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Expression of CD11a, CD11b, CD11c, and CD18 on Neutrophils from Different Clinical Types of Malaria in Malawian Children

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Expression of CD11a, CD11b, CD11c, and CD18 on Neutrophils from Different Clinical Types of Malaria in Malawian Children

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Background: Malaria in individuals who have never had an infection before is usually characterized by an inflammatory response that is linked to the expression of specific activation markers on cells of the innate immune system.

Methods: This study investigated absolute white blood cell (WBC) and neutrophil counts and expression of several adhesion markers on neutrophils from HIV-uninfected children who were suffering from cerebral malaria (n=35), severe malarial anemia (SMA, n=39), and uncomplicated malaria (n=49) and healthy aparasitemic children (n=33) in Blantyre, Malawi.

Results: All clinical malaria groups had higher WBC and neutrophil counts compared to healthy controls, with the acute SMA group having significantly ($p<0.0001$) higher WBC counts than the controls. These elevated counts normalized during recovery. Surprisingly, in all clinical malaria groups, the surface expression of CD11b, CD11c, and CD18 on neutrophils was lower than in healthy controls, again normalizing during convalescence.

Conclusion: In areas where *Plasmodium falciparum* malaria is hyperendemic, such as where this study was conducted, neutrophils have reduced expression of adhesion molecules and activation markers during acute stages of the infection, regardless of the clinical type of malaria. This reduced expression could be due to an individual's past exposure to *P. falciparum* or other parasite-related factors that manifest during active malaria that still need to be investigated.

Keywords: white blood cells, neutrophils, malaria, integrins, receptors

Introduction

Despite significant gains achieved in mitigating the detrimental effect of malaria globally,^{1,2} malaria caused by the *Plasmodium falciparum* parasite still causes death in thousands of children annually, with a majority of these occurring in sub-Saharan Africa.³ Although the actual mechanistic cause of death is yet to be fully elucidated, it is now known that clinical *P. falciparum* malaria can manifest either as uncomplicated malaria (UM) or one of the severe forms of the disease — cerebral malaria (CM), severe malarial anemia (SMA), or respiratory distress — and other complications, with a number of possible overlaps among these syndromes.^{4,5} Of these syndromes, CM is associated with the worst outcomes, contributing to the highest number of deaths,⁴ severe neurological sequelae, and debilitating neurological impairments in survivors.⁶

Neutrophils make up the highest proportion of peripheral leukocytes — 40%–60%.⁷ These cells are known to play a crucial role in the innate immunoresponse during the early stages of infectious diseases, mainly through clearance of pathogens by

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phagocytosis.^{8,9} Studies conducted in Africa have shown that peripheral neutrophil counts increase by as much as 40% during acute UM, with the degree of increase being associated with the level of parasitemia.¹⁰ Furthermore, it is now known that neutrophils are not only the primary circulating cells to respond to an invading pathogen⁹ but that once they are activated, these cells are capable of phagocytosing infected red blood cells (iRBCs).¹¹ Like other circulating immune-system cells, neutrophils too need to express molecules involved in migration (chemotaxis) and adhesion.^{9,12}

During an inflammatory response, as is the case during acute malaria, adhesion molecules work to facilitate pairing between various receptors and their respective ligands and also transmit intrinsic signals, which in turn confer unique effector functions.¹³ It is estimated that four super-families of adhesion molecules are involved in these events, some of which include the selectins (L-selectin, E-selectin, and P-selectin), the integrins, some members of the immunoglobulin superfamily, and cadherins.¹⁴ A large family of heterodimeric glycoproteins, integrins are grouped according to the particular β subunit they possess. Based on this, β_2 integrins are expressed exclusively on leukocytes and participate in leukocyte adhesion to the endothelium. They are also involved in subsequent transmigration through interactions with molecules expressed on the surface of endothelial cells, including ICAM1. These heterodimeric adhesion molecules consist of a common β subunit (CD18) that associates with one of four α subunits: CD11a (LFA1), CD11b (MAC1), CD11c (p150), or CD11d.¹⁵ Based on their amino-acid homology, CD11b and CD11c are quite similar proteins, with close to 50% amino-acid similarity. Several ligands have been described for CD11b/CD18, including iC3b, fibrinogen, heparin, ICAM1 (CD54), ICAM2 (CD102), and ICAM4.¹⁶ However, less is known about CD11c/CD18, which is expressed mainly on monocytes, granulocytes, and activated T and B cells and has been identified as the ligand for ICAM1.¹⁵

During the life-cycle stages of *P. falciparum* that occur inside RBCs, hemoglobin is degraded, but the parasite is unable to catabolize heme. The unprocessed heme ends up aggregating, forming an insoluble polymer called malarial pigment or hemozoin.¹⁷ Various studies have demonstrated that monocytes and macrophages ingest hemozoin or hemozoin-containing parasitized RBCs, which results in a large proportion of these cells being loaded with hemozoin during malaria infection and sometimes for months postinfection.¹⁸ Other studies have further shown that phagocytosis of

hemozoin can actually interfere with the expression of CD11c and CD11b, a receptor for complement components C3b and C4b.¹⁹ In addition, hemozoin-fed monocytes have been observed to be viable but functionally impaired, with hemozoin promoting suspected of contributing to cell dysfunction, downregulation of phagocytosis, and reduced expression of MHC2 and CD54.¹⁹

Although neutrophil numbers are known to increase during acute malaria, little is known about the expression of various migration and adhesion receptors by neutrophils in peripheral. More specifically, not much is known on how neutrophil-adhesion molecules, such as CD11a, CD11b, and CD18, are affected during malaria infection. The goal of this study was thus to determine the absolute neutrophil numbers and characterize the surface expression of leukocyte-adhesion molecules CD11a, CD11b, CD11c, and CD18 on neutrophils in peripheral blood of children acutely suffering from different clinical types of *P. falciparum* malaria in comparison with blood samples from healthy controls.

Methods

Study Participants

Details of the study design and study participants have been reported previously.²⁰ In summary, the study was conducted at the Malawi Liverpool Wellcome Trust Clinical Research Programme and Department of Paediatrics, College of Medicine, University of Malawi, and Blantyre Malaria Project. Study participants were children who were either acutely suffering from malaria at the time of recruitment or had been admitted to Queen Elizabeth Central Hospital with malaria, and children who were medically well at the time of recruitment and were attending surgical outpatient clinics at Queen Elizabeth Central Hospital and Beit Cure International Hospital, both located in Blantyre, Malawi. Study participants were recruited mainly during the malaria season, which usually coincides with the rainy season (November 2005 to April 2006).

Firstly, informed written consent was obtained from the parent or guardian of each participant, then each potential participant was examined by a research nurse and/or a clinical officer. Once the participant had fulfilled the recruitment criteria, baseline demographic data were recorded and a 5 mL peripheral blood sample collected. All participants were then assessed for level of consciousness using the Blantyre Coma Score. This was done

several times, starting from admission and then at 2- to 4-hourly intervals during intensive clinical care. In summary, well over 40 children were prospectively enrolled into each of the three clinical malaria groups, defined by diagnoses of CM, SMA, and UM, and matched the criteria for the healthy control group.

For this study and a previous one on the same participants,²⁰ malaria was defined as a clinical syndrome without any other apparent possible cause, and was confirmed by a thick blood film positive for *P. falciparum* asexual parasites on microscopy. Study participants who presented with CM had a Blantyre Coma Score of two or less at admission and 4 hours later, while participants in the other malaria groups (SMA and UCM) and healthy controls had a score of five at both times.²⁰ Study participants with SMA had a blood-hemoglobin concentration of 5 g/dL or less, whereas those in the other groups had a hemoglobin concentration above this level. All participants who tested positive for HIV infection during the screening stage were excluded from the study and immediately referred to the antiretroviral therapy clinic. Following exclusions, 113 children with malaria (54 with UM, 30 with SMA, and 29 with CM) and 42 healthy controls were recruited. Of the children with malaria, 73 (34 UM, 21 SMA, and 18 CM) were successfully followed up a month after treatment, baseline demographic data recorded, and a 5 mL peripheral blood sample collected for laboratory investigations. Ages and sex ratios of the study participants for each group are provided in [Supplementary Table 1](#).

Ethics Review and Approval

Ethics review and approval for the study was obtained from College of Medicine Research and Ethics Committee. Written informed consent was obtained from the parent or guardian of every child before the child was

recruited into the study. A 5 mL venous blood sample was taken at the time of recruitment and during the recovery period. Blood was collected in tubes that already had EDTA anticoagulant in them, and these samples were used for immunophenotyping. This study was conducted in accordance with the Declaration of Helsinki.

Blood-Sample Analyses

Samples were analyzed as previously reported.²⁰ For each sample, 25 μ L EDTA blood was mixed with 1 μ L of three directly conjugated antibodies, as listed in [Table 1](#). In one tube, 1 μ L of each of the antibodies anti-CD11a-FITC, anti-CD11b-PE, and anti-CD14-PerCP was mixed. In another tube, 1 mL of anti-CD18-FITC, anti-CD11c-PE, and anti-CD14-PerCP was mixed. A third tube was included that was labeled with isotype controls. Samples were incubated for 15 minutes in the dark at room temperature. FACS lysing solution (500 μ L; Becton Dickinson) was added to each tube and incubated in the dark for 10 minutes at room temperature. Cells were washed twice with 2 mL PBS and fixed with 100 μ L PBS–1% formaldehyde. Data were acquired on a BD FACSCalibur flow cytometer and analyzed using CellQuest Pro software. CD14-PerCP was used to set a gate for monocytes, and this simplified the gate setting for neutrophils, which were distinguished by light- and forward-scatter characteristics ([Figure 1](#)).

Data Analysis

Results from the four groups (healthy controls, UCM, SMA, and CM) were analyzed using GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant. The Kruskal–Wallis test was employed in the comparison of geometric mean fluorescent intensity (GMFI) values for the markers CD11a, CD11b, CD11c, and CD18 on neutrophils.

Table 1 Monoclonal antibodies and their corresponding cell population. In each tube, 25 μ L EDTA blood was mixed with 1 μ L of the indicated antibodies. In tube 1, representing the isotype controls, the blood sample was mixed with 1 μ L of each of anti-moG1-FITC, anti-G2a-PE, and anti-CD14-PerCP. In tube 2, the blood sample was mixed with 1 μ L of each of anti-CD11a-FITC, anti-CD11b-PE, and anti-CD14-PerCP. In tube 3, the blood sample was mixed with 1 μ L of each of anti-CD18-FITC, anti-CD11c-PE, and anti-CD14-PerCP. In tube 4, the blood sample was mixed only with anti-CD14-PerCP, since this was the negative control

	FITC	PE	PerCP	
Tube 1	moG1	moG2a	CD14	Isotype controls
Tube 2	CD11a	CD11b	CD14	CD11a- and CD11b-expressing neutrophils
Tube 3	CD18	CD11c	CD14	CD18- and CD11c-expressing neutrophils
Tube 4	—	—	CD14	Negative control

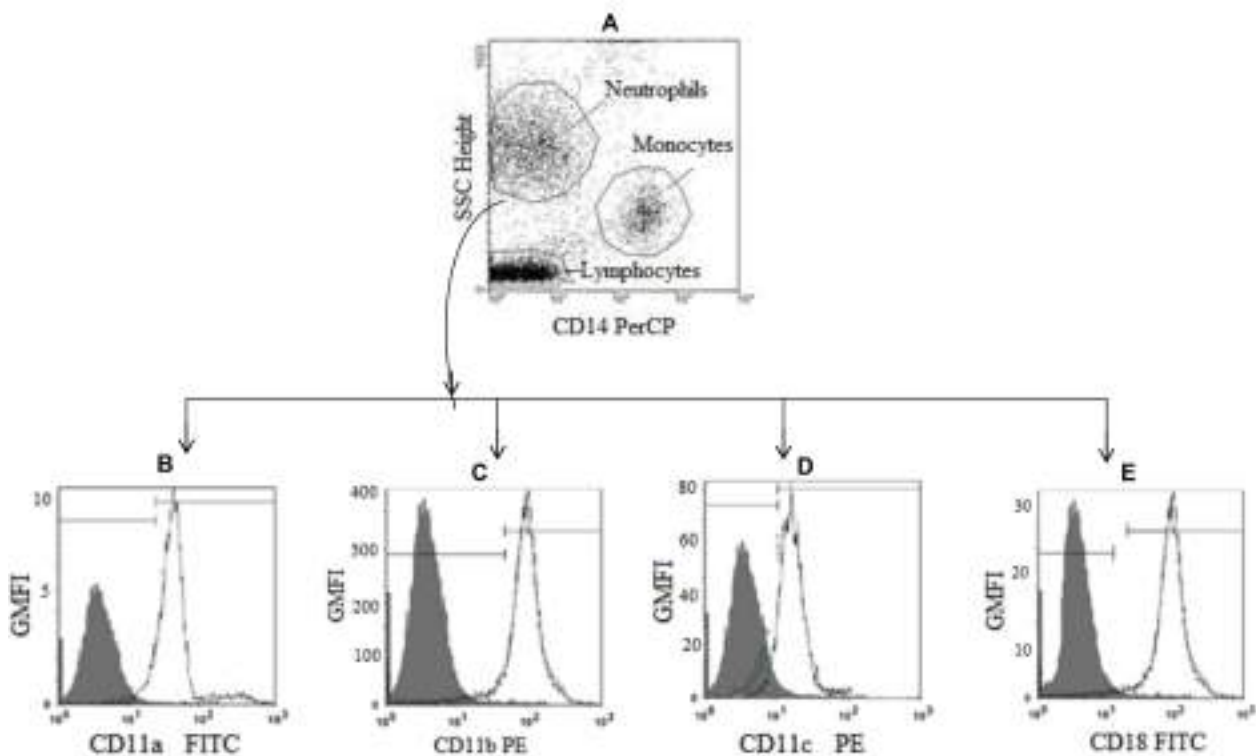


Figure 1 Gating strategy for determining the neutrophil population in a side-scatter/CD14 plot (A), with the neutrophil gate set to exclude monocytes and lymphocytes (A), geometric mean fluorescent intensity (GMFI) values for neutrophil surface markers CD11a (B), CD11b (C), CD11c (D) and CD18 (E). The gray histograms represent the GMFI of the isotype controls, and the white histogram represents the GMFI of the different surface markers.

