly may be confounding data in the differential diagnosis with other entities.[89]

Haematological Complications

Long-term complications in GD patients include a high risk of developing haematological malignancies; this risk has been estimated to be between 14.7-fold and 51.1-fold according to data from the International Collaborative Gaucher Group (ICGG) registry.[90] There is a predominance of hematologic neoplasms derived from the B lymphoid lineage and linked to the monoclonal hypergammaglobulinemia so frequently detected in these patients.[91–98]

Nevertheless, there are discrepancies related to the relative risk according to different studies. B-cell and plasma cell malignancies such as multiple myeloma are the most commonly described. The risk of multiple myeloma is 5.9-fold (95% CI 2.8–10.8) according ICGG data.[90] In other studies, the relative risk was higher at 25-fold (95% CI 9.17–54.40).[91] Table 4 shows the most significant data.

The pathogenesis of these complications has been interpreted as secondary to the chronic immunological disturbances with a predisposition to develop chronic inflammation following chronic B-cell stimulation. An increase in cytokines such as IL-1, IL-6, IL-10, CCL18, and TNF-α has been demonstrated in several experimental studies[81,82] as well as the implication of complement factors[82] and their relationship with the presence of gammopathies in GD1 patients.[99,100] In mouse models of Gaucher's disease-associated gammopathy, monoclonal immunoglobulin has been shown to be reactive against lyso-glucosylceramide.[101,102] The abnormalities in T-cell function could explain the increased incidence of other haematological neoplasias as acute lymphoblastic leukaemia, chronic lymphocytic leukaemia, Hodgkin's disease, and non-Hodgkin's lymphoma.[103–107] In isolated cases, the diagnosis of GD has been reached through the study of a hematological alteration such as monoclonal gammopathy, suspicion of multiple myeloma due to pathological fracture, or thalassemia suspicion. More than 25 isolated cases of haematological neoplasms have been reported, some of them diagnosed simultaneously with GD.[108–111] Most of the cases correspond to B-lineage lymphoid neoplasms, but other sporadic cases of non-neoplastic haematological disorders have been observed[112–115] (Table 5). Co-existence of isolated cases of acute myelogenous leukaemia, chronic myelogenous leukaemia, myelodysplastic syndrome, or myeloproliferative neoplasm have also been described.[116–123]

Table 4 Haematological Complications in GD1

Author Year (Reference)	No Cases	Hyperimmunoglobulin	Haematol Neoplasia	Risk (%)	Others
Shiran et al 1993[85]	48	–	5	10.4	–
Gielchinsky et al 2001[80]	89	–	–		B12 deficiency (36)
Rosenbloom et al 2005[90]	2742 (ICGG)	–	10 (MM)	5.9	–
de Fost et al 2005[93]	131	–	5	12.7*	–
Zimran et al 2005[91]	505	–	8	14.6	–
Wine et al 2007[81]	23 paediatrics	Yes	–	–	–
Taddei et al 2009[94]	403	–	8	3.45**	–
Weinreb et al 2013[96]	184	–	8	14.6	–
Rodic et al 2013[99]	27	Yes	11 (MGUS)	–	–

Notes: *The relative risk for MM 51.1; **The relative risk for MM 25.0.
Abbreviations: GD1, Type 1 Gaucher disease; ICGG, International Collaborative Gaucher Group; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance.

Discussion

Undoubtedly, the haematological manifestations at diagnosis of GD type 1, such as thrombocytopaenia and splenomegaly, are key in the diagnosis of the disease. However, the intensity and variability of these manifestations is very wide due to the heterogeneity of the disease and are therefore not specific.[1–8] In the various screening programmes in which these variables (thrombocytopaenia and splenomegaly) have been used to identify undiagnosed patients, the number of cases detected was low, ie, less than 10% in selected populations and with analyses carried out over long periods of time (between 2 and 4 years).[39–46] Newborn screening programmes also have very low success rates for GD, so they do not appear to be cost-effective outside high-risk populations, with too many probands to identify a very small number of subjects, regardless of the used test (enzyme activity by microfluidics or mass spectrometry, combined with biomarkers or mass genetic analysis).[47] Newborn screening programs are also controversial because of the detection of potentially asymptomatic or oligosymptomatic cases or a high number of false positives, which may have ethical repercussions in terms of parental stress, overmedication or others. This type of search is more applicable in populations with a high prevalence of cases or in family studies. Some approaches have also been used to apply models based on algorithms and methods based on a scoring system by Delphi initiatives in children[57] and adults[58] with a predictive value of around 0.80 in a series of already

diagnosed patients. It seems more effective to insist on training programmes for specialists, both in paediatrics and among primary care physicians, haematologists, internists, rheumatologists, and undergraduates.[124–126]

Splenectomised GD patients generally suffer from increased bone and liver deposits and therefore have increased bone complications and increased susceptibility to infection. It is a therapeutic procedure that should be avoided in GD.[1–4]

Despite the general attention to cytopaenia, we have highlighted the importance of paying attention to the correct assessment of haemostatic parameters.[60] Bleeding is a frequent clinical manifestation in patients with GD and is secondary not only to the thrombocytopaenia that most patients present at diagnosis, but to numerous alterations in coagulation factors, von Willebrand factor, and platelet dysfunction, even with normal platelet counts.[61–68] It is of special interest to keep these data in mind in risky situations such as pregnancy and delivery, surgical interventions, and dental extractions, and to establish correct preventive measures.[62,66,69–72]

In the follow-up of patients, cell counts are mandatory parameters for periodic assessment, whether the patients are on treatment or not.[22,73–76] These are sensitive indicators in the evaluation of the response, but other parameters must also be considered.[77–79] Vitamin B12 deficiency is present more frequently in patients with GD than in the general population, so in situations of anaemia not resolved by treatment, the morphology of red blood cells must be monitored to rule out

Table 5 Cases Reported with Co-Existence of GD and Hematological Diseases

Author Year (Reference)	Haematol Neoplasia	Non-Neoplastic Haematol
Lester et al 1984[115]	–	ITP
Petrides et al 1998[117]	CML	–
Haliloglu et al 1999[112]		Budd-Chiari
Webb et al 2011[122]	Chronic myeloproliferative neoplasia	–
Miri-Moghaddam et al 2011[113]	–	α-thalassaemia
Ranade et al 2013[103]	LAL	
Villarrubia et al 2014[118]	MDS (del5q)	–
Kubo et al 2014[119]	–	Epidural haematoma
Noya et al 2018[120]	CML	–
Kose et al 2019[114]	–	Severe neutropaenia
Ruchlemer et al 2020[121]	MDS & ICUS	–
Maity et al 2021[116]	AML	–

Abbreviations: GD, Gaucher disease; ITP, immune thrombocytopenia; CML, chronic myeloid leukaemia; LAL, lymphoblastic acute leukaemia; MDS, Myelodysplastic syndrome; ICUS, idiopathic cytopenia of undetermined significance; AML, acute myeloid leukaemia.

megaloblastosis, and vitamin B12 and folate levels in the blood must be determined periodically.[80] Also, in cases with persistent thrombocytopaenia, the association of immune thrombocytopaenia must be ruled out.[115]

Regarding the current concerns about the risk of neoplastic processes, eg, haematological neoplasms,[90–98] the origin of this association is still under debate. However, the sphingolipid that accumulates most in this disease, ceramide, is involved in the regulation of cellular signal transduction, in cellular oxidative stress, and in cell death.[127–129] Therefore, the whole cellular regulatory system could be affected, which is a matter of ongoing debate. Moreover, the contribution of the underlying chronic inflammatory state is important in lysosomal

diseases, with permanent activation of the immune system as a result of macrophage activation and the secretion of cytokines, causing immune system dysfunction.[81–83] The recommendation is to monitor the presence of polyclonal and monoclonal gammopathies in patients by performing an annual proteinogram, quantification of immunoglobulins, ferritin, and C-reactive protein levels;[123] the determination of other parameters such as complement factors and lymphocyte populations may help to understand better the functioning of the immune system, but these tests are expensive and not generally available. Also, there are still not enough data for an appropriate interpretation of their impact or changes with therapy.

Recently, the influence of modifier genes in the appearance of neoplasms in GD has also been considered, but this is probably a casual association as occurs in some patients with *BCR/ABL, JAK2,* or *MSH6* mutations and is due to chance.[120,122,123] There is no justification for a systematic panel study of genes related to different neoplasms, except for research purposes.