Wilcoxon matched-pair tests was used to determine the statistical significance of differences in GMFI values observed during acute infection and the recovery stage for UCM, SMA, and CM. The results in Figures 2–4 are presented as medians and 10th and 90th percentiles of absolute cells counts for white BCs (WBCs) and

neutrophils and GMFI values for CD11a, CD11b, CD11c, and CD18 on neutrophils during the acute and convalescent stages of UCM, SMA, and CM. For these scatterplots, the bottom and lines through the middle of the dots correspond to the median value (10th percentile at the bottom to 90th percentile at the top).

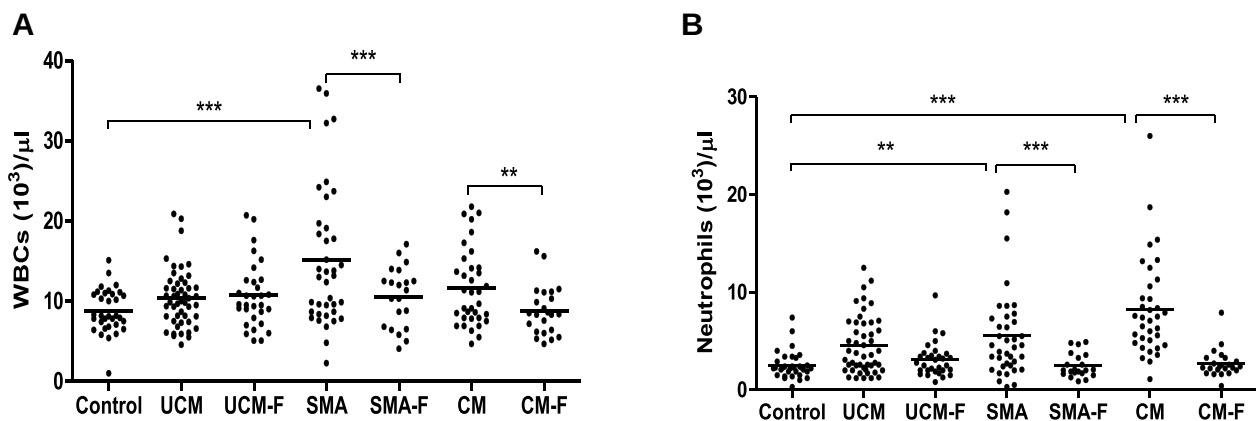


Figure 2 Medians (10th and 90th percentiles) of absolute white blood-cell counts (A) and neutrophil (B) counts in healthy controls and different types of malaria during acute stages of infection (UCM, SMA, CM)* and during convalescence (UCM-F, SMA-F, CM-F)[#] at 30 days after treatment. $p < 0.05$ considered statistically significant. ** $p < 0.01$; *** $p < 0.0001$.

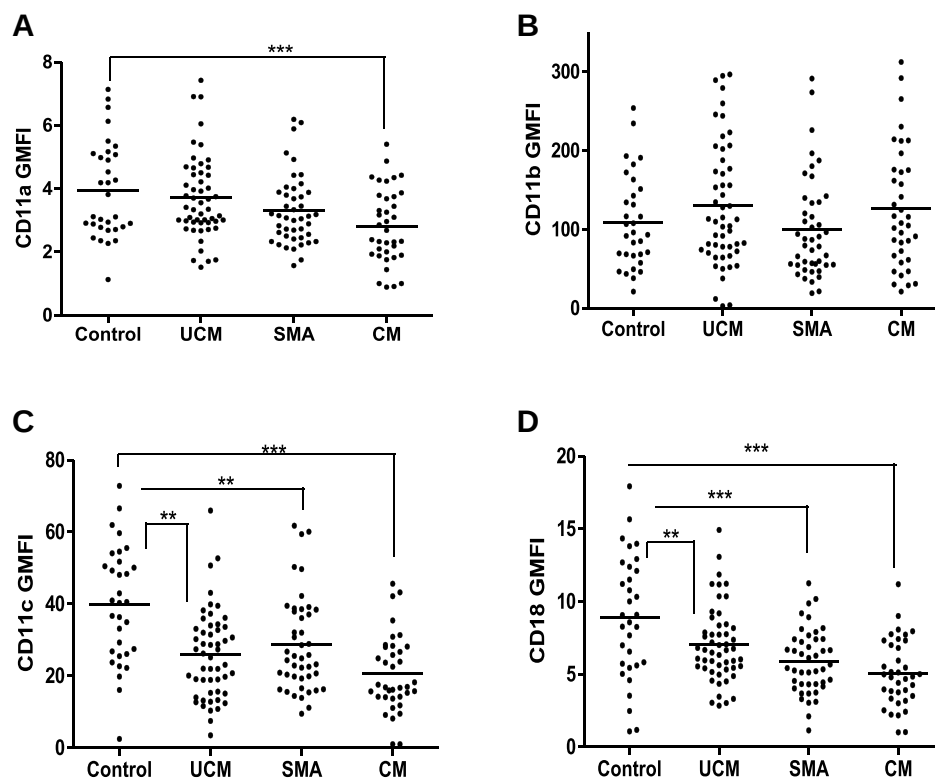


Figure 3 Medians (10th and 90th percentiles) of GMFI of CD11a (A), CD11b (B), CD11c (C), and CD18 (D) on neutrophils during acute stages of different clinical types of malaria (UCM, SMA, and CM)* and in healthy controls. $p < 0.05$ considered statistically significant. ** $p < 0.01$; *** $p < 0.0001$.

Results

Absolute Cell Counts

WBCs (Figure 2A) were higher in all three malaria types when compared to counts in the healthy controls, but the difference was statistically significant ($p = 0.0001$) only between the median counts for SMA ($13.1 \times 10^3/\mu\text{L}$) and for healthy controls ($8.2 \times 10^3/\mu\text{L}$). Comparing cells counts during acute and convalescent stages, WBC counts were significantly ($p = 0.027$) lower (11.4×10^3 cells/ μL) in the convalescent stage of SMA than in acute infection (13.1×10^3 cells/ μL), but this count was still higher than WBC counts in controls, although the difference between the medians was not statistically significant ($p = 0.053$). Neutrophils (Figure 2B) were significantly ($p < 0.0001$) higher (7.4×10^3 cells/ μL) in CM patients than controls (2.30×10^3 cells/ μL), UCM patients (3.7×10^3 cells/ μL), and in SMA patients (4.48×10^3 cells/ μL). The median of neutrophil counts in SMA participants was also significantly ($p = 0.016$) higher than controls. The high neutrophil counts (7.4×10^3 cells/ μL) observed in the acute-CM patients normalized in convalescence (during follow-up, 2.74×10^3 cells/ μL), and the difference between counts in

acute disease and convalescence was significant ($p < 0.0001$, Figure 2B). The high neutrophil counts (4.48×10^3 cells/ μL) observed in acute SMA normalized in convalescence (2×10^3 cells/ μL), and this difference was significant ($p = 0.0036$).

CD11a, CD11b, CD11c, and CD18 Expression on Neutrophils During Acute Malaria

In acute infection, the expression of CD11a (Figure 3A) — but not CD11b (Figure 3B) — CD11c (Figure 3C), and CD18 (Figure 3D) decreased in all malaria types. The decrease in the expression of CD11a was significant ($p = 0.0023$) in acute CM, and the expression of CD18 was also significantly lower for acute UCM ($p = 0.0319$), SMA ($p = 0.0006$) and CM ($p < 0.0001$) than healthy controls. The expression of CD11c (Figure 3C) was significantly lower for UCM and CM (both $p < 0.0001$) and for SMA ($p = 0.0015$) than healthy controls. There were no significant differences in expression of CD11b among any of the three malaria types (Figure 3B) or in comparison with healthy controls.

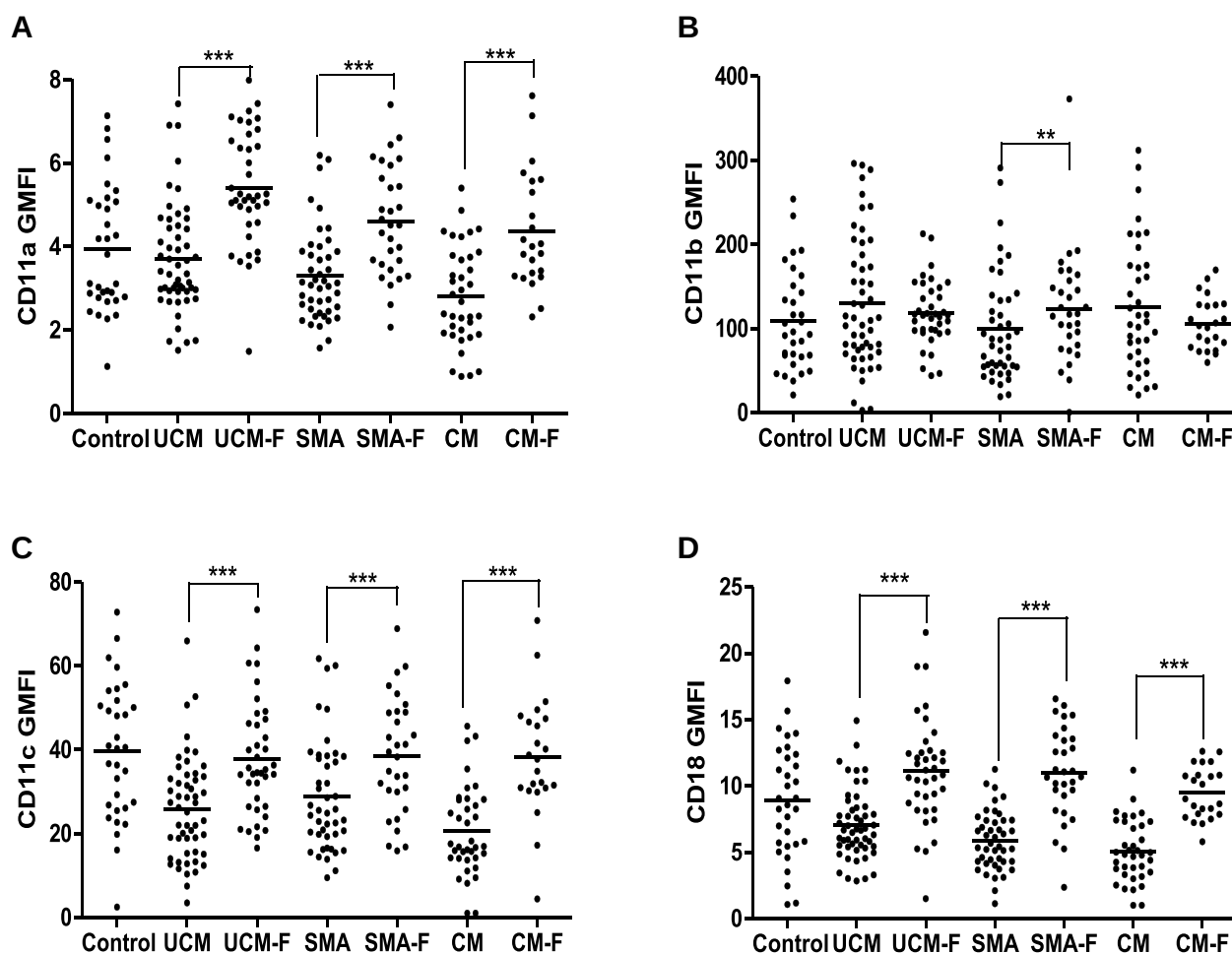


Figure 4 Medians (10th and 90th percentiles) of GMFI of CD11a (A), CD11b (B), CD11c (C), and CD18 (D) on neutrophils in healthy controls and in acute stages of infection (UCM, SMA, and CM)* and during convalescence (UCM-F, SMA-F and CM-F)[#] when at 30 days after treatment. $p<0.05$ considered statistically significant. ** $p<0.01$; *** $p<0.0001$.

Abbreviations: UCM, uncomplicated malaria; CM, cerebral malaria; SMA, severe malarial anemia; UCM-F, convalescent stage of uncomplicated malaria, CM-F, convalescent stage of cerebral malaria; SMA-F, convalescent stage of severe malarial anemia.

CD11a, CD11b, CD11c, and CD18 Expression on Neutrophils During Recovery Stage

The expression of CD11a (Figure 4A) — but not CD11b (Figure 4B) — CD11c (Figure 4C), and CD18 (Figure 4D) was significantly ($p<0.0001$) higher during recovery for all malaria types than in acute malaria. Although increases were observed in the expression of CD11b in convalescence (Figure 4B), the difference between acute and convalescent stages was significant ($p=0.0380$) only in SMA patients. Overall, the expression of all these markers in convalescence increased to almost identical levels to those observed in healthy controls (Figure 4A–D).

Discussion

In a previous study on a Malawian cohort,²¹ WBC counts were observed to decrease with age from 18 months to adulthood. The observation in the current study of higher WBC counts in all malaria groups than controls suggests that the increase in WBC counts was in response to acute malaria infection. The high WBC counts observed in severe malaria (CM and SMA) were not consistent with studies conducted elsewhere,^{22,23} which reported lower-than-normal total WBC counts during acute severe *P. falciparum* malaria. However, this study's results are consistent with other studies^{24–26} that also reported higher-than-normal WBC counts in *P. falciparum* patients.

One of those studies²⁴ found higher leukocyte counts in Kenyan children admitted with malaria than aged-matched healthy controls. It also found higher-than-normal leukocyte counts were linked to severe anemia and death and that children who died as a result of malaria had significantly higher numbers of leukocytes than those who survived. Studies finding decreased leukocytes in acute *P. falciparum* malaria patients^{22,23,27} had recruited patients aged >15 years who presented with UCM, while studies that found similar results to those of this report^{24–26} had investigated *P. falciparum* malaria in children aged ≤5 years who presented with severe malaria, similar to this report. Although other factors, such as geographical location, malaria endemicity,²⁸ and different malaria strains,²⁹ may play a crucial role, our results suggest that the age of the malaria patient is a crucial factor that determines how leukocytes are affected by malaria infection. Similarly, although neutrophil counts increase with age in healthy individuals,^{30,31} the neutrophil counts observed in acute CM were much higher than the highest count (2,800 cells/μL) observed in healthy children aged 3–5 years. This observation also suggests that the neutrophilia in acute SMA and CM could be as a result of the malaria infection itself. The neutrophilia observed in children with acute CM in this study was similar to what had been observed in children who presented with severe malaria in the Gambia,³² although the clinical malaria type was not clearly stated.