Conclusion

In this review, an extensive search of the literature was performed. Haematological manifestations are without any doubt a universal feature of GD patients. The variability of their presentation is related to the age of the patient, but mostly with the severity of the disease. Despite the presence of cytopaenia, coagulation complications can also appear in a non-small number of patients and treating physicians need to be aware of it, especially in situations such as pregnancy or surgery. Massive screening strategies are expensive, but programmes that include a minimum of clinical suspicion with haematological parameters are more cost-effective; moreover, awareness strategies among the different medical specialties are a continuous need. There are still unmet needs in the understanding and managing of haematological complications, such as the interpretation and evaluation of the risk for long-term complications or the immune imbalance before and during therapy.

Acknowledgments

Disclosure

References

1. Cox TM, Schofield JP. Gaucher's disease: clinical features and natural history. *Baillieres Clin Haematol*. 1997;10:657–689. doi:10.1016/S0950-3536(97)80033-9

2. Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. *Lancet*. 2008;372:1263–1271. doi:10.1016/S0140-6736(08)61522-6

3. Pastores GM, Hughes DA. Gaucher disease. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. *GeneReviews® [Internet]*. Seattle (WA): University of Washington, Seattle; 1993–2021. July 27, 2000 [updated June 21, 2018].

4. Stirnemann J, Belmatoug N, Camou F, et al. A review of Gaucher disease pathophysiology, clinical presentation and treatments. *Int J Mol Sci*. 2017;18:441. doi:10.3390/ijms18020441

5. Nalysnyk L, Rotella P, Simeone JC, et al. Gaucher disease epidemiology and natural history: a comprehensive review of the literature. *Hematology*. 2017;22:65–73. doi:10.1080/10245332.2016.1240391

6. Baris HN, Cohen IJ, Mistry PK. Gaucher disease: the metabolic defect, pathophysiology, phenotypes and natural history. *Pediatr Endocrinol Rev*. 2014;12(Suppl 1):72–81.

7. Grabowski GA, Zimran A, Ida H. Gaucher disease types 1 and 3: phenotypic characterization of large populations from the ICGG Gaucher registry. *Am J Hematol*. 2015;90(Suppl 1):S12–8. doi:10.1002/ajh.24063

8. Andrade-Campos M, Capablo JL, Fraile JJ, et al. Twenty-five years diagnosing Gaucher's disease in Spain, what we have learned? *Blood*. 2018;132(Supplement 1):1110. doi:10.1182/blood-2018-99-115474

9. Gordon S, Plüddemann A, Martinez Estrada F. Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol Rev*. 2014;262:36–55. doi:10.1111/imr.12223

10. Boven LA, van Meurs M, Boot RG, et al. Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages. *Am J Clin Pathol*. 2004;122:359–369. doi:10.1309/BG5VA8JRDQH1M7HN

11. Gören Şahin D, Üsküdar Teke H, Karagülle M, et al. Gaucher cells or pseudo-Gaucher cells: that's the question. *Turk J Haematol*. 2014;31:428–429. doi:10.4274/tjh.2014.0019

12. Medrano-Engay B, Irun P, Gervas-Arruga J, et al. Iron homeostasis and inflammatory biomarker analysis in patients with type 1 Gaucher disease. *Blood Cells Mol Dis*. 2014;53:171–175. doi:10.1016/j.bcmd.2014.07.007

13. Regenboog M, van Kuilenburg AB, Verheij J, et al. Hyperferritinemia and iron metabolism in Gaucher disease: potential pathophysiological implications. *Blood Rev*. 2016;30:431–437. doi:10.1016/j.blre.2016.05.003

14. Arends M, van Dussen L, Biegstraaten M, Hollak CE. Malignancies and monoclonal gammopathy in Gaucher disease; a systematic review of the literature. *Br J Haematol*. 2013;161:832–842. doi:10.1111/bjh.12335

15. Limgala RP, Ioanou C, Plassmeyer M, et al. Time of initiating enzyme replacement therapy affects immune abnormalities and disease severity in patients with Gaucher disease. *PLoS One*. 2016;11:e0168135. doi:10.1371/journal.pone.0168135

16. Mitrovic M, Elezovic I, Miljic P, Suvajdzic N. Acquired von Willebrand syndrome in patients with Gaucher disease. *Blood Cells Mol Dis*. 2014;52:205–207. doi:10.1016/j.bcmd.2013.11.001

17. Spectre G, Roth B, Ronen G, et al. Platelet adhesion defect in type I Gaucher disease is associated with a risk of mucosal bleeding. *Br J Haematol*. 2011;153:372–378. doi:10.1111/j.1365-2141.2011.08613.x

18. Mehta A. Gaucher disease: unmet treatment needs. *Acta Paediatr*. 2008;97:83–87. doi:10.1111/j.1651-2227.2008.00653.x

19. Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *PLoS Med*. 2021;18:e1003583. doi:10.1371/journal.pmed.1003583

20. Medoff AS, Bayrd ED. Gaucher's disease in 29 cases: hematologic complications and effect of splenectomy. *Ann Intern Med*. 1954;40:481–492.

21. Giraldo P, Pérez-López J, Núñez R, et al. Patients with type 1 Gaucher disease in Spain: a cross-sectional evaluation of health status. *Blood Cells Mol Dis*. 2016;56:23–30. doi:10.1016/j.bcmd.2015.10.001

22. Charrow J, Esplin JA, Gribble TJ, et al. Gaucher disease: recommendations on diagnosis, evaluation, and monitoring. *Arch Intern Med*. 1998;158:1754–1760. doi:10.1001/archinte.158.16.1754

23. Essabar L, Meskini T, Lamalmi N, et al. Gaucher's disease: report of 11 cases with review of literature. *Pan Afr Med J*. 2015;20:18. doi:10.11604/pamj.2015.20.18.4112

24. Niederau C, Häussinger D. Gaucher's disease: a review for the internist and hepatologist. *Hepatogastroenterology*. 2000;47:984–997.

25. Niederau C, Rolfs A, Vom Dahl S, et al. Diagnose und Therapie des Morbus Gaucher. Aktuelle Empfehlungen der deutschen Therapiezentren im Jahr 2000 [Diagnosis and therapy of Gaucher disease. Current recommendations of German therapy centers in the year 2000]. *Med Klin (Munich)*. 2001;96:32–39. doi:10.1007/PL00002150

26. Belmatoug N, Caubel I, Stirnemann J, Billette de Villemeur T. La maladie de Gaucher [Gaucher's disease]. *J Soc Biol*. 2002;196:141–149. doi:10.1051/jbio/2002196020141

27. Giraldo P, Pocoví M, Pérez-Calvo J, et al. Report of the Spanish Gaucher's disease registry: clinical and genetic characteristics. *Haematologica*. 2000;85:792–799.

28. Weinreb NJ, Camelo JS Jr, Charrow J, et al.; International Collaborative Gaucher Group (ICGG) Gaucher Registry (NCT00358943) investigators. Gaucher disease type 1 patients from the ICGG Gaucher Registry sustain initial clinical improvements during twenty years of imiglucerase treatment. *Mol Genet Metab*. 132;2021:100–111. doi:10.1016/j.ymgme.2020.12.295

29. Zimran A, Altarescu G, Rudensky B, et al. Survey of hematological aspects of Gaucher disease. *Hematology*. 2005;10:151–156. doi:10.1080/10245330500067181

30. Mistry PK, Batista JL, Andersson HC, et al. Transformation in pretreatment manifestations of Gaucher disease type 1 during two decades of alglucerase/imiglucerase enzyme replacement therapy in the International Collaborative Gaucher Group (ICGG) Gaucher Registry. *Am J Hematol*. 2017;92:929–939. doi:10.1002/ajh.24801

31. Thomas AS, Mehta AB, Hughes DA. Diagnosing Gaucher disease: an on-going need for increased awareness amongst haematologists. *Blood Cells Mol Dis*. 2013;50:212–217. doi:10.1016/j.bcmd.2012.11.004

32. Hughes D. Gaucher disease: hematologic and oncologic implications. *Clin Adv Hematol Oncol*. 2011;9:771–772.

33. Linari S, Castaman G. Hematological manifestations and complications of Gaucher disease. *Expert Rev Hematol*. 2016;9:51–58. doi:10.1586/17474086.2016.1112732

34. Zimran A, Belmatoug N, Bembi B, et al. Demographics and patient characteristics of 1209 patients with Gaucher disease: descriptive analysis from the Gaucher Outcome Survey (GOS). *Am J Hematol*. 2018;93:205–212. doi:10.1002/ajh.24957

35. Puri RD, Kapoor S, Kishnani PS, et al.; Gaucher Disease Task Force. Diagnosis and management of Gaucher disease in India - consensus guidelines of the Gaucher Disease Task Force of the Society for Indian Academy of Medical Genetics and the Indian Academy of Pediatrics. *Indian Pediatr*. 2018;55:143–153. doi:10.1007/s13312-018-1249-9

36. Hughes DA, Pastores GM. Haematological manifestations and complications of Gaucher disease. *Curr Opin Hematol*. 2013;20:41–47. doi:10.1097/MOH.0b013e32835a9148

37. Andrade-Campos MM, de Frutos LL, Cebolla JJ, et al. Identification of risk features for complication in Gaucher's disease patients: a machine learning analysis of the Spanish registry of Gaucher disease. *Orphanet J Rare Dis*. 2020;15:256. doi:10.1186/s13023-020-01520-7

38. Revel-Vilk S, Szer J, Zimran A. Hematological manifestations and complications of Gaucher disease. *Expert Rev Hematol*. 2021;14:347–354. doi:10.1080/17474086.2021.1908120

39. Wasserstein MP, Caggana M, Bailey SM, et al. The New York pilot newborn screening program for lysosomal storage diseases: report of the first 65,000 infants. *Genet Med*. 2019;21:631–640. doi:10.1038/s41436-018-0129-y

40. Navarrete-Martínez JI, Limón-Rojas AE, Gaytán-García MJ, et al. Newborn screening for six lysosomal storage disorders in a cohort of Mexican patients: three-year findings from a screening program in a closed Mexican health system. *Mol Genet Metab*. 2017;121:16–21. doi:10.1016/j.ymgme.2017.03.001

41. Burlina AB, Polo G, Salviati L, et al. Newborn screening for lysosomal storage disorders by tandem mass spectrometry in North East Italy. *J Inherit Metab Dis*. 2018;41:209–219. doi:10.1007/s10545-017-0098-3

42. Burton BK, Charrow J, Hoganson GE, et al. Screening for lysosomal storage disorders in Illinois: the initial 15-month experience. *J Pediatr*. 2017;190:130–135. doi:10.1016/j.jpeds.2017.06.048

43. Elliott S, Buroker N, Cournoyer JJ, et al. Pilot study of newborn screening for six lysosomal storage diseases using Tandem Mass Spectrometry. *Mol Genet Metab*. 2016;118:304–309. doi:10.1016/j.ymgme.2016.05.015

44. Kang L, Zhan X, Gu X, Zhang H. Successful newborn screening for Gaucher disease using fluorometric assay in China. *J Hum Genet*. 2017;62:763–768. doi:10.1038/jhg.2017.36

45. Liao HC, Chiang CC, Niu DM, et al. Detecting multiple lysosomal storage diseases by tandem mass spectrometry–a national newborn screening program in Taiwan. *Clin Chim Acta*. 2014;431:80–86. doi:10.1016/j.cca.2014.01.030

46. Camargo Neto E, Schulte J, Pereira J, et al. Neonatal screening for four lysosomal storage diseases with a digital microfluidics platform: initial results in Brazil. *Genet Mol Biol*. 2018;41:414–416. doi:10.1590/1678-4685-gmb-2017-0227

47. Sanders KA, Gavrilov DK, Oglesbee D, et al. A comparative effectiveness study of Newborn screening methods for four lysosomal storage disorders. *Int J Neonatal Screen*. 2020;6:44. doi:10.3390/ijns6020044

48. Málaga DR, Brusius-Facchin AC, Siebert M, et al. Sensitivity, advantages, limitations, and clinical utility of targeted next-generation sequencing panels for the diagnosis of selected lysosomal storage disorders. *Genet Mol Biol*. 2019;42(1 suppl 1):197–206. doi:10.1590/1678-4685-gmb-2018-0092

49. Lei K, Zhao Y, Sun L, et al. A pilot screening of high-risk Gaucher disease children using dried blood spot methods in Shandong province of China. *Orphanet J Rare Dis*. 2018;13:48. doi:10.1186/s13023-018-0782-x

50. Huang Y, Jia X, Tang C, et al. High risk screening for Gaucher disease in patients with splenomegaly and/or thrombocytopenia in China: 55 cases identified. *Clin Chim Acta*. 2020;506:22–27. doi:10.1016/j.cca.2020.03.016

51. Motta I, Filocamo M, Poggiali E, et al.; Splenomegaly Gaucher Disease study group. A multicentre observational study for early diagnosis of Gaucher disease in patients with Splenomegaly and/or Thrombocytopenia. *Eur J Haematol*. 2016;96:352–359. doi:10.1111/ejh.12596

52. Miyamoto T, Iino M, Komorizono Y, et al. Screening for Gaucher disease using dried blood spot tests: a Japanese Multicenter, Cross-sectional Survey. *Intern Med*. 2021;60:699–707. doi:10.2169/internalmedicine.5064-20

53. Andrade-Campos M, Alfonso P, Irun P, et al. Diagnosis features of pediatric Gaucher disease patients in the era of enzymatic therapy, a national-base study from the Spanish Registry of Gaucher Disease. *Orphanet J Rare Dis*. 2017;12:84. doi:10.1186/s13023-017-0627-z

54. Giraldo P, López de Frutos L, Cebolla JJ. Biomarker combination is necessary for the assessment of Gaucher disease? *Ann Transl Med*. 2018;6(Suppl 1):S81. doi:10.21037/atm.2018.10.69

55. Fuller M, Tucker JN, Lang DL, et al. Screening patients referred to a metabolic clinic for lysosomal storage disorders. *J Med Genet*. 2011;48:422–425. doi:10.1136/jmg.2010.088096

56. Tang C, Jia X, Tang F, et al. Detection of glucosylsphingosine in dried blood spots for diagnosis of Gaucher disease by LC-MS/MS. *Clin Biochem*. 2021;87:79–84. doi:10.1016/j.clinbiochem.2020.10.011

57. Mistry PK, Cappellini MD, Lukina E, et al. A reappraisal of Gaucher disease-diagnosis and disease management algorithms. *Am J Hematol*. 2011;86:110–115. doi:10.1002/ajh.21888

58. Di Rocco M, Andria G, Deodato F, et al. Early diagnosis of Gaucher disease in pediatric patients: proposal for a diagnostic algorithm. *Pediatr Blood Cancer*. 2014;61:1905–1909. doi:10.1002/pbc.25165

59. Mehta A, Kuter DJ, Salek SS, et al. Presenting signs and patient co-variables in Gaucher disease: outcome of the Gaucher Earlier Diagnosis Consensus (GED-C) Delphi initiative. *Intern Med J*. 2019;49:578–591. doi:10.1111/imj.14156

60. Billett HH, Rizvi S, Sawitsky A. Coagulation abnormalities in patients with Gaucher's disease: effect of therapy. *Am J Hematol*. 1996;51:234–236. doi:10.1002/(SICI)1096-8652(199603)51:3<234::AID-AJH9>3.0.CO;2-Y

61. Hollak CE, Levi M, Berends F, et al. Coagulation abnormalities in type 1 Gaucher disease are due to low-grade activation and can be partly restored by enzyme supplementation therapy. *Br J Haematol*. 1997;96:470–476. doi:10.1046/j.1365-2141.1997.d01-2076.x

62. Katz K, Tamary H, Lahav J, et al. Increased operative bleeding during orthopaedic surgery in patients with type I Gaucher disease and bone involvement. *Bull Hosp Jt Dis*. 1999;58:188–190.