Results of work in mice depleted of peripheral neutrophils and infected with *P. berghei* ANKA showed that the depletion of neutrophils much earlier in the infection ended up preventing the development of experimental CM (ECM) and greatly reduced the level of microhemorrhage in the brain,³³ suggesting that neutrophils are somehow involved in the development of ECM although this possible link has not been proven in human CM as yet. However, results of another study have shown that neutrophils, or possibly a subset of neutrophils, interact with *P. falciparum* iRBCs through the PfEMP1 and ICAM1 receptors.³⁴ Considering the role these two markers play in the interaction between iRBCs and the endothelium of the brain microvasculature,^{35,36} it is possible that activated neutrophils are involved directly or indirectly in the cytoadherence of iRBCs in brain blood vessels, which is one of the signature characteristics of human CM,³⁷ or in some way contribute to malaria-associated pathology.^{38,39}

One study reported that the neutrophil-specific activation marker lipocalin was elevated in plasma collected from patients presenting with severe malaria.⁴⁰ Others

observed that lipocalin was upregulated in the brains of mice just before the onset of ECM syndrome.⁴¹ This has led some to the hypothesis that neutrophils recruited to the inflammation site are a likely source of such cytokines as IL12, IL18, IFN γ , and TNF α , which contribute to malaria pathogenesis.⁴¹ Various studies,^{27,42} including one of ours,²⁰ have shown that T cells and other immune-system cells are highly activated during acute malaria. These results are in support of the hypothesis that malaria infection stimulates cells of the innate and acquired immune systems to secrete proinflammatory cytokines with the aim of clearing the malaria parasites from the circulation.⁴³ It is thus surprising to observe a general downregulation of the expression of integrins by neutrophils in acute stages of all clinical malaria types.

However, the significantly lower expression of CD11c in acute malaria we observed in this study has been observed by other investigators¹⁹ who investigated how membrane antigens directly involved in the immunoresponse and phagocytic process are expressed. That investigation involved mixing PBMCs prepared from healthy unexposed adults with *P. falciparum* hemozoin extracts. The investigators found that expression of CD54 (ICAM1) and CD11c was decreased in hemozoin-loaded monocytes, although expression of CD11b was not affected in any way.¹⁹ This observation led them to suggest that hemozoin loading might contribute to impairment of the immunoresponse, especially antigen presentation.¹⁹ A previous study by our group⁴⁴ showed lower expression of CD11a, CD11b, and CD18 on monocytes from blood samples of Malawian children suffering from acute CM, SMA, and UCM, which then normalized in convalescence.

Contrary to the results of this study, in vitro exposure of leukocytes from naïve unexposed adults to isolated malaria pigment derived from ruptured schizonts has resulted in a significant increase in the expression of CD11b/CD18.¹⁸ The earliest response of CD11b/CD18 expression was detected 10 minutes following exposure to infected malaria pigment, a plateau reached after 60 minutes, and a decline starting after 90 minutes.¹⁸ The low expression of CD11b and CD18 observed in this study may be due to the fact that we were studying a population that had already been exposed to *P. falciparum* antigens on a number of occasions prior to the infection, which resulted in the children being recruited. In addition, an in vitro study¹⁸ assessed the expression of these markers over 90 minutes, whereas the children in this study presented most likely after they

had been infected with blood-stage malaria for hours or days. Therefore, the period of exposure may explain the marked differences between the results of this study and those of the *in vitro* study. Surprisingly, there seems to be no studies that have assessed the expression of these markers *in vivo* in different clinical types of malaria, as this study has done. Other studies have found that neutrophils isolated from malaria-infected children are capable of phagocytosing schizonts *in vitro*,⁴⁵ whereas neutrophils from US servicemen infected with *P. falciparum* but no documented previous exposure to *P. falciparum* malaria were not.^{46,47} One study measured the expression of CD11a/CD18 on lymphocytes in peripheral blood of Sudanese malaria patients, finding that expression of these markers was significantly higher on lymphocytes of malaria patients than lymphocytes from healthy controls. However, the study did not determine the expression of these markers on neutrophils.

This study has several limitations, one of which is the failure to include a neutrophil-specific marker in the antibody cocktail used in labeling the blood samples, as others have done using such markers as CD66b.^{48,49} However, other studies^{9,50} have used a similar gating strategy to that used in this study for characterizing neutrophils in whole blood. Secondly, we were restricted to studying only the expression of a limited number of neutrophil receptors due to the type of monoclonal antibodies available at the time. Future studies could include more markers, such as FcγRI, FcγRII, and FcγRI, and complement receptors (CR1 and CR3). Lastly, if this study measured hemozoin loading in the neutrophils in the different groups, as we had previously done with monocytes,⁴⁴ we could have established a more convincing link between hemozoin load and the lower-than-normal expression of the molecules observed.

Conclusion

Expression of the integrins CD11a, CD11b, CD11c, and CD18 on neutrophils was markedly lower during the acute stages of all three malaria types than in blood samples from controls. The expression of all four integrins was higher in convalescence than during acute infection. One possible explanation for these observations is that the hemozoin loading that occurs during acute infection contributes to a reduction in expression of the surface integrins, since the reduction in molecule expression decreased with malaria severity.

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Disclosure

The author declares no conflicts of interest.

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Phenotype, Allele and Genotype Frequency of ABO and Rhesus D Blood Groups of Blood Donors at the North Gondar District Blood Bank, Northwest Ethiopia

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Phenotype, Allele and Genotype Frequency of ABO and Rhesus D Blood Groups of Blood Donors at the North Gondar District Blood Bank, Northwest Ethiopia

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Background: Knowledge of the ABO and RhD group distribution is essential for blood banks inventory and assuring quality blood transfusion services. The objective of this study was to determine the frequency of ABO and RhD phenotype, allele, and genotype among blood donors at North Gondar District Blood Bank from 2010 to 2012, Northwest Ethiopia.

Methods: The data of the current study were obtained from registration logbooks of blood donors registered. The ABO and RhD grouping was done by using commercially available monoclonal antibodies (anti-A, anti-B and anti-D) by slide methods. Results with no agglutination by anti-D antibody were confirmed using anti-human globulin test. Descriptive statistics were analyzed using SPSS version 20. The allele and genotype frequency of the donors was determined by Hardy–Weinberg equilibrium assumption. The difference between the observed and expected frequency was tested by online Chi-square calculator. P-value of <0.05 was considered statistically significant.

Results: Among 6471 blood donors, 82.1%, 94.1% and 55.4% were males, replacement donors and in the age group of 21–30 years, respectively. Blood group O (47.04%) and blood group AB (4.81%) were the dominant and least common, respectively. The distribution of the RhD negative blood group was 5.76%. The distribution of A, B and O alleles was 0.1714, 0.1433 and 0.6859, respectively. Moreover, the genotype frequency of AA, AO, BB, BO, AB and OO was 0.0294, 0.2350, 0.0205, 0.1966, 0.0491 and 0.4704, respectively. The genotype frequency of DD, Dd and dd was 0.5774, 0.3649 and 0.0576, respectively. The result showed that there was no statistically significant difference between observed and expected allele and genotype frequency (P-value >0.05).

Conclusion: Blood group O and AB were the most and least prevalent, respectively. The allele and genotype frequency of the population was fulfilled the Hardy–Weinberg equilibrium assumption. This finding might be useful for blood transfusion services.

Keywords: ABO blood group, blood donor, Ethiopia, RhD

Introduction

Blood is essential for transporting oxygen, nutrients, wastes and hormones in the body. The ABO blood group system is the most clinically important blood group system, which was discovered by Karl Landsteiner in 1900¹ and awarded the Nobel Prize in 1930. The fourth type of blood group was also discovered by Alfred Von Decastello and Adriano Sturli and named blood group AB, in 1902.² The rhesus

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(Rh) blood group system is the second most significant blood group system. Depending on the presence of the D antigen on the red cell, the blood group system has two phenotypes; RhD positive and RhD negative. The ABO and RhD genes are found at chromosomes 9 and 1, respectively. Proteins and carbohydrates, bided to lipids or protein, are the blood group determining red blood cells antigens.³

Even there are about 100 blood group systems with 500 antigens, the ABO blood group systems are the most clinically important blood group systems in blood transfusion and organ transplantation services.^{4,5} A and B antigens are highly antigenic and have naturally occurring antibodies in human plasma which are missing the corresponding antigen and can cause hemolysis in vivo. The Rh blood group system is the second most clinically significant blood group with regard to transfusion. In addition to this, they are also important in the context of genetic studies, identifying of medico-legal issues and tracing family history.⁵

The presence of ABO antigens in red blood cells is depending on glycosyltransferases, which add sugars to the antecedent substance. However, a specific sets of epitopes and the RhD protein make up the D antigen.⁶

The ABO blood groups are determined by using anti-sera (anti-A, anti-B and anti-AB) to detect the presence of A and B antigens. Additionally, known red cells antigens can be used to diagnose anti-A and anti-B in the plasma and called backward grouping. ABO and Rh phenotypes, allele and gene frequency differ broadly according to races and geographical borders.^{3,7-9}

The frequency and distribution of ABO and Rh blood groups differ by ethnicity even in the same region. In Ethiopia, the frequencies of ABO and Rh blood groups have been investigated in some areas of the country. These studies showed that the frequency of the O blood group was the dominant followed by A, B and AB.¹⁰⁻¹³ Concerning to Rh blood group, about 7.0 –19.37% of the donors were RhD negative¹⁴ with the highest prevalence in Gambella (Ethiopia) (19.37%).¹⁰

Detection of the Rh system is imperative to avoid the erythroblastosis fetalis; which frequently occurred when an RhD negative mother carries an RhD positive fetus and during the second birth causes the death of the fetus. Knowing the frequency of ABO and RhD blood groups in a particular population is crucial for efficient management of blood bank inventor, even for local transfusion services.⁹ Thus, the main objective of the current study

was to determine the frequency and distribution of ABO and Rh blood groups among blood donors at the North Gondar district blood bank.

Methods and Materials

Study Area, Design and Population

This study was a retrospective study carried out at the University of Gondar hospital's distinct blood bank. The distinct blood bank is located at Gondar Town which was found 727 Km north of the capital city of Ethiopia, Addis Ababa. The blood groups of donors of either sex, donating during the period of three years (2010–2012) were studied. The blood bank provides transfusion services to approximately about five million people in North Gondar and neighboring districts. Annually, the blood bank collects about 2500 units of blood. The majority of the collected blood is used for emergency, surgical and gynecological cases.

The source population was all replacement and voluntary blood donors who donate at the University of Gondar Hospital's distinct blood bank and the study populations were donors who had donated between the year of 2010 and 2012. They are eligible for blood donation if donors are apparently healthy, none anemic (greater 12g/dl and 13g/dl hemoglobin for female and male, respectively) and aged between 18 and 65 years. The donors should be 45 kilograms and above for 350-millimeter unit donation and greater than 50 kilograms for 450-millimeter unit donation. Moreover, they should have normal vital signs (temperature <37°C, blood pressure; diastolic; 70–90 mmHg, systolic; 100–150 mmHg) and be free from any sexually transmitted infections including human immune virus, hepatitis B virus, hepatitis C virus and syphilis (Blood Donor Medical Assessment Guideline, National Blood Bank Service of Ethiopia, 2017).

Sample Size and Sampling Technique

All blood donors who donated blood at the North Gondar District Blood Bank from 2010 to 2012 were study populations. A total of 6471 blood donors were included. All the blood donors were selected by the censuses sampling technique.

Laboratory Tests

Blood grouping was determined by using commercially accessible blood grouping anti-sera; anti-A, anti-B and anti-D (ERYCLONE anti-A, anti-B and anti-D

monoclonal antibodies) by using the tile method. A drop of donor blood was placed on three places on a clean white tile. Then each antiserum was added and mixed with each blood drop with the help of an applicator stick. The presence of agglutination was indicating the presence of the corresponding Blood groups antigen. Hardy–Weinberg equilibrium assumption was used for the determination of the frequencies of alleles A, B, O, D and d, and expressed as a proportion.

Data Collection and Analysis

Demographic data of the donor like age, sex and occupation were obtained from the blood bank registration log book. The collected data were entered into Epi Info 3.5.1 and then transformed to SPSS Version 20 for analysis. The descriptive statistics were presented in the form of tables. The observed phenotype, allelic and genotypic frequency of the ABO and Rh blood group was compared by using chi-square test (calculated by online Chi-square calculator; available from <https://www.icalcu.com/stat/chisqtest.html>) considering the Hardy–Weinberg equilibrium assumption. According to Hardy–Weinberg assumption, the allelic and genotypic frequencies of the population will remain stable from generation to generation, provided that there is no mutation, no migration, and no natural selection in a large population with random mating. Therefore, if the locus of ABO with three alleles are, “O”, “A” and “B” and then the frequency of the alleles “O”, “A” and “B” will be designated by r , p and q , respectively and the frequencies of the O, A, B, and AB phenotypes are r^2 , $(p^2 + 2pr)$, $(q^2 + 2qr)$, and $2pq$, respectively.

So, the allele frequency of O, A and B can be estimated from the ABO phenotype by the following formula.

$$r = \sqrt{(\text{frequency of O phenotype})}$$

$$p = \sqrt{(\text{frequency of A + O phenotype})} - r$$

$$q = \sqrt{(\text{frequency of B + O phenotype})} - r$$

The allele frequency obtained by this formula from the observed phenotype frequency is called observed or unadjusted allele frequency. It is clear that the sum of all allelic frequencies should be one. However, it is true if the observed frequencies of the phenotypes had no deviation from the expected values. However, due to some factors, the observed phenotype frequency may deviate from the expected value. Hence, the summation of the allele frequencies can also deviate from one. The deviation (d) can

be calculated as follows; $d = 1 - (p + q + r)$; then the allelic frequencies should be corrected by:

$pc = p(1 + \frac{d}{2})$, $qc = q(1 + \frac{d}{2})$, and $rc = 1 - (pc + qc)$ (corrected or adjusted allele frequency). The phenotype and genotype frequency, recalculated from the corrected allele frequency is called expected phenotype and genotype frequency, respectively and can be estimated from corrected allelic frequencies by the following formula.