63. Giona F, Palumbo G, Amendola A, et al. Platelet function and coagulation abnormalities in type 1 Gaucher disease patients: effects of enzyme replacement therapy (ERT). *J Thromb Haemost*. 2006;4:1831–1833. doi:10.1111/j.1538-7836.2006.02037.x

64. Deghady A, Marzouk I, El-Shayeb A, Wali Y. Coagulation abnormalities in type 1 Gaucher disease in children. *Pediatr Hematol Oncol*. 2006;23:411–417. doi:10.1080/08880010600623232

65. Mitrovic M, Antic D, Elezovic I, et al. Haemostatic abnormalities in treatment-naïve patients with type 1 Gaucher's disease. *Platelets*. 2012;23:143–149. doi:10.3109/09537104.2011.597526

66. Givol N, Goldstein G, Peleg O, et al. Thrombocytopenia and bleeding in dental procedures of patients with Gaucher disease. *Haemophilia*. 2012;18:117–121. doi:10.1111/j.1365-2516.2011.02540.x

67. Komninaka V, Repa K, Marinakis T, et al. Platelet function defects in patients with Gaucher disease on long term ERT-implications for evaluation at bleeding challenges. *Blood Cells Mol Dis*. 2020;80:102371. doi:10.1016/j.bcmd.2019.102371

68. Rosenbaum H. Hemorrhagic aspects of Gaucher disease. *Rambam Maimonides Med J*. 2014;5(4):e0039. doi:10.5041/RMMJ.10173

69. Mitrovic M, Elezovic I, Grujicic D, et al. Complex haemostatic abnormalities as a cause of bleeding after neurosurgery in a patient with Gaucher disease. *Platelets*. 2015;26:260–262. doi:10.3109/09537104.2014.903471

70. Clarkson CP, Magann EF, Siddique SA, Morrison JC. Hematological complications of Gaucher's disease in pregnancy. *Mil Med*. 1998;163:499–501. doi:10.1093/milmed/163.7.499

71. Granovsky-Grisaru S, Belmatoug N, Vom Dahl S, et al. The management of pregnancy in Gaucher disease. *Eur J Obstet Gynecol Reprod Biol*. 2011;156:3–8. doi:10.1016/j.ejogrb.2010.12.024

72. Rosenbaum H. Management of women with Gaucher disease in the reproductive age. *Thromb Res*. 2015;135(Suppl 1):S49–51. doi:10.1016/S0049-3848(15)50443-X

73. Hollak CE, Maas M, Aerts JM. Clinically relevant therapeutic endpoints in type I Gaucher disease. *J Inherit Metab Dis*. 2001;24 (Suppl 2):97–105;87–8. doi:10.1023/A:1012492429191

74. Pastores GM, Weinreb NJ, Aerts H, et al. Therapeutic goals in the treatment of Gaucher disease. *Semin Hematol*. 2004;41(4 Suppl 5):4–14. doi:10.1053/j.seminhematol.2004.07.009

75. Weinreb NJ, Finegold DN, Feingold E, et al. Evaluation of disease burden and response to treatment in adults with type 1 Gaucher disease using a validated disease severity scoring system (DS3). *Orphanet J Rare Dis*. 2015;10:64. doi:10.1186/s13023-015-0280-3

76. Biegstraaten M, Cox TM, Belmatoug N, et al. Management goals for type 1 Gaucher disease: an expert consensus document from the European working group on Gaucher disease. *Blood Cells Mol Dis*. 2018;68:203–208. doi:10.1016/j.bcmd.2016.10.008

77. Shemesh E, Deroma L, Bembi B, et al. Enzyme replacement and substrate reduction therapy for Gaucher disease. *Cochrane Database Syst Rev*. 2015;3:CD010324.

78. Mankin HJ, Trahan CA, Barnett NA, et al. A questionnaire study for 128 patients with Gaucher disease. *Clin Genet*. 2006;69:209–217. doi:10.1111/j.1399-0004.2006.00573.x

79. Piran S, Roberts A, Patterson MA, Amato D. The clinical course of untreated Gaucher disease in 22 patients over 10 years: hematological and skeletal manifestations. *Blood Cells Mol Dis*. 2009;43:289–293. doi:10.1016/j.bcmd.2009.08.002

80. Gielchinsky Y, Elstein D, Green R, et al. High prevalence of low serum vitamin B12 in a multi-ethnic Israeli population. *Br J Haematol*. 2001;115:707–709. doi:10.1046/j.1365-2141.2001.03156.x

81. Wine E, Yaniv I, Cohen IJ. Hyperimmunoglobulinemia in pediatric-onset type 1 Gaucher disease and effects of enzyme replacement therapy. *J Pediatr Hematol Oncol*. 2007;29:451–457. doi:10.1097/MPH.0b013e31806451d3

82. Sender SU, Limgala RP, Ivanova MM, et al. Persistent immune alterations and comorbidities in splenectomized patients with Gaucher disease. *Blood Cells Mol Dis*. 2016;59:8–15. doi:10.1016/j.bcmd.2016.02.003

83. Gervas-Arruga J, Cebolla JJ, de Blas I, et al. The influence of genetic variability and proinflammatory status on the development of bone disease in patients with Gaucher disease. *PLoS One*. 2015;10(5):e0126153. doi:10.1371/journal.pone.0126153

84. Pandey MK, Burrow TA, Rani R, et al. Complement drives glucosylceramide accumulation and tissue inflammation in Gaucher disease. *Nature*. 2017;543:108–112. doi:10.1038/nature21368

85. Shiran A, Brenner B, Laor A, Tatarsky I. Increased risk of cancer in patients with Gaucher disease. *Cancer*. 1993;72:219–224. doi:10.1002/1097-0142(19930701)72:1<219::AID-CNCR2820720139>3.0.CO;2-Y

86. Reardon M, Cotter P, Kealy WF, Duggan PF. Gaucher's disease with haematological complications. *Ir Med J*. 1993;86:39–40.

87. de Lonlay P, Fenneteau O, Touati G, et al. Manifestations hématologiques dans les erreurs innées du métabolisme [Hematologic manifestations of inborn errors of metabolism]. *Arch Pediatr*. 2002;9:822–835. doi:10.1016/S0929-693X(02)00005-2

88. Elias AF, Johnson MR, Boitnott JK, Valle D. Neonatal cholestasis as initial manifestation of type 2 Gaucher disease: a continuum in the spectrum of early onset Gaucher disease. *JIMD Rep*. 2012;5:95–98.

89. Vom Dahl S, Mengel E. Lysosomal storage diseases as differential diagnosis of hepatosplenomegaly. *Best Pract Res Clin Gastroenterol*. 2010;24:619–628. doi:10.1016/j.bpg.2010.09.001

90. Rosenbloom BE, Weinreb NJ, Zimran A, et al. Gaucher disease and cancer incidence: a study from the Gaucher Registry. *Blood*. 2005;105:4569–4572. doi:10.1182/blood-2004-12-4672

91. Zimran A, Liphshitz I, Barchana M, et al. Incidence of malignancies among patients with type I Gaucher disease from a single referral clinic. *Blood Cells Mol Dis*. 2005;34:197–200. doi:10.1016/j.bcmd.2005.03.004

92. Costello R, O'Callaghan T, Baccini V, Sébahoun G. Aspects hématologiques de la maladie de Gaucher [Hematological aspects of Gaucher disease]. *Rev Med Interne*. 2007;28(Suppl 2):S176–9. doi:10.1016/S0248-8663(07)78877-X

93. de Fost M, Vom Dahl S, Weverling GJ, et al. Increased incidence of cancer in adult Gaucher disease in Western Europe. *Blood Cells Mol Dis*. 2006;36:53–58. doi:10.1016/j.bcmd.2005.08.004

94. Taddei TH, Kacena KA, Yang M, et al. The underrecognized progressive nature of N370S Gaucher disease and assessment of cancer risk. *Am J Hematol*. 2009;84:208–214. doi:10.1002/ajh.21362

95. Lo SM, Stein P, Mullally S, et al. Expanding spectrum of the association between Type 1 Gaucher disease and cancers: a series of patients with up to 3 sequential cancers of multiple types–correlation with genotype and phenotype. *Am J Hematol*. 2010;85:340–345. doi:10.1002/ajh.21684

96. Weinreb NJ, Lee RE. Causes of death due to hematological and non-hematological cancers in 57 US patients with type 1 Gaucher Disease who were never treated with enzyme replacement therapy. *Crit Rev Oncog*. 2013;18:177–195. doi:10.1615/CritRevOncog.2013005921