Expected frequency of phenotype A: $p_c^2 + 2p_c r_c$ (AA genotype + 2*AO genotype)

Expected frequency of phenotype B: $q_c^2 + 2q_c r_c$ (BB genotype + 2*BO genotype)

Expected frequency of phenotype O: r_c^2 (OO genotype)

Expected frequency of phenotype AB: $2p_c q_c$ (2*AB genotype)

Concerning the Rh blood groups, there are only two phenotypes; RhD positive (DD + Dd) and RhD negative (dd). Therefore, if D and d alleles have p and q frequencies respectively, the frequency of RhD negative is equal to q^2 and the allele frequencies will be $\sqrt{q^2}$ and $p = 1 - q$. However, we cannot investigate the deviation, because we have no degree of freedom.¹⁵

Data Quality Assurance

The data quality was assured by following standard operation procedures, double entry. In addition, the quality of reagents used to determine the blood groups were checked by running the known blood sample.

Result

About 6471 blood donors have participated in the study. From these study participants 5311 (82.1%), 3586 (55.4%), 6089 (94.1%) and 1842 (28.5%) were male, 21–30 age group, replacement donor and students, respectively (Table 1).

ABO and Rh Phenotype, Allele, and Genotype Frequency

Out of the study participants 47.04%, 26.44%, 21.71% and 4.81% were had blood group O, A, B and AB phenotypes, respectively. The expected ABO phenotype distribution was 46.98%, 26.42%, 21.69% and 4.91% which was similar to the observed frequency (Chi-squared (3, N = 6471) = 0.16, P-value: 0.984). The current result also revealed that 94.24% of the donors were RhD positive. The observed and adjusted (corrected) allele distribution of A, B and O were 0.1714, 0.1433 and 0.6859; and 0.1713, 0.1433 and 0.6854,

Table 1 The Socio Demographic Characteristics of Blood Donors at University of Gondar Comprehensive Specialized Hospital Blood Bank (N = 4671)

Variables		Frequency	
		Number (n)	Percent (%)
Sex	Male	5311	82.1
	Female	1160	17.9
Age category	18–20	969	15.0
	21–30	3586	55.4
	31–40	1252	19.3
	41–50	508	7.9
	>50	156	2.4
Donor type	Voluntary	382	5.9
	Replacement	6089	94.1
Occupation	Student	1842	28.5
	Farmer	1728	26.7
	Government employed	947	14.6
	Private employed	296	4.6
	Self employed	1068	16.5
	Unemployed	590	9.1

respectively. The result showed that there was no significant difference between the observed and corrected allele frequency (Chi-squared (2, N = 6471) = 0; P-value: 1).

The observed ABO genotype frequency of the donors was 0.0294, 0.2350, 0.0205, 0.1966, 0.0491 and 0.4704 for AA, AO, BB, BO, AB and OO, respectively. On the other hand, the corrected ABO genotype frequency was 0.0293, 0.2348, 0.0205, 0.1964, 0.0491 and 0.4698 for AA, AO, BB, BO, AB and OO, respectively (had no significant difference from the observed genotype frequency; Chi-squared (2, N = 6471) = 0; P-value: 1). The Rh genotype frequency was 0.5774, 0.3649 and 0.0576 for DD, Dd and dd, respectively (Table 2).

Phenotype distribution of ABO and Rh blood group of donors also showed that there was no significant ABO and Rh phenotype distribution difference with age (chi-squared (28, N = 6471) = 27.86, P-value = 0.472). Moreover, the result showed that the ABO and Rh phenotype distribution had no statistical significance difference between male and female (chi-squared (7, N = 6471) = 7.649, P-value = 0.365) (Table 3).

Discussion

Knowing the distribution of ABO and RhD blood groups is imperative for blood bank inventory and transfusion

services. It also helps to decide the way of mobilization of voluntary blood donors. In addition, understanding of ABO and RhD blood groups are valuable in studying population genetics, resettlement trend and settling certain medico-legal problems specifically disputed parentage and taking protective measures against blood group-associated diseases.¹⁶

The current result showed that most of the donors (89.7%) were from young age groups (<40 years old). The highest frequency of young donors in the current study might be due to over concentration on the selection of students' donors. The finding was similar to studies found in Madagascar¹⁷ and Saudi Arabia¹⁸ which reported that most of the donors were the youngest age groups. The least donors were found in >50 years old age group. This might be due that the elderly age groups might defer frequently than the young age groups. Studies revealed that deferral significantly increased as age increased due to abnormal blood pressure.^{19,20}

In the current finding, most of the donors (82.1%) were males. This was comparable with other studies conducted in Madagascar¹⁷ and India.^{21–23} The reason might be the fact that males might be easily qualifying through the selection processes in donor recruitment. The other reason might be the difference in blood donation practice between males and females. Studies conducted in Ethiopia,^{24,25} Nigeria²⁶ and Saudi Arabia²⁷ were supported that males had more blood donation practice than women. Indeed, other studies supported males and females had no significant difference in blood donation practice.^{20,28,29} The other reason might be due to the high deferral rate in females. Studies showed that females were more defer than males^{19,30,31} due to being more anemic compared to males.^{19,20}

World Health Organization and other international and national organizations advised that blood donations must be voluntary and unpaid. This ensures the availability of a constant and safe blood supply.³² However, this finding showed that the majority (94.1%) of the donors were replacement donors. This might be due to the poor blood donation practice in Ethiopia. Other researches done in the area demonstrated that blood donation practice was low.^{25,33} The finding was similar to the studies conducted in Tanzania,⁴ Egypt³⁴ and Saudi Arabia,¹⁸ but, contradicted to a study conducted in India where the voluntary blood donors accounted for 87.75% of the donors.³⁵ The discrepancy might be attributed to difference in knowledge, attitude and practice of blood donation between the populations.

Table 2 Phenotype, Allele, and Genotype Frequency of ABO and Rh Among Blood Donors at University of Gondar Comprehensive Specialized Hospital Blood Bank (N = 6471)

ABO Phenotype	Observed Phenotype Frequency	Expected Phenotype Frequency	Chi Squared; P value	Unadjusted Allele Frequency	Adjusted Allele Frequency	Chi Squared; P value	Genotype Frequency			Chi Squared; P value
							Genotype	Unadjusted Frequency	Adjusted Frequency	
A	1711 (0.2644)	1710 (0.2642)	0.16; 0.984	0.1714	0.1713	0; 1	AA	0.0294	0.0293	0; 1
							AO	0.2350	0.2348	
B	1405 (0.2171)	1405 (0.2169)		0.1433	0.1433		BB	0.0205	0.0205	
							BO	0.1966	0.1964	
AB	311 (0.0481)	318 (0.0491)		–	–		AB	0.0491	0.0491	
O	3044 (0.4704)	3040 (0.4698)		0.6859	0.6854		OO	0.4704	0.4698	
Rh D										
RhD positive	6098 (0.9424)	6098 (0.9424)	–	0.7599	0.7599	–	DD	0.5774	0.5774	–
							Dd	0.3649	0.3649	
RhD negative	373 (0.0576)	373 (0.0576)		0.2401	0.2401		dd	0.0576	0.0576	

Table 3 ABO Blood Group Distribution Regarding to Age and Sex (N = 6471)

Variables	Frequency	ABO and RhD Phenotype							
		A ⁺ N (%)	A ⁻ N (%)	B ⁺ N (%)	B ⁻ N (%)	AB ⁺ N (%)	AB ⁻ N (%)	O ⁺ N (%)	O ⁻ N (%)
Age									
18–20	969	259 (26.7)	11 (1.1)	210 (21.7)	11 (1.1)	33 (3.4)	1 (0.1)	419 (43.2)	25 (2.6)
21–30	3586	897 (25.1)	60 (1.7)	715 (19.9)	45 (1.2)	176 (4.9)	7 (0.1)	1586 (44.2)	100 (2.8)
31–40	1252	305 (24.4)	16 (1.3)	249 (19.9)	19 (1.5)	61 (4.8)	3 (0.2)	561(44.8)	38 (3.0)
41–50	508	110 (21.7)	5 (0.1)	111(21.9)	9 (1.8)	20 (3.9)	4 (0.8)	239 (47.0)	10 (2.0)
>50	156	46 (29.5)	2 (1.3)	34 (21.8)	2 (1.3)	6 (3.8)	0 (0.0)	61 (39.1)	5 (3.2)
Test statistics	Chi squared: 27. 86, degrees of freedom:28, P value: 0.472								
Sex									
Male	5312	1314 (24.7)	79 (1.5)	1074 (20.2)	68 (1.3)	247 (4.6)	15 (0.3)	2374 (44.7)	141 (2.7)
Female	1159	303 (26.1)	15 (1.3)	245 (21.1)	18 (1.6)	49 (4.2)	0 (0.0)	492 (42.5)	37 (3.2)
Test statistics	Chi squared: 7. 649, degrees of freedom:7, P value: 0.365								
Total	6471	1617 (25.0)	94 (1.5)	1319 (20.4)	86 (1.3)	296 (4.6)	15 (0.2)	2866 (44.3)	178 (2.8)

The genes accountable for the ABO blood groups have always taken a meticulous prototype for its circulation. In the current finding, the O blood group was the dominant ABO blood group with a frequency of 47.04%, followed by the A blood group (26.44%), B blood group (21.71%) and AB blood group (4.8%), which concluded that O>A>B>AB. Various other findings were showed that the O blood group was the most dominant blood group with the order of O>A>B>AB.^{11,36–40} Of course, there was a difference in the proportion of the phenotype frequency. For example, the prevalence of the O blood group was 61.82% in Mexican Population³⁸ where as in the current study it was 47.04%. The variation might be due to race differences. In contrast to this finding, studies done in India⁴¹ and Pakistan⁴² revealed that the B blood group was the dominant phenotype, followed by O, A and AB blood group. On the other hand, studies done in Nepal⁴³ and Egypt⁴⁴ indicated that A blood group phenotype was the dominant blood group followed by O, B and AB. Moreover, studies in some European countries (Switzerland, Portugal and Greek) showed that A blood group was the dominant phenotype followed by O, B and AB.^{45–47}

The current finding also showed that 94.24% and 5.76% of blood donors were RhD positive and RhD

negative, respectively. This was similar to a study conducted in Kumaon,¹⁶ Mexico³⁸ and Ethiopia.¹¹ However, this finding was different from the other findings done in Uganda,⁴⁸ Egypt,⁴⁴ Kenya,⁴⁰ Sudan⁴⁹ and Gambella (Ethiopia).¹⁰ It showed that the RhD negative frequency was higher than Kenya (3.9% vs.5.76%) Sudan (2% vs 5.76% and Uganda (2% vs 5.76%) and it was lower than the t study done in Egypt (14.4% vs 5.76%) and Gambella (Ethiopia) (19.37% vs 5.76%).

In this study, the distribution of the allele A, B, and O were 0.1714, 0.1433 and 0.6859, respectively (allele frequency of O>A>B). This sequence was similar to the study done in Nigeria,⁵⁰ Cameroon⁵¹ and Sudan.⁵² However, it was different from the studies done in Guinea,⁵³ Nigeria^{54,55} and Madagascar¹⁷ where B allele frequency was higher than A allele frequency (O>B>A). On the other hand, the genotype frequency of AA, AO, BB, BO, AB and OO were 0.0294, 0.2350, 0.0205, 0.1966, 0.0491 and 0.4704, respectively. The finding revealed that the phenotypic distribution of the blood groups in the general study population groups were in agreement with Hardy–Weinberg equilibrium expectations (P-value > 0.05). The finding was supported by the other studies done in Ethiopia¹¹ and Cameroon.⁵¹ However, a study done in Lagos (Nigeria) found significant

difference between the expected and the observed phenotypic frequencies of the blood groups, which violated Hardy–Weinberg law.⁵⁶

Limitation of the Study

The method of blood group determination was done by using the tile slide method and this might miss the detection of weak antigens.

Conclusion

The current finding showed that the majority blood group was “O” and the least frequent was AB. Regarding Rh blood group, RhD positive takes the great share over RhD negative blood groups. The females’ blood donation practice was very low and it is important to improve it by improving health status and the knowledge about blood donation. The current finding might provide knowledge about the genetic distribution and polymorphism of ABO and RhD blood groups among the blood donors at the University of Gondar hospital’s distinct blood bank. This would be valuable to those who are interested in the genetic study, physicians and other stakeholders, particularly those who are involved in blood transfusion program activities.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

This study was conducted per the Declaration of Helsinki. The study was conducted after obtaining ethical clearance from the Ethical review board of the School of Biomedical and Laboratory Sciences, College of Medicine and Health Science, University of Gondar. Permission was also obtained from the University of Gondar Hospital Laboratory head before the data collection. The data was accessed and complied with relevant data protection and privacy regulations by using anonymous. However, since the study was a retrospective review of blood donors’ records, informed consent from the study participants was not sought.

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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.

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Disclosure

The authors declare that there have no conflicts of interest.

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DIC in Pregnancy – Pathophysiology, Clinical Characteristics, Diagnostic Scores, and Treatments

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Abstract: Obstetrical hemorrhage and especially DIC (disseminated intravascular coagulation) is a leading cause for maternal mortality across the globe, often secondary to underlying maternal and/or fetal complications including placental abruption, amniotic fluid embolism, HELLP syndrome (hemolysis, elevated liver enzymes and low platelets), retained stillbirth and acute fatty liver of pregnancy. Various obstetrical disorders can present with DIC as a complication; thus, increased awareness is key to diagnosing the condition. DIC patients can present to clinicians who may not be experienced in a variety of aspects of thrombosis and hemostasis. Hence, DIC diagnosis is often only entertained when the patient already developed uncontrollable bleeding or multi-organ failure, all of which represent unsalvageable scenarios. Beyond the clinical presentations, the main issue with DIC diagnosis is in relation to coagulation test abnormalities. It is widely believed that in DIC, patients will have prolonged prothrombin time (PT) and partial thromboplastin time (PTT), thrombocytopenia, low fibrinogen, and raised D-dimers. Diagnosis of DIC can be elusive during pregnancy and requires vigilance and knowledge of the physiologic changes during pregnancy. It can be facilitated by using a pregnancy specific DIC score including three components: 1) fibrinogen concentrations; 2) the PT difference – relating to the difference in PT result between the patient’s plasma and the laboratory control; and 3) platelet count. At a cutoff of ≥ 26 points, the pregnancy specific DIC score has 88% sensitivity, 96% specificity, a positive likelihood ratio (LR) of 22, and a negative LR of 0.125. Management of DIC during pregnancy requires a prompt attention to the underlying condition leading to this complication, including the delivery of the patient, and correction of the hemostatic problem that can be guided by point of care testing adjusted for pregnancy.

Keywords: DIC, hyperfibrinolysis, thrombin, pregnancy specific DIC score, maternal mortality, placental abruption

Introduction

The life threatening thrombo-hemorrhagic condition known as disseminated intravascular coagulation (DIC), is generally more common in men than women apart from the reproductive years when pregnancy and its complications increase its prevalence.¹ DIC during pregnancy constitutes one of the leading causes for maternal mortality worldwide,^{2,3} and its rate varies from 0.03%⁴ to 0.35%.⁵ This complication is often secondary to underlying maternal and/or fetal complications including placental abruption,^{6–8} postpartum hemorrhage, amniotic fluid embolism, acute fatty liver of pregnancy, preeclampsia and Hemolysis, Elevated Liver enzymes and Low Platelets (HELLP) syndrome,⁹ and retained stillbirth.¹⁰ DIC is characterized by a concomitant over-activation of the coagulation and fibrinolytic

systems,¹¹ leading to widespread microvascular thrombosis, disruption of blood supply to different organs, ischaemia, and multi-organ failure. This extensive activation of the coagulation cascade leads to consumption and depletion of platelets and coagulation proteins, which can provoke concurrent severe bleeding.¹² Of note, obstetric DIC more typically presents with bleeding complications, rather than thrombotic complications. The current review discusses the clinical characteristics, pathophysiology, diagnostic scores, and treatments of DIC in pregnancy.

DIC – Definitions and Types

The Scientific and Standardization Committee (SSC) on DIC of the International Society on Thrombosis and Haemostasis (ISTH) defined DIC as

an acquired syndrome characterized by the intravascular activation of coagulation with a loss of localization arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe, can produce organ dysfunction.¹³

This definition highlights three important aspects: 1) it is always secondary to other causes, one of them being an obstetric related, such as abruption placentae, pre-eclampsia, or intrauterine fetal death.¹⁴ 2) DIC represents systemic pathological activation of coagulation. Normal haemostasis refers to the formation of a thrombus in response to endothelial damage and, in these cases, the clotting and fibrinolytic processes are limited to the site of endothelial injury.¹⁵ However, in DIC, there is uncontrolled dissemination of this localised thrombotic process which is clearly pathological. In obstetrical DIC, there is extraplacental dissemination of the activated coagulation system which is usually localised to the placenta.¹⁶ 3) The third crucial aspect of the DIC definition is the role of microvasculature. DIC is a process which originates in the microvasculature, or the vascular endothelium, the most ubiquitous organ in the human body. Excessive and dysregulated thrombin generation due to (and causing) marked endothelial dysfunction can result in organ damage from microthrombi.¹⁷ If microvascular dysfunction led to organ damage, it would be considered DIC according to the ISTH definition. If in obstetrical disorders, microthrombi can lead to kidney impairment or cerebrovascular ischaemia, this would be considered as a manifestation of DIC. Microthrombi in the placental vessels can be one of the pathophysiological processes which lead to the complications associated with pre-eclampsia including foetal

growth retardation and organ impairment. The microthrombi can arise from localised coagulation activation with platelet aggregation being a significant contributory factor. Most inflammatory disorders like pre-eclampsia are associated with the release of large amounts of Von Willebrand factor into the circulation. In normal circumstances, the ultra large multimer forms of the Von Willebrand factor is cleaved by a metalloprotease enzyme, ADAMTS-13. But in severe inflammation, the enzyme is quenched by the inordinate amount of ultra-large multimers released and the consequence is binding to platelets causing platelet aggregation and microthrombi. In support of this mechanism,^{18,19} Miodownik et al²⁰ have shown that congenital thrombotic thrombocytopenic purpura can present with clinical complications during pregnancy and maternal vascular lesions of under-perfusion in the placenta.

The clinical presentation of DIC can be either thrombosis and/or bleeding as it is a thrombohemorrhagic disorder.²¹ This may be explained by the extremely rapid pace of thrombin generation in the thrombotic phenotype, while it is relatively slower in the haemorrhagic presentation of this complication (Figure 1). The “hyperfibrinolytic” form of DIC as often observed in postpartum DIC, and abruption²² results from an extremely rapid burst of thrombin generation, whereas a “procoagulant” form of DIC (eg, pre-eclampsia related DIC) may result from an excessive but slower and more gradual form of thrombin generation.²³ Although both procoagulant and hyperfibrinolytic processes may proceed simultaneously in DIC, the clinical presentation of thrombosis or bleeding is determined by the predominant mechanism at a particular time.²⁴

Various obstetrical disorders can present with DIC as a complication; thus, increased awareness is key to diagnosing the condition. DIC patients can present to clinicians who may not be experienced in a variety of aspects of thrombosis and haemostasis. Hence, DIC diagnosis is often only entertained when the patient already developed uncontrollable bleeding or multi-organ failure, all of which represent unsalvageable scenarios.²⁵ Beyond the clinical presentations, the main issue with DIC diagnosis is in relation to coagulation test abnormalities. It is widely believed that in DIC, patients will have thrombocytopenia, prolonged prothrombin time (PT) and partial thromboplastin time (PTT), low fibrinogen and raised D-dimers.²⁶ These lab markers were considered as diagnostic of DIC in an era when the DIC diagnosis was predominantly made

The different types of DIC

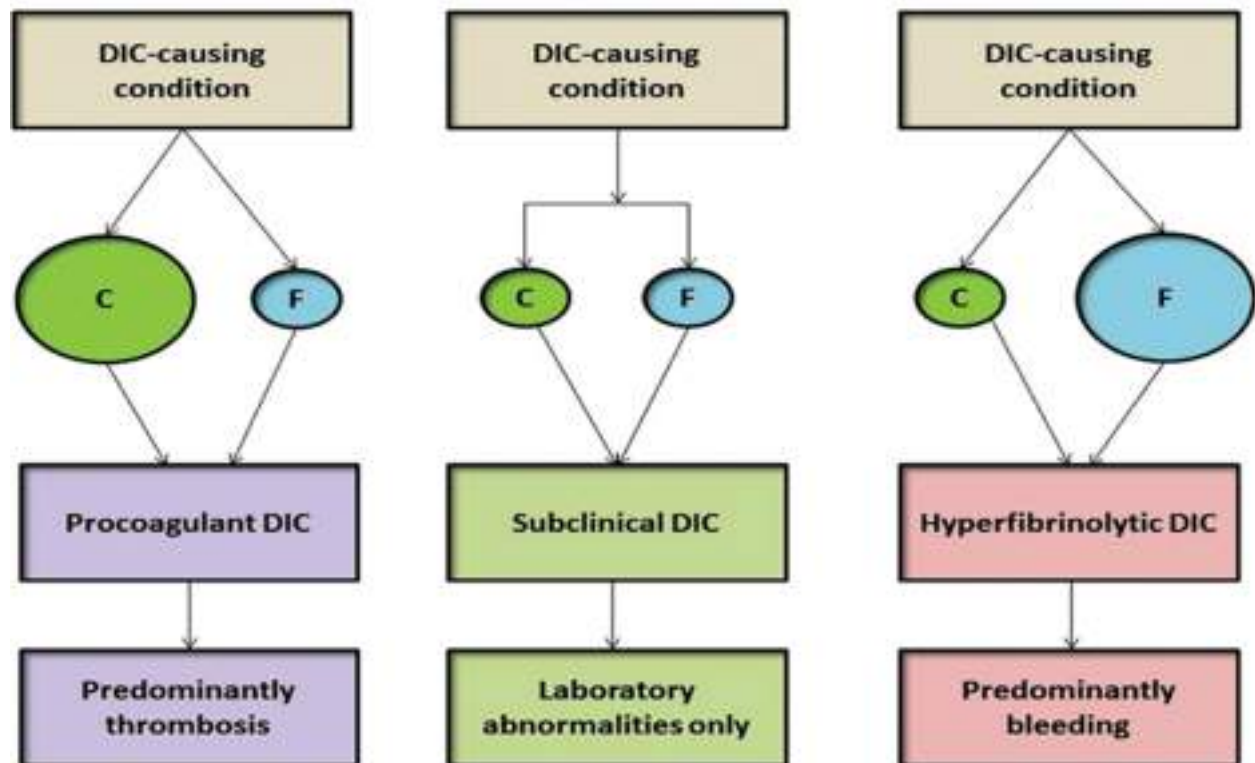


Figure 1 The different types of DIC and their clinical presentation. If there is predominance of coagulation pathway activation (denoted as C), in comparison with the fibrinolytic pathways (denoted as F), procoagulant DIC is the result. While the reverse leads to hyperfibrinolytic DIC.

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post-mortem or in extremely ill patients.⁹ The introduction of DIC scoring discussed below generates cut off values of laboratory tests that enable the clinicians to diagnose DIC at early stages of the disease with a more subtle abnormalities of the laboratory tests.

Epidemiology of DIC in Pregnancy

DIC has a higher lifetime prevalence in men than in women, the only period where the incidence of this disorders is similar between the genders is during women's reproductive years.¹ The prevalence of obstetrical DIC differs worldwide ranging from as low as 0.03% in North America to 0.35% in developing countries.^{2–5,27–29} Differences in quality and accessibility to advance maternity care, as well as inconsistent definitions and diagnostic criteria led to variations in the reported incidence of DIC. Indeed, a cohort from the Nationwide Inpatient Sample from USA,³⁰ reported that the prevalence of DIC during

pregnancy from 1998 to 2009, increased by 35% from 0.092% to 0.125%³⁰ and linked with nearly a quarter of maternal deaths. Furthermore, DIC was the second most common severe maternal morbidity indicator in the USA between 2010 to 2011, affecting 32 per 10,000 delivery hospitalizations.³¹

DIC is always secondary to an underlying disorder. Indeed, it is associated with pregnancy complications such as placental abruption, HELLP syndrome, preeclampsia, retained stillbirth, sepsis, post-partum hemorrhage (PPH), acute fatty liver, and amniotic fluid embolism (Figure 2). The proportions of the contribution of each risk factor varies among countries. Indeed, the most frequent pregnancy complication associated with DIC according to Rattray et al⁴ was placental abruption followed by PPH and preeclampsia. Similarly, Erez et al⁵ reported that placental abruption was the leading underlying obstetrical disorder leading to DIC especially when it

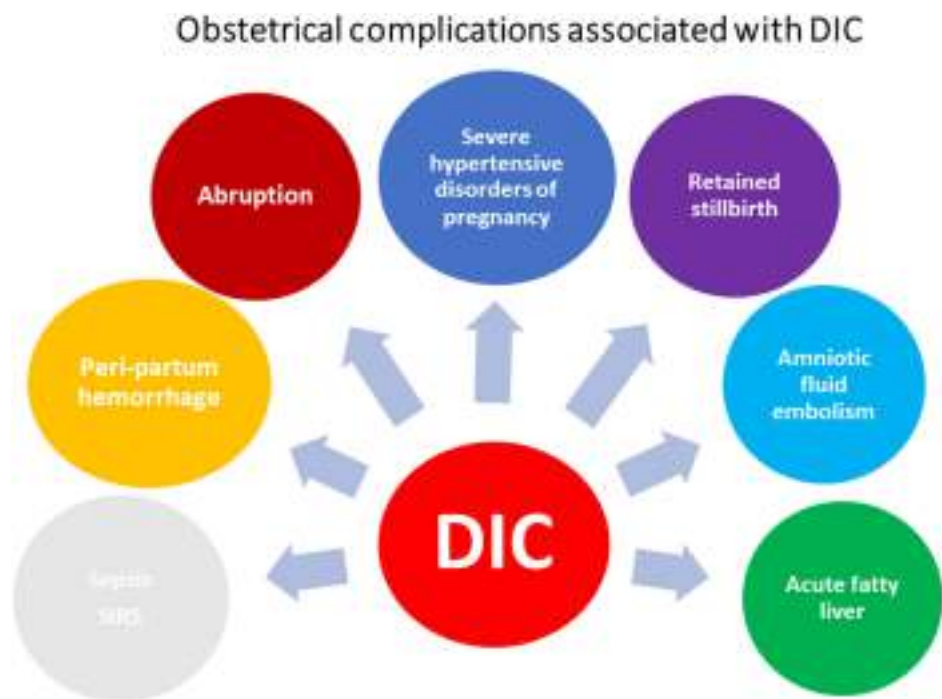


Figure 2 Obstetrical complications associated with DIC in pregnancy.

was accompanied by fetal death. Other groups reported preeclampsia³² and fetal death²⁹ to be the leading obstetrical complications associated with DIC (Figure 3). This

suggests that early recognition and treatment of complications such as preeclampsia or stillbirth may prevent the deterioration to DIC in these patients. Moreover, in countries

Variation in pregnancy complications resulting in DIC in different countries

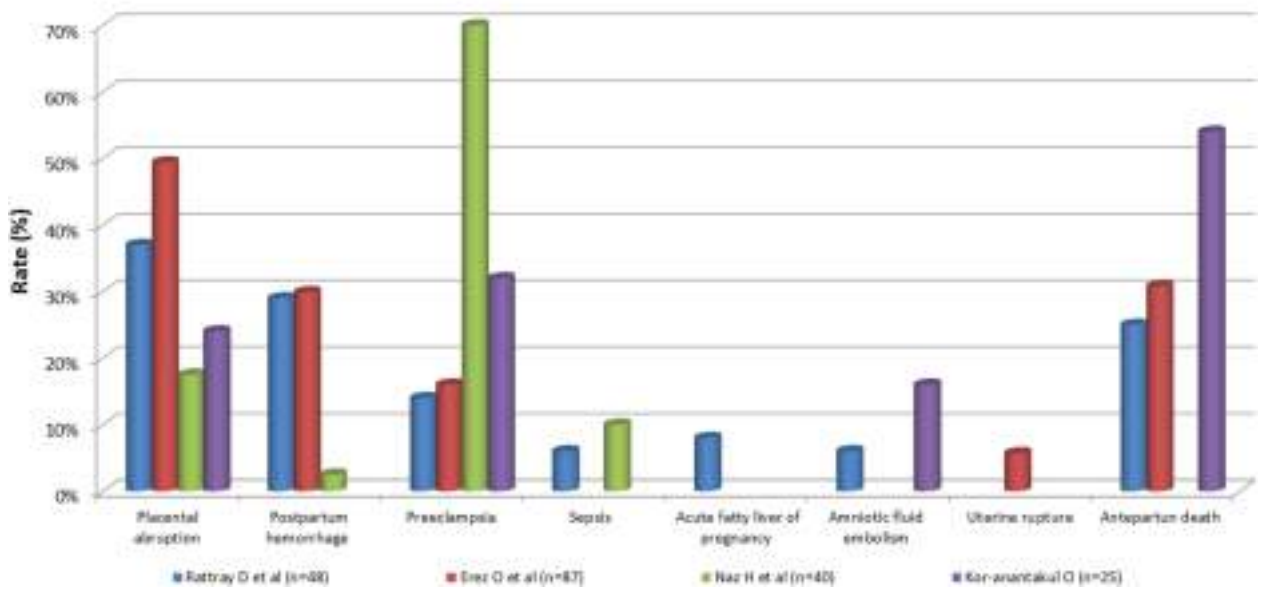


Figure 3 Global distribution of obstetrical complications associated with DIC in pregnancy.
Note: Data was modified from references 4, 5, 26 and 32.

with early recognition and treatment of preeclampsia and stillbirth, placental abruption (usually unexpected and non-preventable), becomes the leading obstetrical complication associated with DIC.