97. Weinreb NJ, Barbouth DS, Lee RE. Causes of death in 184 patients with type 1 Gaucher disease from the United States who were never treated with enzyme replacement therapy. *Blood Cells Mol Dis*. 2018;68:211–217. doi:10.1016/j.bcmd.2016.10.002

98. Mistry PK, Taddei T, Vom Dahl S, Rosenbloom BE. Gaucher disease and malignancy: a model for cancer pathogenesis in an inborn error of metabolism. *Crit Rev Oncog*. 2013;18:235–246. doi:10.1615/CritRevOncog.2013006145

99. Rodic P, Pavlovic S, Kostic T, et al. Gammopathy and B lymphocyte clonality in patients with Gaucher type I disease. *Blood Cells Mol Dis*. 2013;50:222–225. doi:10.1016/j.bcmd.2012.11.012

100. Cox TM, Rosenbloom BE, Barker RA. Gaucher disease and comorbidities: b-cell malignancy and parkinsonism. *Am J Hematol*. 2015;90(Suppl 1):S25–8. doi:10.1002/ajh.24057
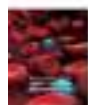
101. Nair S, Branagan AR, Liu J, et al. Clonal immunoglobulin against lysolipids in the origin of myeloma. *N Engl J Med*. 2016;374:555–561. doi:10.1056/NEJMoa1508808

102. Nair S, Bar N, Xu ML, et al. Glucosylsphingosine but not Saposin C, is the target antigen in Gaucher disease-associated gammopathy. *Mol Genet Metab*. 2020;129:286–291. doi:10.1016/j.ymgme.2020.01.009

103. Ranade A, Chintapatla R, Varma M, Sandhu G. Hematologic manifestations and leukemia in Gaucher's disease. *Clin Adv Hematol Oncol*. 2013;11:253–255.

104. Shvidel L, Hurwitz N, Shtalrid M, et al. Complex IgA gammopathy in Gaucher's disease. *Leuk Lymphoma*. 1995;20:165–168. doi:10.3109/10428199509054770

105. Martinez-Redondo C, Ortuño FJ, Lozano ML, et al. IgM monoclonal component associated with type I Gaucher disease resolved after enzyme replacement therapy: a case report. *J Inherit Metab Dis*. 2009;32(Suppl 1):S265–7. doi:10.1007/s10545-009-1207-8

106. Perales M, Cervantes F, Cobo F, Montserrat E. Non-Hodgkin's lymphoma associated with Gaucher's disease. *Leuk Lymphoma*. 1998;31:609–612. doi:10.3109/10428199809057621

107. Shvidel L, Sigler E, Shtalrid M, et al. Parotid gland involvement, the presenting sign of high grade non-Hodgkin lymphoma in two patients with Gaucher disease and sicca syndrome. *J Inherit Metab Dis*. 2007;30:825. doi:10.1007/s10545-007-0610-2

108. Zimran A, Ruchlemer R, Revel-Vilk S. A patient with Gaucher disease and plasma cell dyscrasia: bidirectional impact. *Hematology Am Soc Hematol Educ Program*. 2020;2020:389–394. doi:10.1182/hematology.2020000123

109. Monge J, Chadburn A, Gergis U. Synchronous multiple myeloma and Gaucher disease. *Hematol Oncol Stem Cell Ther*. 2020;13:42–45. doi:10.1016/j.hemonc.2019.07.001

110. Hawkesford MP, Bowey AJ, Rao J, Meara NJ. Synchronous presentation of Gaucher disease and solitary plasmacytoma with progression to multiple myeloma. *Scott Med J*. 2011;56:236. doi:10.1258/smj.2011.011178

111. Kim MJ, Suh JT, Lee HJ, et al. Simultaneous detection of Gaucher's disease and renal involvement of non-Hodgkin's lymphoma: the first Asian case report and a review of literature. *Ann Clin Lab Sci*. 2012;42:293–301.

112. Haliloglu M, Hoffer FA, Haight AE, Cunningham JM. Budd-Chiari syndrome caused by Gaucher's disease. *Pediatr Radiol*. 1999;29:908–910. doi:10.1007/s002470050723

113. Miri-Moghaddam E, Velayati A, Naderi M, et al. Coinheritance of Gaucher disease and α-thalassemia resulting in confusion between two inherited hematologic diseases. *Blood Cells Mol Dis*. 2011;46:88–91. doi:10.1016/j.bcmd.2010.08.007

114. Kose MD, Canda E, Kağnıcı M, et al. Coexistence of Gaucher disease and severe congenital neutropenia. *Blood Cells Mol Dis*. 2019;76:1–6. doi:10.1016/j.bcmd.2018.07.001

115. Lester TJ, Grabowski GA, Goldblatt J, et al. Immune thrombocytopenia and Gaucher's disease. *Am J Med*. 1984;77:569–571. doi:10.1016/0002-9343(84)90124-4

116. Maity P, Roy S, Das R, et al. Acute myeloid leukemia along with Gaucher disease in a child. *Indian J Case Rep*. 2021;5:211–213. doi:10.32677/IJCR.2021.v07.i05.012

117. Petrides PE, leCoutre P, Müller-Höcker J, et al. Coincidence of Gaucher's disease due to a private mutation and Ph' positive chronic myeloid leukemia. *Am J Hematol*. 1998;59:87–90. doi:10.1002/(SICI)1096-8652(199809)59:1<87::AID-AJH17>3.0.CO;2-Z

118. Villarrubia J, Rodríguez-Gambarte JD, Abalo L, et al. Gaucher disease and myelodysplastic syndrome with isolated del(5q). *Br J Haematol*. 2014;165:164. doi:10.1111/bjh.12798

119. Kubo T, Shimose S, Fujimori J, et al. Extraosseous extension caused by epidural hematoma in Gaucher disease mimicking malignant bone tumor. *JIMD Rep*. 2014;14:67–70.

120. Noya M, Andrade-Campos M, Irun P, et al. Gaucher disease and chronic myeloid leukemia: first reported patient receiving enzyme replacement and tyrosine kinase inhibitor therapies simultaneously. *Clin Case Rep*. 2018;6:887–892. doi:10.1002/ccr3.1460

121. Ruchlemer R, Mittelman M, Zimran A. Gaucher disease, myelodysplastic syndrome and ICUS. *Blood Cells Mol Dis*. 2020;80:102373. doi:10.1016/j.bcmd.2019.102373

122. Webb BD, Weinreb NJ, Botti AC, et al. JAK2V617F mutation and myeloproliferative malignancy in a patient with Type 1 Gaucher disease. *Blood Cells Mol Dis*. 2011;46:103–104. doi:10.1016/j.bcmd.2010.09.007

123. Lo SM, Choi M, Liu J, et al. Phenotype diversity in type 1 Gaucher disease: discovering the genetic basis of Gaucher disease/hematologic malignancy phenotype by individual genome analysis. *Blood*. 2012;119:4731–4740. doi:10.1182/blood-2011-10-386862

124. Hughes D, Cappellini MD, Berger M, et al. Recommendations for the management of the haematological and onco-haematological aspects of Gaucher disease. *Br J Haematol*. 2007;138:676–686. doi:10.1111/j.1365-2141.2007.06701.x

125. Mehta A, Belmatoug N, Bembi B, et al. Exploring the patient journey to diagnosis of Gaucher disease from the perspective of 212 patients with Gaucher disease and 16 Gaucher expert physicians. *Mol Genet Metab*. 2017;122:122–129. doi:10.1016/j.ymgme.2017.08.002

126. Deriaz S, Serratrice C, Lidove O, et al. Parcours diagnostique des patients atteints de maladie de Gaucher de type 1: enquête auprès de médecins internistes et hématologues [Diagnostic journey of type 1 Gaucher Disease patients: a survey including internists and hematologists]. *Rev Med Interne*. 2019;40:778–784. doi:10.1016/j.revmed.2019.07.011

127. Mistry PK, Sadan S, Yang R, et al. Consequences of diagnostic delays in type 1 Gaucher disease: the need for greater awareness among hematologists-oncologists and an opportunity for early diagnosis and intervention. *Am J Hematol*. 2007;82:697–701. doi:10.1002/ajh.20908

128. Barth BM, Shanmugavelandy SS, Tacelosky DM, et al. Gaucher's disease and cancer: a sphingolipid perspective. *Crit Rev Oncog*. 2013;18:221–234. doi:10.1615/CritRevOncog.2013005814

129. Murugesan V, Chuang W-L, Liu J, et al. Glucosylsphingosine is a key biomarker of Gaucher disease. *Am J Hematol*. 2016;91(11):1082–1089. doi:10.1002/ajh.24491

Hypercoagulable State Induced Spinal Cord Stroke After Coronavirus Disease 19 Infection

Lisda Amalia

Published online: 14 Dec 2021.