Placental abruption seems to be the underlying mechanism for the development of DIC when it is associated with other obstetrical complications including fetal death and HELLP syndrome.^{7,33–39}

Thus, pregnancy is a time point in women's life in which she is at increased risk to develop DIC as a result from different complications of pregnancy, and especially placental abruption. Prompt attention and treatment of underlying obstetrical disorders and rapid diagnosis and management of placental abruption can reduce the risk for DIC substantially.

Pathophysiology of DIC in Pregnancy

DIC has a complex pathophysiology, the understanding of the normal haemostasis and the highly orchestrated balance involving the various pathways including pro-coagulant/anticoagulant, fibrinolytic/antifibrinolytic and platelet/von Willebrand factor axis during pregnancy, is essential for the characterisation of the haemostatic abnormalities observed during DIC.¹⁷

Excessive and uncontrolled *in vivo* thrombin generation is a key to the development of DIC.²¹ The excess thrombin generated will activate these pathways in differential manners. Hence, if the procoagulant and antifibrinolytic pathways are both activated "in excess", then the result would be thrombosis, especially if the natural anticoagulants have also been consumed.²⁵ If the excess thrombin generated drives the fibrinolytic pathway "in excess" of the other pathways, bleeding will commence.⁹

How does this translate to obstetrical cases? In the placental circulation, maintenance of the blood flow is paramount to ensure adequate oxygenation and nutrition of the foetus.¹⁶ At the same time there is local physiological activation of coagulation in the placental bed, in preparation to quench large amounts of bleeding anticipated during parturition.¹⁶ Despite the heightened coagulation, clots are not formed in the placental bed except in cases of abnormal placental pathologies.⁴⁰ In obstetrical disorders, the much-enhanced coagulation process could lead to microvascular clots which impair the appropriate development of the fetus.⁴⁰ In addition, if the activated procoagulant pathway does not come to a halt, this would spill into the maternal circulation causing systemic coagulopathy, otherwise termed as DIC.²⁴ While, if there is

a brisk fibrinolytic response, then this could lead to haemorrhage wherein formation of firm and dense clots is prevented by fibrinolytic proteins.⁴¹ Furthermore, D-dimers and fibrin-degradation products that results from fibrinolysis interfere with platelet activation and can impair myometrial contractility.⁴²

Pathological Mechanisms of DIC in Pregnancy

Endothelial Dysfunction and Platelet Activation

Normal pregnancy is associated with activation of maternal leukocytes⁴³ into a state akin to sepsis.⁴⁴ Nevertheless, placental trophoblast maintain a balanced systemic maternal inflammation during gestation⁴⁵ by inactivation of maternal leukocytes. However, infectious agents, septic abortion, and amniotic fluid embolism⁴⁶ leading to sepsis perturbed this balance and can lead to the development of maternal DIC (Figure 4).

During sepsis the coagulation system can be activated by Endothelial cells or leucocytes (either intact, dysfunctional, or activated), platelets, and remnants of cell surfaces, inflammatory mediators and coagulation proteins participate in the processes leading to uncontrolled activation of coagulation cascade and DIC⁴⁷ (Figure 5). These cells release pro-inflammatory cytokines such as tumor necrosis factor- α , interleukin-1 (IL-1) and interleukin-6 (IL-6), along with propagation of tissue factor (TF) expression on the surface of endothelial cell and leukocytes^{22,48–54} that can initiate an uncontrolled activation of the coagulation cascade via the TF/factor VIIa pathway leading to thrombin generation if this coagulation response is uncontrolled it will eventually lead to DIC.⁵⁵ The most significant source of TF is not completely clear as this activator of the coagulation cascade is expressed not only in mononuclear cells in response to pro-inflammatory cytokines (mainly IL-6), but also by vascular endothelial (Figure 5), cancer cells,^{53,54,56} decidua and placenta.

Despite the potency of TF as a trigger of coagulation, potent anticoagulation pathways can control and limit its activity. However, all the natural anticoagulant pathways (ie, anti-thrombin III, protein C system, and TF pathway inhibitor [TFPI]) are compromised during sepsis mediated DIC.⁵⁷ Indeed, during DIC antithrombin III concentration are markedly reduced, due to its consumption,⁵⁸ degradation (by elastase from activated neutrophils),⁵⁹ and impaired synthesis.⁴⁹ Moreover, protein C system inhibition due to decreased protein synthesis, cytokine-mediated down-regulation of endothelial thrombomodulin and a drop in the concentration of the free fraction of protein S (protein C co-factor) may further

Schematic representation of pathogenic pathways in DIC

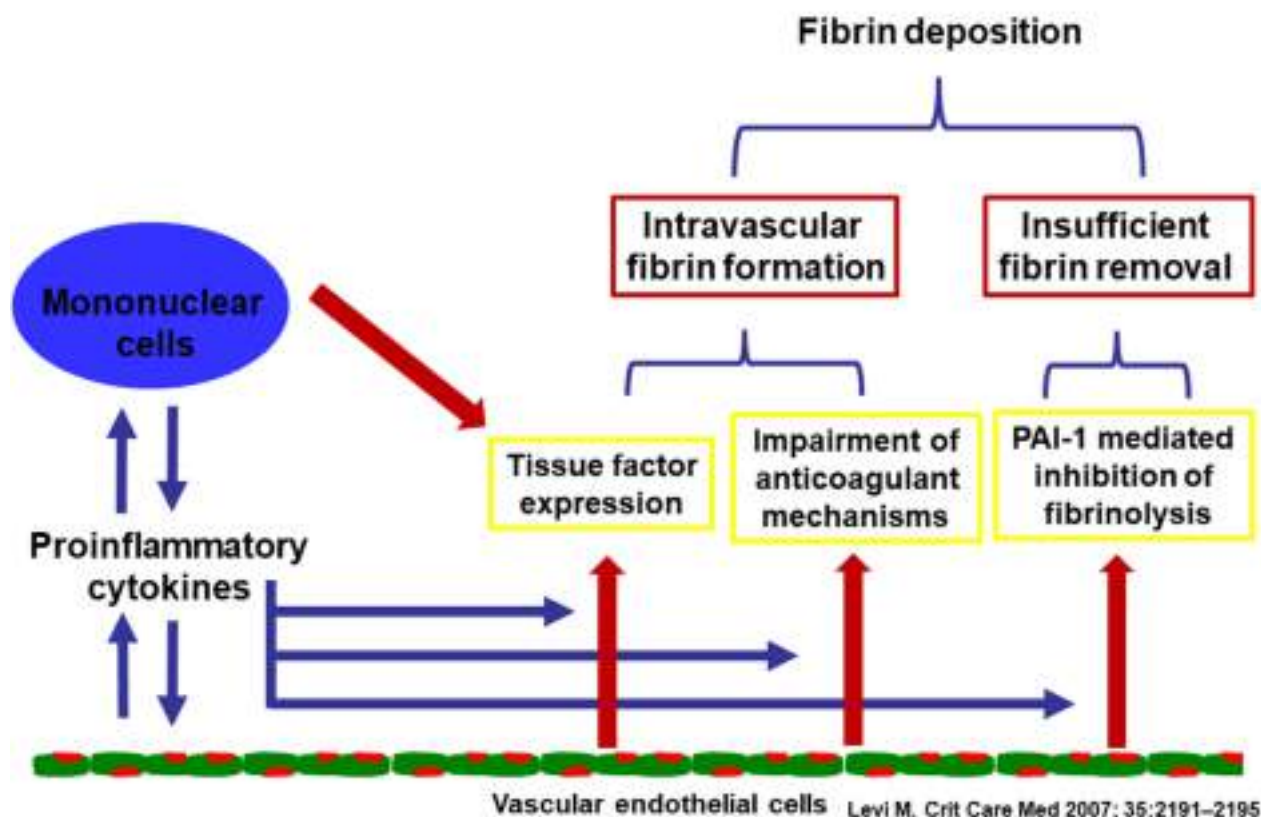


Figure 4 Schematic representation of pathogenic pathways in DIC.

Notes: Adapted from: Levi M. Disseminated intravascular coagulation. *Crit Care Med*. <https://journals.lww.com/ccmjournal/pages/default.aspx>. 2007;35(9):2191–2195.⁴⁹ With permission. © 2007 by the Society of Critical Care Medicine and Lippincott Williams & Wilkins.

compromise an adequate regulation of thrombin generation.⁶⁰ Lastly, reduce TFPI function cannot address the increased activation of the extrinsic coagulation cascade.⁶¹

Fibrin formation is increased during DIC at the time of maximal activation of coagulation due to the following: 1) there is a continuous elevation of plasminogen activator inhibitor-1 (PAI-1) that suppresses fibrinolysis;^{49,62} 2) platelets activation⁶³ increases TF expression on monocytes especially in the presence of granulocytes in a P-selectin dependent reaction.⁵⁶ This effect can be attributed to the induction of nuclear factor kappa B (NF- κ B) activation following the binding of activated platelets to neutrophils and mononuclear cells.⁶⁴ Similar mechanisms are activated during DIC in pregnancy resulting from sepsis or systemic maternal inflammation.

Systemic inflammatory response resulting from maternal infection is not unique only for bacterial infection. Indeed, maternal DIC associated with COVID-19 infection

was previously reported.⁶⁵ Unlike the procoagulant DIC in the non-pregnant state,⁶⁶ during pregnancy, COVID-19 coagulopathy in the third trimester was associated with a clinical manifestation mimicking normotensive HELLP syndrome associated with thrombocytopenia and elevated liver enzymes⁶⁵ and the coagulopathy was of the hyperfibrinolytic type with fibrinogen concentrations and bleeding.⁶⁷ This issue is further discussed in a Communication regarding COVID-19 coagulopathy in pregnancy from the ISTH SSC for Women's Health.

Trophoblast Deportation and Systemic Activation of Maternal Coagulation Cascade

During normal gestation, the trophoblasts face two contradicting hemostatic functions: 1) sustaining of laminar flow of maternal blood in the intervillous space without clotting; and 2) to prevent bleeding at the maternal fetal interface.⁹ This led to the acquirement of endothelial

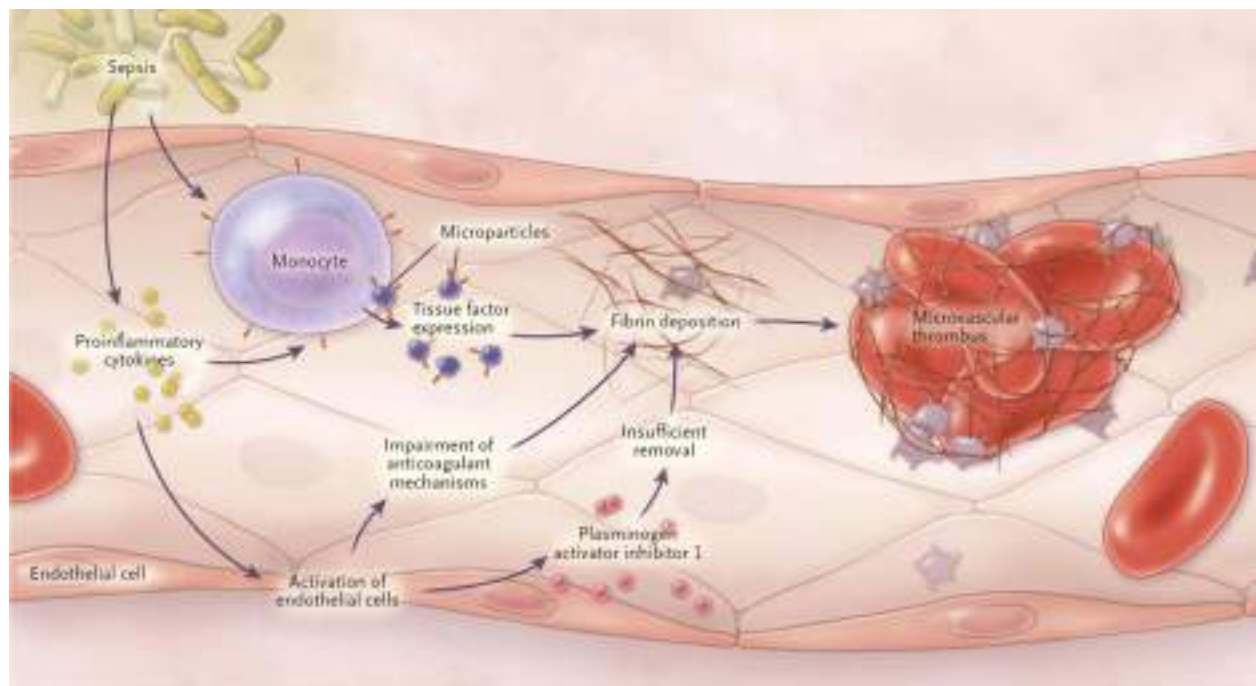


Figure 5 Mechanisms of DIC in sepsis involving endothelial dysfunction and platelet activation.

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cell like properties to the syncytiotrophoblast along with the expression of TF to maintain hemostatic function. Placental TF has higher activity in comparison to that expressed on human umbilical vein endothelial cells. The syncytiotrophoblast has also anti coagulation properties as it is able to synthesize Protein C, Protein S and Protein Z as well as pregnancy specific inhibitor of the tissue factor pathway known as TFPI-2 (Placental Protein 5)^{68–70} to prevent uncontrolled activation of the coagulation cascade.^{71–74} The prevention of fibrinolysis by the placenta is achieved by placental production of PAI-2 during normal pregnancy.⁷⁵ These physiological changes during pregnancy are associated with relatively constant tissue plasminogen activator (t-PA) concentrations, leading to a prothrombotic state related in part to reduced clot lysis.⁷⁶

Disruption of trophoblast/decidua integrity would lead to a discharge of potent TF into maternal circulation, and activation of coagulation cascade that in large quantities can lead to uncontrolled systemic thrombin generation and DIC^{22,77} (Figure 6).

Abruptio of the placental is the classica example for this mechanism, especially in cases of concealed abruptio or when it is complicated by stillbirth. Even though some

regard that consumption coagulopathy as the underlying mechanism of placental abruptio associated DIC, it seems that there is more to it. Indeed, women with a retro placental clot have a faster developing and more severe form of DIC than those with PPH although they have overall lower blood loss. Indeed, patients who develop placental abruptio suffer from a combined coagulopathy incorporating consumption coagulopathy and substantial release of thromboplastin (tissue factor) into the maternal circulation.^{9,49} In addition, local hypoxia and hypovolemia trigger endothelial response leading to increased expression of vascular endothelial growth factor, which causes an increased endothelial expression of TF.⁷⁸ This view is supported by the following: 1) Erez et al²² reported that among women with stillbirth, those with abruptio, have higher amniotic fluid TAT complexes concentrations and 2) mice who were injected with placental extracts developed DIC and died. The authors also demonstrated that administration of heparin prevented the death of the injected mice.⁷⁹ Further study suggested that thromboplastin triggers this response in mice via its effect on clotting time and chemical properties.⁷⁹ Recent advances in the understanding of the mechanisms of DIC in HELLP syndrome suggest that abruptio rather than liver dysfunction

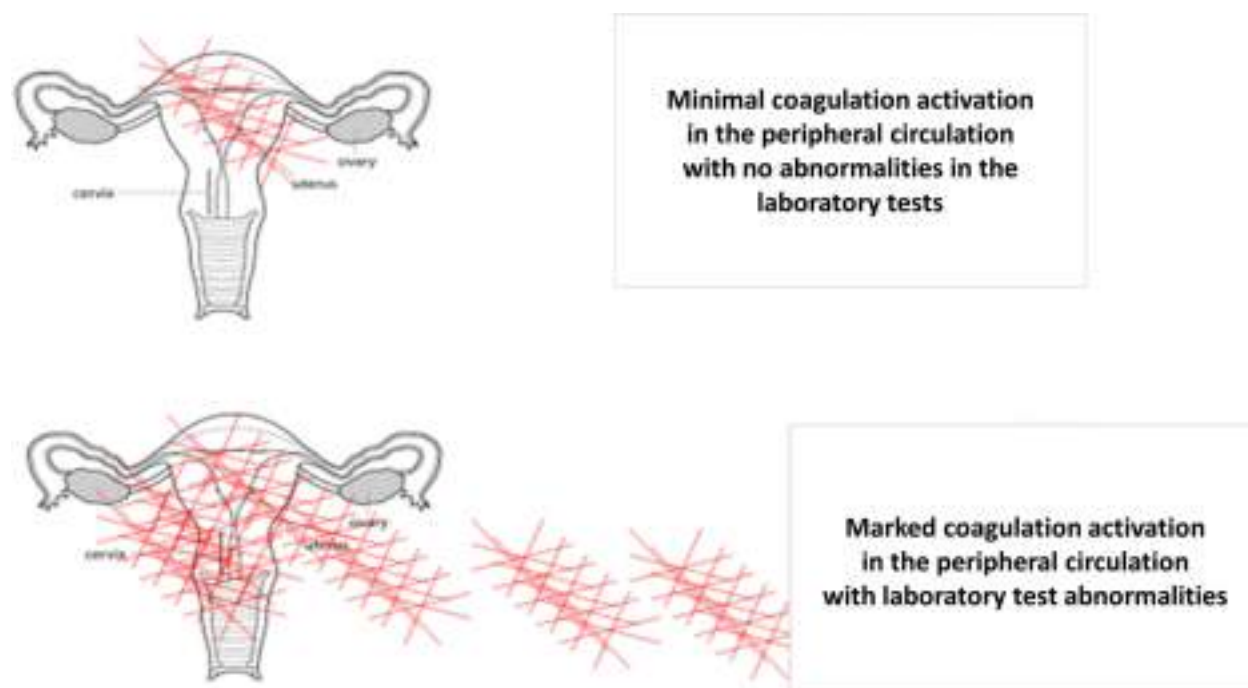


Figure 6 Trophoblast deportation and systemic activation of maternal coagulation cascade. Coagulation system is activated in the placental circulation in the normal pregnant state. This activated coagulation can spill into the peripheral circulation in patients who have obstetrical complications like pre-eclampsia. If coagulation activation in the peripheral circulation becomes uncontrolled, it manifests as DIC.

is the driving force for the development of DIC in these patients.³⁹ Indeed, the rate of placental abruption was 5.6% in women with HELLP syndrome without DIC in comparison to 42.9% among those with DIC,³⁹ suggesting that the main underlying mechanism leading to DIC in HELLP syndrome is related to trophoblast deportation into maternal circulation rather than to an intrinsic liver dysfunction.

Amniotic fluid embolism, although rare, is a major cause for maternal DIC, though a combined mechanism of activation of coagulation by the TF rich amniotic fluid debris that enter the maternal circulation and the acute systemic maternal inflammatory response further propagating the activation of the coagulation cascade. These patients have a fibrinolytic type of DIC⁸⁰ observed as unlike placental abruption autopsies of women who died due to amniotic fluid embolism lack evidence of fibrin deposition.⁸¹ Moreover, Beller et al⁸² studied the clotting and fibrinolytic systems of their patient in three blood samples: the first was drawn on admission, the second at the onset of shock and the third at the start of cardiac massage following cardiac arrest. Clotting parameters on admission were normal. The blood in the third sample did not clot even after adding thrombin in excess. Moreover,

the clotting time remained prolonged after fibrinogen was added in the presence of excess thrombin indicating that anti-thrombin activity was present. However, protamine sulfate did not restore the clotting time to normal, indicating that the antithrombin activity was not attributable to heparin. The contribution of systemic maternal inflammation to the development of DIC in women with amniotic fluid embolism is highlighted in the report of three cases of amniotic fluid embolism in which the patients had elevated maternal circulation TNF α upon admission prior to the clinical presentation of this complication.⁴⁶

Obstetrical Hemorrhage

Hemorrhage, especially post-partum is regarded by many^{4,9,50,83–87} as one of the major causes for DIC in pregnancy. Classically related to PPH, this type of consumption coagulopathy is a complication of uterine atony or rupture, retained placenta or membranes, placenta accreta, or severe lacerations of the birth canal (cervical or vaginal).^{5,88,89} The characteristics of the DIC developing in these cases are the rapid maternal loss of a large blood volume along with its coagulation factors, resulting in patients who are hemodynamically compromised. Currently, there is a debate whether this form of

consumption coagulopathy is truly DIC or just a massive blood loss that depletes the patient's coagulation factors and can lead to death due to exsanguination.⁹⁰ However, maternal coagulopathy associated with PPH is not just the loss of blood volume and coagulation factor but rather a more complex complication that can be considered as DIC. This can be attributed to the procoagulant state and increased thrombin generation associated with parturition attributed to the release of TF to the maternal circulation following the separation of the membranes and the placenta,⁹¹ along with the systemic inflammation accompanies the process of labor and delivery.^{92,93} Indeed, women who had PPH are considered at high risk for development of deep vein thrombosis during the puerperium.⁹⁴ The evidence brought herein, that parturient with PPH have a higher activation of coagulation cascade, beyond physiologic levels for normal pregnancy, suggest that the clinicians who treat these patients must consider them as high risk groups for DIC, even though the fundamental pathology is considered to be the rapid and massive loss of blood and coagulation factors.⁷⁷ Therefore, PPH requires a careful and prompt assessment and to be treated pharmacologically and/or surgically, and by blood products as well as volume expanders to sustain the maternal circulation in an attempt to prevent the subsequent development of DIC.

Disruption of Liver Function

Acute fatty liver of pregnancy is severe, but a rare unique complication of pregnancy mostly observed in nulliparous patients toward the end of the third trimester.^{95–97} Clinicopathological features of this complication include progressive loss of liver function,⁹⁸ abnormal renal function⁹⁹ and DIC.¹⁰⁰ The liver presents accumulation of fatty microvascular infiltration of hepatocytes with, without alteration of the overall structure of the liver.⁹⁸ Nevertheless, the synthetic function of the liver is hampered leading to lower production of fibrinogen and coagulation factors and along with the hemorrhage observed in this syndrome lead to DIC. Indeed, in one of the largest series of acute fatty liver published thus far including 51 patients,¹⁰¹ and 80% of these women according the ISTH DIC score^{102,103} had DIC similar to the report of Vigil-De Gracia et al¹⁰⁰ in which DIC was present in >70% of the patients. The characteristics of the hemostatic parameters of women with DIC in the study of Nelson et al¹⁰¹ demonstrated that patients with DIC had persistent decreased plasma fibrinogen for several days following delivery and mild to moderately elevated fibrin

degradation products.¹⁰¹ Unlike women with abruption that their hemostatic parameters recover within hours after delivery those with acute fatty liver requires several days for this to happen as a function of the recovery of the synthetic function of their liver. Thus, DIC is a central component of acute fatty liver and in a way reflects the severity of the hepatic injury.

Collectively, the alteration in maternal physiology associated with pregnancy is further augmented by different pregnancy complications leading to perturbation of the hemostatic system and the development of DIC. It is important to understand the underlying mechanisms associated with the development of DIC in each complication as it will assist the clinician in the prevention and management of DIC in these cases.

Diagnosis of DIC in Pregnancy: Clinical Definitions, Laboratory Findings and Scoring Systems

The determination whether obstetrics coagulopathy results from consumption of coagulation factors, their loss due to uncontrolled bleeding, or both. Isolated intravascular consumption of coagulation factor is a true DIC, whereas their loss due to perfused obstetrical hemorrhage can be regarded as dilutional/consumption coagulopathy. However, it is very hard to make such distinction.²⁵

The hallmark of successful management of this dire complication depends on prompt and accurate recognition of DIC. Unfortunately, often the diagnosis of DIC by the attending physicians is based mainly on clinical presentation of the patient and often made relatively late in the course of the disease. During pregnancy, there is a physiologic change in the maternal plasma concentrations of many of the coagulation parameters leading to a false perception that the status of the coagulation system is normal in cases when the patient is already developing coagulopathy. Moreover, often there is underestimation of the amount of bleeding and the relevant laboratory test are performed too late when the patient is already compromised. It is important to emphasize that there is no single laboratory or clinical test that is sensitive and specific enough to diagnose DIC and the risk to develop DIC is not evident in all cases.^{4,9,40,46,50,90,104} DIC is a dynamic situation that requires a continuous assessment of the clinical and laboratory parameters including decreasing concentration of fibrinogen and platelet count, prolongation of prothrombin time, and increased concentration of

fibrin split products or d-dimers^{14,105,106} until it resolves. For those reasons, there is a need to: 1) develop means for early diagnosis of DIC by clinicians; and 2) establish a universal definition of DIC in pregnancy. The development of DIC scores was an effort to address both challenges. Such scores need to be simple, easy to perform, and readily available to clinicians worldwide. Indeed, these scores use simple and readily available coagulation tests including platelet count, prothrombin time (PT) prolongation, fibrinogen, and fibrin split products/D-dimer concentrations.

The use of DIC scoring systems was introduced to facilitate the diagnosis of DIC. The ISTH DIC score was proposed in 2001¹⁰² followed by that of the Japanese Association for Acute Medicine (JAAM) in 2005,¹⁰⁷ both scores have good prediction performance for the diagnosis of DIC and the identification of critically ill non-pregnant patients and their prognosis in intensive care units.^{103,108–110} None of these scores is adjusted for the physiologic hemostatic changes occurring in pregnancy, limiting their applicability during gestation.

Pregnancy is associated with adaptive changes in the coagulation system that are aimed to address the challenges posed by the need to have extra circulatory maternal blood flow through the placental bed. Thus, the mother has to protect herself from a life-threatening bleeding especially during labor and delivery on one hand, and secure a continuous blood flow through the placental bed to nourish the developing fetus on the other hand.^{91,111–114} The adaptive changes in the hemostatic system are observed in the maternal circulation, placental bed and amniotic fluid as

summarized in Table 1. Women with a normal pregnancy have: 1) excessive thrombin generation;^{114,115} 2) increased agonist derived platelets aggregation,^{116,117} 3) two to three fold increase in fibrinogen concentrations; and 4) towards term they experience a 20% to 1000% increase in factors VII, VIII, IX, X, and XII,¹¹⁸ as well as up to 400% increase in von Willebrand factor.¹¹⁸ By contrast, factors XIII and XI concentrations decrease during gestation and those of factors II & V unchanged.¹¹⁹ The excessive thrombin generation observed during normal pregnancy,^{114,115} is supported by the observations of elevation in circulating maternal fibrinopeptide A, prothrombin fragments (PF) 1 and 2, and thrombin–antithrombin (TAT) III complexes during pregnancy,^{91,120–122} especially at the time of and after normal labor^{123,124} and delivery,^{121,124} subsequently, in the course of the puerperium their concentrations decrease.¹²⁴ Pregnancy is also associated with changes in the anticoagulation proteins concentrations. Indeed, there is a 55% decline in free protein S plasma concentration reaching a nadir at birth leading to an increase in resistance to activated protein C^{118,125}. This process is exacerbated by cesarean delivery and infection.^{118,126} The concentrations of PAI-1 increase by 3 to 4-fold during pregnancy while plasma PAI-2 values, which are negligible before pregnancy reach concentrations of 160 mg/L at delivery.¹¹⁸ Thus, pregnancy is associated with increased clotting potential, as well as decreased anticoagulant properties, and fibrinolysis.⁹² In addition to the changes in maternal circulation, pregnancy is associated with changes in the local hemostatic mechanisms. Indeed, there is an increase in decidual and myometrial tissue factor.^{93,127–129}

Table 1 Adaptive Changes of the Coagulation System During Pregnancy

Category	Increased	Decreased	No Change
Systemic changes			
Procoagulant factors	I, V, VII, VIII, IX, X	XI	
Anticoagulation proteins	Soluble TM	PS	PC
Adhesive proteins	vWF		
Fibrinolytic proteins	PAI-1, PAI-2	t-PA	TAFI
Microparticles and antiphospholipid antibodies	MP		ALPA
Local placental changes	TF	TFPI	

Notes: Reprinted from: Brenner B. Haemostatic changes in pregnancy. *Thromb Res.* 2004;114(5–6):409–414.¹⁹⁸ With permission from Elsevier. doi: 10.1016/j.thromres.2004.08.004.