Submit your article to this journal 🗗

Article views: 136

View related articles 🗗

View Crossmark data 🗗

Citing articles: 4 View citing articles 🗗

CASE REPORT

Hypercoagulable State Induced Spinal Cord Stroke After Coronavirus Disease 19 Infection

Lisda Amalia (ORCID)

Department of Neurology, Faculty Medicine, Universitas Padjadjaran/RSUP Dr. Hasan Sadikin Bandung, Bandung, Indonesia

Background: Spinal cord stroke after coronavirus disease 19 (COVID-19) infection is rare, and limited cases have been reported. Spinal cord ischemia after COVID-19 infection is related to increased coagulopathy with thromboembolic consequences. Patients with COVID-19 may have a hypercoagulable state and an increased rate of thromboembolic events, such as occlusion in the spinal artery.

Case Presentation: We report a male case with confirmed COVID-19 infection, aged 60 years, with flaccid paraplegia, hyporeflexia, loss of sensation below the 12th thoracic level, loss of autonomic function, bilateral positive Babinski sign 14 days after the onset of flu-like symptoms, and elevated serum D-dimer and fibrinogen levels. There was stenosis of the spinal artery at the 12th thoracic level in magnetic resonance imaging and magnetic resonance angiography. He showed improvement in motor strength of the lower limb (walking with assistance), numbness and pain, and urine and fecal retention after receiving a subcutaneous anticoagulant.

Conclusion: COVID-19 can damage endothelial cells and activate thrombotic pathways, which can lead to clinical thromboembolic complications, such as occlusion in the spinal artery, resulting in spinal cord stroke.

Keywords: COVID-19 infection, hypercoagulable state, spinal cord stroke

Introduction

In late December 2019, reports of a severe acute respiratory illness emerged from Wuhan (China), which was established as coronavirus disease 2019 (COVID-19); it involved a severe acute respiratory syndrome and rapidly spread to other regions.[1] The World Health Organization (WHO) declared COVID-19 as a pandemic on March 11, 2020, because of the rapid global spread of the disease, and on October 14, 2021, over 239 million confirmed cases of COVID-19 were reported globally, which included over 4.8 million deaths.[2] The first two cases of COVID-19 in Indonesia were reported on March 2, 2020. By January 12, 2021, there were 846,765 confirmed COVID-19 cases and 24,645 deaths in Indonesia.[3]

Interestingly, several reports of COVID-19 cases indicated neurological symptoms of both the central (CNS) and peripheral nervous systems, and the most common serious CNS complications are cerebrovascular diseases.[4] Spinal cord stroke after COVID-19 infection is rare, and limited cases have been reported.[5–7]

We discuss a case report of spinal cord stroke following acute COVID-19 pneumonia caused by anterior spinal artery occlusion. Written informed consent to publish case details and any accompanying images was provided by the patient. Dr. Hasan Sadikin General Hospital Bandung Human Research Ethics Committee approved this consent process.

Correspondence: Lisda Amalia
Department of Neurology, Faculty Medicine, Universitas Padjadjaran/RSUP Dr. Hasan Sadikin Bandung, Jl. Pasteur 38, Bandung, 40161, Indonesia
Email dr.lisda@gmail.com

Case Presentation

A 60-year-old male was admitted to the emergency unit with weakness and numbness in both lower limbs with girdle-like pain at the lower thoracic level after 14 days of fever and upper respiratory tract infection. Two days later, symptoms progressed to complete lower-limb paralysis, with loss of sensation below the 12th thoracic level. He also had urine and fecal retention. Coronary artery disease as a comorbidity was recorded 10 months earlier, and the patient underwent a primary coronary intervention procedure because of three-vessel coronary artery occlusion. He had hypertension, which was regularly controlled, and had ceased smoking for 20 years. Routine medication before admission was 81 mg aspirin once daily, 2.5 mg bisoprolol once daily, and 20 mg atorvastatin once daily.

Upon examination, the patient was fully alert, and the cranial nerves were unaffected. Both lower limbs had complete paralysis, with hypotonia, hyporeflexia, and a positive Babinski sign bilaterally. Sensation to light touch diminished below the 12th thoracic level, with loss of pricking pain, temperature sensation, and vibration (tested by a tuning fork).

Laboratory tests were positive for severe acute respiratory syndrome-coronavirus-type 2 (SARS-CoV-2) based on a polymerase chain reaction test using a nasopharyngeal swab. Serum D-dimer (4.06 mg/L), fibrinogen (663 mg/dL) (normal values: D-dimer up to 0.55 mg/L and fibrinogen 238–498 mg/dL), and erythrocyte sedimentation rate (ESR) in the first hour (98 mm/hour; reference ESR value <15 mm/h) were elevated. White blood cell (WBC) count $7.9 \times 10^9/\mu L$, red blood cell (RBC) count $4.18 \times 10^6/\mu L$, hemoglobin 11.70 g/dL, platelet count $188 \times 10^3/\mu L$, neutrophils 80.9% (the neutrophil count was high), lymphocytes 15% (low), eosinophils 0.70 (low), basophils 0%, and monocytes 2% (normal values: WBCs $4–10 \times 10^3/\mu L$, hemoglobin 12–15 g/dL, platelet count $140–450 \times 10^3/\mu L$, neutrophils 40%–75%, lymphocytes 20%–45%, monocytes 2%–10%, eosinophils 2–6%, and basophils 0–1%). All electrolytes were within the normal limit. Liver and renal functions were also within the normal limit. A sagittal T2-weighted magnetic resonance imaging (MRI)-magnetic resonance angiography image of the thoracic and lumbar spine (Figure 1A and B) showed a drop in signal of the anterior spinal artery at the 12th thoracic level. He was treated with heparin 5000 IU every 8 hours, 81 mg aspirin once daily, 2.5 mg bisoprolol once daily, 20 mg atorvastatin once daily, 5 mg lisinopril once daily, 500 mg mecobalamin every 8 hours, and 300 mg gabapentin once daily for neuropathic pain symptoms.

After 44 days of hospitalization, he showed an improvement in lower limb motor strength (walking with assistance) and exhibited numbness, pain, and urine and fecal retention.

Discussion

COVID-19 infection can involve rapid multiorgan dysfunction and varies in presentation from asymptomatic to severe.[1] Interestingly, several reports of COVID-19 cases have shown neurological symptoms of both the CNS and peripheral nervous system,[8] and the most common serious CNS complications are cerebrovascular diseases, which indicate direct and immune-mediated effects of the virus on the nervous system.[4]

Stroke is reported in 2%–6% of hospitalized patients with COVID-19, and acute cerebrovascular disease is the most severe complication of COVID-19 due to abnormalities in cascade coagulation according to cohort studies.[1] SARS-CoV-2 can induce inflammatory response syndrome, higher production of pro-inflammatory mediators (cytokines and chemokines), activation of thrombotic pathways, and a hypercoagulation state due to abnormalities in cascade coagulation, which contributes to the cerebrovascular event.[9] These responses may be because the viral invasion of the vascular endothelium (endotheliitis) contributes to vascular ischemia of the spinal cord.[9] Spinal cord stroke after COVID-19 infection is rare, and limited cases have been reported.[5–7]

Tumor necrosis factor-α, interleukin (IL)-6, and IL-1 are important cytokines that inhibit intrinsic anticoagulation pathways in COVID-19 patients. This reaction facilitates coagulation activation and thrombin formation, which induces occlusion in the vascular system.[10] Furthermore, local apoptosis and potent inflammatory cytokines are induced by inflammation in pericytes and infection in endothelial cells, and intravascular coagulopathy is activated following the inflammatory process in the pulmonary alveoli. Patients with COVID-19 may have a hypercoagulable state and an increased rate of thromboembolic events, such as occlusion in the spinal artery.[10]

In this case report, we presented a typical ischemic pattern of spinal cord stroke after COVID-19 infection, with an acute onset, a complete neurological problem at the 12th thoracic level, and a hypercoagulable state confirmed by elevated D-dimer levels and hyperfibrinogenemia. Intravascular thrombosis pathways leading to microvascular and macrovascular thrombosis complications occur after intravascular thrombosis pathways are activated because of systemic cytokine production. Cytokines and platelets interact with neutrophils, which

Figure 1 Magnetic resonance imaging (MRI). (**A**) Magnetic resonance angiography showing a drop in signal at the 12th thoracic level. (**B**) MRI showing no ischemic or demyelinating lesion.

stimulate thrombin production and fibrin deposition. Excess fibrin deposition and fibrinolysis shutdown lead to intravascular thrombosis and eventually clinical thromboembolic complications, such as occlusion in the spinal artery, which leads to spinal cord stroke.[11] However, further studies are needed to explore the causal relationship.

Limitation of the Study

This case study has several limitations. MRI was not repeated following the administration of the anticoagulant or following neurological deficit recovery.

Conclusion

Some COVID-19 cases exhibit neurological symptoms, with the most common serious CNS complications being cerebrovascular diseases, such as spinal cord stroke. SARS-CoV-2 can damage endothelial cells and activate inflammatory and thrombotic pathways due to an abnormal coagulation cascade, which eventually result in clinical thromboembolic complications, such as occlusion in the spinal artery, resulting in spinal cord stroke. Complications following COVID-19 infection are increasing and are often unfamiliar to healthcare professionals. Thus, clinicians, especially neurologists, should be informed of such severe complications to enable prompt diagnoses and appropriate treatments to decrease spinal cord stroke-related morbidity and reduce public health burdens.

Funding

Disclosure

The author reports no conflicts of interest for this work.

References

1. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;395:497–506. doi:10.1016/S1040-6736(20)30183-5
2. World Health Organization. Rolling updates on coronavirus disease (COVID-19). Geneva: World Health Organization; 2020 [cited January 13, 2021]. Available from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/events-as-they-happen. Accessed December 7, 2021.
3. Ministry of Health Republic of Indonesia. COVID-19 crisis center. Jakarta: Ministry of Health Republic of Indonesia; 2021 [Cited January 12, 2021] Available from: https://www.pusatkrisis.kemkes.go.id. Accessed December 7, 2021.
4. Ellul MA, Benjamin L, Singh B, et al. Neurological associations of COVID-19. *Lancet Neurol*. 2020;19:767–783. doi:10.1016/s1474-4422(20)30221-0
5. AlKetbi R, AlNuaimi D, AlMulla M, et al. Acute myelitis as a neurological complication of COVID-19: a case report and MRI findings. *Radiol Case Rep*. 2020;15:1591–1595. doi:10.1016/j.radcr.2020.06.001
6. Munz M, Wessendorf S, Koretsis G, et al. Acute transverse myelitis after COVID-19 pneumonia. *J Neurol*. 2020;267:2196–2197. doi:10.1007/s00415-020-09934-w
7. Sarma D, Bilello LA A case report of acute transverse myelitis following novel coronavirus infection. *Clin Pract Cases Emerg Med*. 2020;4:321–323. doi: 10.5811/cpcem.2020.5.47937.
8. Sotoca J, Rodríguez-álvarez Y. COVID-19-associated acute necrotizing myelitis. *Neurol Neuroimmunol Neuroinflamm*. 2020;7:e803. doi:10.1212/nxi.0000000000000803
9. Reddy ST, Garg T, Shah C, et al. Cerebrovascular disease in patients with COVID-19: a review of the literature and case series. *Case Rep Neurol*. 2020;12:199–209. doi:10.1159/000508958
10. Getu S, Tiruneh T, Andualem H, et al. Coagulopathy in SARS-CoV-2 infected patients: implication for the management of COVID 19. *J Blood Med*. 2021;12:635–643. doi:10.2147/JBM.S304783
11. Ortega-Paz L, Capodano D, Montalescot G, Angiolillo DJ. Coronavirus disease 2019–associated thrombosis and coagulopathy: review of the pathophysiological characteristics and implications for antithrombotic management. *J Am Heart Assoc*. 2021;10:e019650. doi:10.1161/JAHA.120.019650

Pruritus as a Presenting Symptom of *FIP1L1-PDGFRA*-Positive Chronic Eosinophilic Leukemia

Mohammad Abu-Tineh, Esra'a Aljaloudi & Mohamed A Yassin

CASE REPORT

Pruritus as a Presenting Symptom of *FIP1L1-PDGFRA*-Positive Chronic Eosinophilic Leukemia

Mohammad Abu-Tineh ⓘ[1]
Esra'a Aljaloudi[2]
Mohamed A Yassin ⓘ[1]

[1]Department of Oncology- Hematology and BMT Section, National Center for Cancer Care and Research, Hamad Medical Corporation, Doha, Qatar; [2]Department of Family Medicine, Hamad Medical Corporation, Doha, Qatar

Abstract: Eosinophilia can be found in a variety of benign and malignant conditions, and a persistent eosinophilic count of more than 1500/mm^3 necessitates additional investigation. Patients with FIP1L1-PDGFRA-positive chronic eosinophilic leukemia might present as asymptomatic or in a catastrophic state with multi-organ involvement. We present the case of a young male patient who was diagnosed with FIP1L1-PDGFRA chronic eosinophilic leukemia after a long history of recurrent cutaneous symptoms with no systemic signs.
Keywords: chronic eosinophilic leukemia, *FIP1L1-PDGFRA*, eosinophilia, imatinib

Introduction

FIP1L1-PDGFRA-positive chronic eosinophilic leukemia is a rare myeloproliferative neoplasm marked by a continuous increase in circulating eosinophils ($\geq$1.5 x 109/L). Secondary causes should be ruled out before verifying clonality and confirming the CEL diagnosis.[1] The infiltration of eosinophils into many organs, as well as the production of eosinophilic granules and cytokines, cause severe damage and probable organ malfunction. Patients can report anything from serious symptoms like restricted cardiomyopathy to long-term eosinophilia with no obvious symptoms. According to patients with FIP1L1-PDGFRA-positive chronic eosinophilic leukemia, the gastrointestinal system, lungs, and skin are the most affected organs.[1] We present the case of a 30-year-old male patient who was diagnosed with FIP1L1-PDGFRA-positive CEL, which manifested mostly as severe pruritus of the skin.

Case Presentation

A 29-year-old male patient with a background of newly developed attacks of cough and shortness of breath of a few months' duration was diagnosed with intermittent asthma, with no similar history during childhood. The patient was referred to the Dermatology team with complaints of multiple skin lesions related to sun exposure along with persistent pruritus. The patient had an initial assessment of common causes of allergies which came back negative, thus he was referred for further evaluation. A few months later the patient presented with the same complaints, but the later episode was associated with watery diarrhea and a history of subjective weight loss, along with a history of severe persistent pruritus. CBC showed elevated WBC counts with marked eosinophilia for which the patient was referred for further workup. CBC showed WBC of 22 x10^3/μL normal value (4.0–10.0 x10^3/μL), Hb: 14.6 gm/dL normal value (13.0–17.0 gm/dL), PLT: 168 x10^3/

Correspondence: Mohammad Abu-Tineh
Department of Oncology- Hematology and BMT Section- National Center for Cancer Care and Research, Alrayan Street, Doha, Qatar
Tel +97455003969
Email Abutineh90@gmail.com

μL normal value (150–400 x10^3/μL), ANC: 4.8 x10^3/μL normal values (2.0–7.0 x10^3/μL), eosinophil count: 14.1 x10^3/μL normal values (0.0–0.5 x10^3/μL). Peripheral smear

showed normocytic normochromic red cells, leukocytosis with marked eosinophilia, composed mostly of mature forms, the majority of eosinophils showed abnormal nuclear segmentation and or sparse cytoplasmic granulation, platelets were adequate.

Chest X-ray was unremarkable, abdomen US was significant for mild hepatosplenomegaly.

IgE levels were within normal limits. Bone marrow was remarkably hypercellular (almost 100% cellularity) with granulocytic hyperplasia and remarkably increased eosinophilic cells with adequate erythropoiesis and megakaryocytes with some dysplastic forms. CD117 immunostain performed on bone marrow showed increased mast cells, including some spindle-forms. These mast cells were positive for mast cell tryptase and aberrantly positive for CD 25. No increase in CD34-positive cells was noted. Reticulin stain showed areas of mildly increased reticulin fibers (MF0-1). FISH (fluorescence in situ hybridization) analysis using the *FIP1L1/CHIC2/PDGFRA (4q12)* probe was consistent with a deletion of *CHIC2* resulting in the fusion of *PDGFA* and *FIP1L1* in 76% of nuclei. There was no BCR/ABL1 gene rearrangement. Molecular genetics revealed no *KIT* mutation. The molecular studies for JAK-2, CALR, and KIT D816V mutation were negative.

The patient was started on imatinib 100 mg orally once a day, and his numbers were normalized within a month, along with a considerable improvement in his skin manifestations in terms of frequency, episodes, and intensity.

Discussion

Eosinophilia is described as an increase in the number of eosinophils in the peripheral blood, with an absolute count of greater than 500 eosinophils x103μL. Hypereosinophilic syndrome is defined as an elevated eosinophil count (>1500 eosinophils x103/L) that lasts longer than 6 months (HES). Nonetheless, in 2011, the working conference on eosinophil disorders and syndromes proposed that at least two episodes of eosinophilia separated by at least four weeks can be classified as persistent, indicating that there is no identifiable etiology for eosinophilia and that patients had signs and symptoms of organ involvement.[1,2]

Chronic unexplained eosinophilia greater than 1500/mm3 is a hallmark of PDGFRA-associated chronic eosinophilic leukemia, a type of blood cancer. Persistent eosinophilia (1.5 x 109/L) in the absence of reactive causes,

followed by a positive peripheral blood screening for the FIP1L1-PDGFRA gene fusion (via RT-PCR or interphase/metaphase FISH), is classified as myeloid/lymphoid neoplasms associated with eosinophilia and PDGFRA rearrangement, according to the WHO 2017 classification. The real incidence of chronic eosinophilic leukemia, not otherwise described, is unknown due to the difficulties in separating it from the idiopathic hypereosinophilic syndrome.[1]

CEL is classified as a myeloproliferative subtype of hypereosinophilic syndrome. Eosinophilic myeloid and lymphoid neoplasms with PDGFRA, PDGFRB, and FGFR1 rearrangements were classified as a distinct class in the 2008 WHO classification;[3] these neoplasms are defined by overexpression of an abnormal tyrosine kinase caused by a mutation or a particular fusion gene. A mutant pluripotent (myeloid-lymphoid) stem cell has been identified as the cell of origin.[4] Clonal eosinophilia is caused by gene rearrangements or point mutations in the PDGFRA, PDGFRB, or FGFR1 genes, with the creation of a FIP1L1-PDGFRA fusion gene being the most common,[5] accounting for 10–20% of patients with unexplained eosinophilia in Western countries. As a result of the interstitial deletion of 4q12 that leads to FIP1L1-PDGFRA fusion, approximately 70% of patients with PDGFRA rearrangement have eosinophilia.[6]

The FIP1L1-PDGFRA fusion protein is a platelet-derived growth factor receptor that remains active even when platelet-derived growth factor is present because the first 29 amino acids of the FIP1L1 protein can activate the PDGFRα kinase domain. The activation of the entire subsequent signaling pathway is triggered by the continual phosphorylation of the receptor on a tyrosine, causing the change of hematopoietic cells to an endless growth state.[7]

The FIP1L1-PDGFRA fusion gene is considered the most important molecular biomarker, and it has recently been shown to be susceptible to tyrosine-kinase inhibitor medications like imatinib.

The clinical manifestation is caused by a rapid increase in eosinophils and their distribution in organs such as the skin. Cough, dyspnea, generalized weakness, skin rash, and rhinitis are the most common symptoms.[8] Persistent eosinophilia can affect any organ; one well-known example is cardiac involvement, notably endomyocardial fibrosis, which increases the risk of death. Lung fibrosis, thromboembolism, and eosinophilic gastritis are some of the most serious symptoms.[8] In addition to an enlarged spleen and higher tryptase levels in the blood, those with PDGFRA-associated chronic eosinophilic leukemia may have an enlarged spleen.

Our patient's symptoms were mostly cutaneous, with several skin lesions reported as recurrent skin abscesses connected to intermittent sun exposure, as well as recurrent pruritus that became prominent and severe at the time of diagnosis. There were no major systemic manifestations. Eczema-like symptoms, angioedema, and numerous mucosal ulcers are all common cutaneous signs of eosinophilic leukemia.[9] Currently, many studies have demonstrated a significant outcome with complete hematologic and molecular remission in FIP1L1-PDGFRA-positive patients after initiating imatinib therapy of 100 to 400 mg daily.[2] A maintenance dose of 100–200 mg weekly can maintain total metabolic remission, with the majority of patients achieving complete molecular remission with 100 mg daily.[10]

Conclusion

FIP1L1-PDGFRA-positive CEL can manifest primarily as a skin symptom rather than a systemic disease; this case was reported to raise physician awareness of a common skin symptom that could be a sign of a serious illness; unusual presentations of skin symptoms that do not improve with standard treatment methods should prompt further evaluation.

Abbreviations

PDGFRA, platelet-derived growth factor receptor A; PDGFRB, platelet-derived growth factor receptor Beta; FGFR1, fibroblast growth factor receptor 1; TKI, tyrosine kinase inhibitor; DM, diabetes mellitus; ANC, absolute neutrophil count; FISH, fluorescence in situ hybridization; HES, hypereosinophilic syndrome; CML, Chronic Myelogenous Leukemia; CBC, complete blood count; MPN, myeloproliferative neoplasm.

Statement of Ethics

The case was approved by Hamad Medical Corporation Research Center with reference number MRC-04-20-997.

Consent

Written informed consent was obtained from our patient to allow the publication of information.

Disclosure

The authors report no conflict of interest in this work.

References

1. Gotlib J. World Health Organization-defined eosinophilic disorders: 2017 update on diagnosis, risk stratification, and management. *Am J Hematol.* 2017;92(11):1243–1259. doi:10.1002/ajh.24880
2. Abu-Tineh M, Amer A, Ibrahim F, Ibrahim WH, Alhasson H, Yassin MA. FIP1L1-platelet-derived growth factor receptor A-positive chronic eosinophilic leukemia showing dramatic response to imatinib: a case report. *Medicine.* 2021;2(1):e0019.
3. Bain BJ. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1. *Haematologica.* 2010;95(5):696. doi:10.3324/haematol.2009.021675
4. Wang SA. The diagnostic work-up of hypereosinophilia. *Pathobiology.* 2019;86(1):39–52. doi:10.1159/000489341
5. Savage N, George TI, Gotlib J. Myeloid neoplasms associated with eosinophilia and rearrangement of PDGFRA, PDGFRB, and FGFR 1: a review. *Int J Lab Hematol.* 2013;35(5):491–500. doi:10.1111/ijlh.12057
6. Vandenberghe P, Wlodarska I, Michaux L, et al. Clinical and molecular features of FIP1L1-PDFGRA (+) chronic eosinophilic leukemias. *Leukemia.* 2004;18(4):734–742. doi:10.1038/sj.leu.2403313
7. Gotlib J, Cools J, Malone JM, Schrier SL, Gilliland DG, Coutré SE. The FIP1L1-PDGFRα fusion tyrosine kinase in hypereosinophilic syndrome and chronic eosinophilic leukemia: implications for diagnosis, classification, and management. *Blood.* 2004;103 (8):2879–2891. doi:10.1182/blood-2003-06-1824
8. Roufosse F, Weller PF. Practical approach to the patient with hypereosinophilia. *J Allergy Clin Immunol.* 2010;126(1):39–44. doi:10.1016/j.jaci.2010.04.011
9. Leiferman KM, Gleich GJ, Peters MS. Dermatologic manifestations of the hypereosinophilic syndromes. *Immunol Allergy Clin North Am.* 2007;27(3):415–441. doi:10.1016/j.iac.2007.07.009
10. Reiter A, Gotlib J. Myeloid neoplasms with eosinophilia. *Blood.* 2017;129(6):704–714. doi:10.1182/blood-2016-10-695973