Similarly, changes are observed in the chorioamniotic membranes (mainly the amnion) and in the amniotic fluid.^{91,130–133}

To address the changes of pregnancy in the definition of DIC, Erez et al,⁵ developed a pregnancy specific DIC score by using platelet count, fibrinogen concentrations and the PT difference – relating to the difference between the patient's PT and the laboratory control. This diagnostic performance of this score for DIC at a cutoff of ≥ 26 points were: 88% sensitivity, 96% specificity, a positive likelihood ratio (LR) of 22, and a negative LR of 0.125 (Table 2). To validate the score vs the ISTH DIC score, Erez et al⁵ compared the diagnostic parameters of both scores in women with placental abruption (n= 684); of them, 21.93% (150/684) needed blood products transfusion and 6.29% (43/684) developed DIC. The pregnancy modified DIC score had 88% sensitivity, 96% specificity, while the modified ISTH score had at a cutoff point of 0.5 had 74% sensitivity, and 95% specificity of for the diagnosis of DIC. An independent validations of

these results was reported in a population of French women with obstetrical hemorrhage who were admitted to intensive care unit.¹³⁴ Among women with liver rupture or hematoma, pregnancy specific DIC score >26 was associated with increased blood product transfusions requirement, longer hospitalizations, and lower neonatal 1 and 5 minutes Apgar scores.¹³⁵ Moreover, a retrospective analysis of blood product transfusion requirements in patients with PPH revealed that according to the pregnancy specific DIC score, blood transfusion was unnecessary in 179 of the 279 postpartum women (64.1%) suggesting that its use may prevent unnecessary transfusions and their related risks and complications.¹³⁶ Recently, Clark et al,¹³⁷ suggested an additional modified version of the ISTH DIC score. Erez et al¹³⁸ tested the performance of this suggested scoring system on their cohort and found that this score had 14.9% sensitivity, 99.9% specificity, and a LR+ score of 14.9. Despite the good likelihood ratio score, the DIC score presented by Clark et al¹³⁷ could identify only 13 out of 87 cases of DIC in

Table 2 An Effect of Components of the New DIC Score – Results of Logistic Regression

	Effect of Individual Analytes		Effect of Individual Analytes Adjusted to Other Tests		Assigned Weight ^a
	Relative Risk	p-value	Relative Risk	p-value	
PT difference (seconds)					
<0.5	1.0		1.0		0
0.5–1	12.7	0.031	29.3	<0.001	5
1.0–1.5	27.7	0.005	68.8	<0.001	12
>1.5	60.3	<0.001	558.1	<0.001	25
Platelets (10 ⁹ /L)					
<50	3.1	0.06	89.2	<0.001	1
50–100	5.2	<0.001	56.2	<0.001	2
100–185	2.9	0.001	12.8	<0.001	1
>185	1.0		1.0		0
Fibrinogen (g/L)					
<3.0	59.0	<0.001	662.9	<0.001	25
3.0–4.0	13.4	<0.001	59.1	<0.001	6
4.0–4.5	2.4	0.320	6.8	0.03	1
>4.5	1.00		1.0		0

Notes: ^aWeight was calculated as relative risk of each of the adjusted factors to the relative risk of a factor with minimal effect. Adapted from: Erez O, Novack L, Beer-Weisel R, et al. DIC score in pregnant women—a population based modification of the International Society on Thrombosis and Hemostasis score. *PLoS One*. 2014;9:e93240.⁵ Copyright: © 2014 Erez et al. Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/legalcode>)

this cohort, casting a heavy shadow on the clinical utility of this score. Thus, following the study by Erez et al⁵ coagulation parameters standards of non-pregnant patients can no longer use for the diagnosis of DIC in pregnant women, as “Normal” values of coagulation tests are different during pregnancy, parturition, and puerperium than in the non-pregnant state (Figure 7).

Other DIC scores in pregnancy were suggested by: 1) Terao et al¹³⁹ in 1987 developed a DIC score that included three categories, etiology, clinical manifestation, and laboratory tests (PT, fibrinogen, FDP, and platelets). The study included 77 women with DIC recruited from 100 centers in Japan. A DIC score ≥ 7 was considered positive and identified 90% (70/77) of the patients. Of note, this score was not validated in comparison to the normal

obstetric population, and it is currently not in clinical use outside of Japan. 2) the utilization of the fibrinogen/C-reactive protein (CRP) ratio as a tool to diagnose the development of DIC among women with HELLP syndrome by Windsperger et al¹⁴⁰ demonstrated a good sensitivity and perform better than fibrinogen concentrations alone in these patients.

The incorporation of pregnancy specific scoring system to diagnose DIC in pregnant women is an accurate and easy to use and may assist clinicians in real time during at the Labor and Delivery wards. However, there is a need for an international consensus on the diagnostic criteria and scoring for DIC in pregnancy, this will facilitate standardization of the definition and will support international research effort in this dire complication of pregnancy.

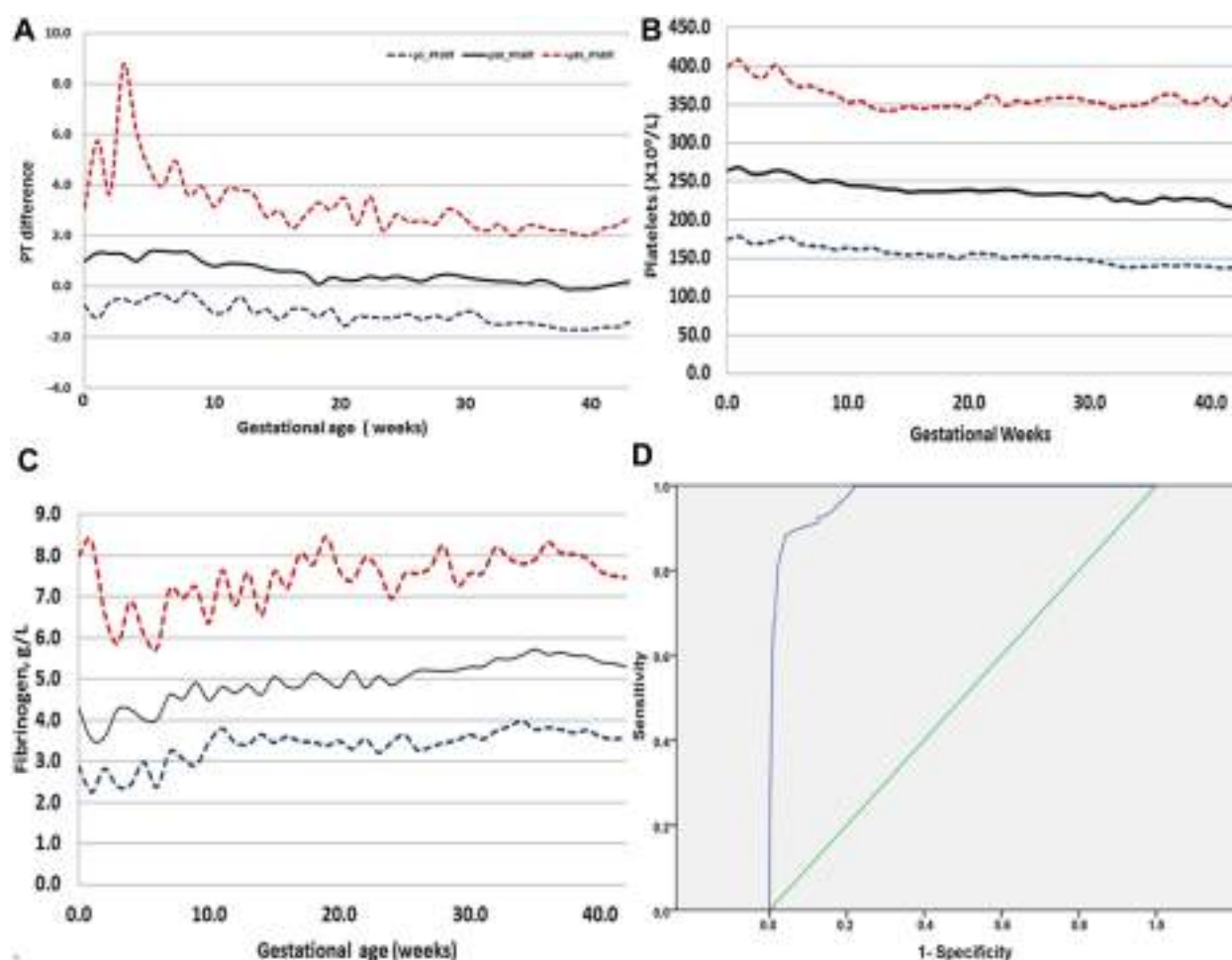


Figure 7 The changes in the major components of the pregnancy modified DIC score: (A) PT difference: (stands for the difference between the patients PT results and the laboratory control); (B) platelets; (C) fibrinogen, with advancing gestations in women with advancing gestation. (D) ROC curve analysis for the association of the pregnancy specific DIC score with the development of DIC.

Notes: Adapted from: Erez O, Novak L, Beer-Weisel et al. DIC score in pregnant women—a population based modification of the International Society on Thrombosis and Hemostasis score. *PLoS One*. 2014;9(4):e93240.⁵ Copyright: © 2014 Erez et al. Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/legalcode>).

Point of Care Testing in the Management of DIC During Pregnancy and Parturition

Standard laboratory tests have limitations, including absence of real-time data and incapacity to determine the functionality of the hemostatic system of whole blood (ie the strength of blood clot, and platelet function). Point of care viscoelastic tests Thromboelastography (TEG); and Rotational Thromboelastometry (ROTEM) are the most widely studied viscoelastic tests which provide a rapid assessment of in vivo coagulation. Specific TEG/ROTEM values were defined for pregnancy^{16,26,84,141,142} and at the time of delivery.¹⁴³ Indeed, in comparison to the non-pregnant state, the characteristics of TEG differ significantly between pregnant and non-pregnant women, displaying a shorter R and K a higher α angle and maximum amplitude suggesting that clot formation is faster and bigger during pregnancy especially during the third trimester.¹⁴⁴ The ROTEM parameters display similar changes in comparison to the non-pregnant state.¹⁴⁵ Moreover, in a study by Rigouzzo et al¹⁴⁶ the TEG parameters (the maximum amplitude of K ≤ 63.5 mm and time to maximum rate of thrombus generation) had excellent diagnostic performance for fibrinogen concentration ≤ 2 g/L and Platelet count $\leq 80,000/\text{mm}^3$ in women with postpartum hemorrhage. Suggesting that TEG can be an excellent point of care testing to identify women with severe PPH at risk to develop DIC. Encouraging data show these tests may enable early detection of dysfunctional coagulation and hyperfibrinolysis,¹⁴⁷ allowing adequate surveillance and prompt intervention.^{14,148} A study of 21 patients classified according to the ISTH DIC score, proposed a thromboelastographic score had 95.2% sensitivity, 81.0% specificity, and an AUC of 0.957 for identifying overt DIC¹⁴⁹ thus allowing, along other diagnostic and prognostic modalities (ie DIC scores), an adequate surveillance and, eventually, a prompt intervention during the early stages of DIC in pregnancy.¹⁵⁰

Treatment of DIC in Pregnancy

Prevention, early diagnosis and treatment of obstetrical hemorrhage are the heart of midwifery.

Prevention of obstetrical hemorrhage is based on identification of patients at risk for peripartum bleeding, active management of the third stage of labor and prompt diagnosis and management of obstetrical complications that may be associated with the development of DIC even in the absence

of labor, the interested reader is referred to the guidelines of the professional committees and selected reviews (ref).

The basic principles for treating obstetrical DIC are presented in Figure 8 and include the following:^{9,14,106,151} 1) treatment and resolution of the underlying condition leading to DIC; 2) fast and prompt delivery or termination of pregnancy (before the threshold of viability). The delivery options should be discussed by a multidisciplinary team and consider the safest mode of delivery to the mother, how fast she is expected to deliver, what are the resources of blood products and other supportive mechanisms available, and can she sustain a surgery; 3) supportive treatment with blood product transfusion, surgical care and related measures; 4) rigorous clinical and laboratory patients surveillance; 5) prompt involvement of needed consultant such as hematologists, gynecological surgeons, anesthesiologists and others; and 6) in small to medium size health care facilities it is important to estimate whether their blood bank can support a massive blood transfusion and, if necessary, contact regional or larger hospitals for assistance or for transferring the patient.

Treatment algorithms for obstetric DIC always involve simultaneous blood product transfusion as a replacement of women's blood loss. Specific transfusion protocols have been previously published and the interested reader is referred to the specific papers on the subject.¹⁵² The harmonized guidance from the ISTH^{151,153} give a fine definition for the thresholds for blood products transfusion, and massive transfusion protocols with fixed RBC: plasma: platelet ratios,^{83,152} are mostly being used, but this recommendation is under debate.¹⁵⁴ The response to blood component therapy should be monitored both clinically and with repeated assessments of the platelet count and coagulation parameters. For further reading, the reader is referred to the published guidelines for blood component replacement from national professional societies^{155–159} and the scientific subcommittees on Women's Health Issues in Thrombosis and Hemostasis and on DIC of the ISTH.^{41,160}

Viscoelastic Hemostatic Assays Guided Transfusion Protocols

During DIC there is a rapid consumption of fibrinogen and coagulation factors, making the monitoring and maintenance of proper blood products administration by conventional laboratory tests challenging, as rapid correction of blood component deficiencies is required at a rate faster than their depletion. ROTEM/TEG allow early detection and dynamic monitoring of clotting abnormalities, and

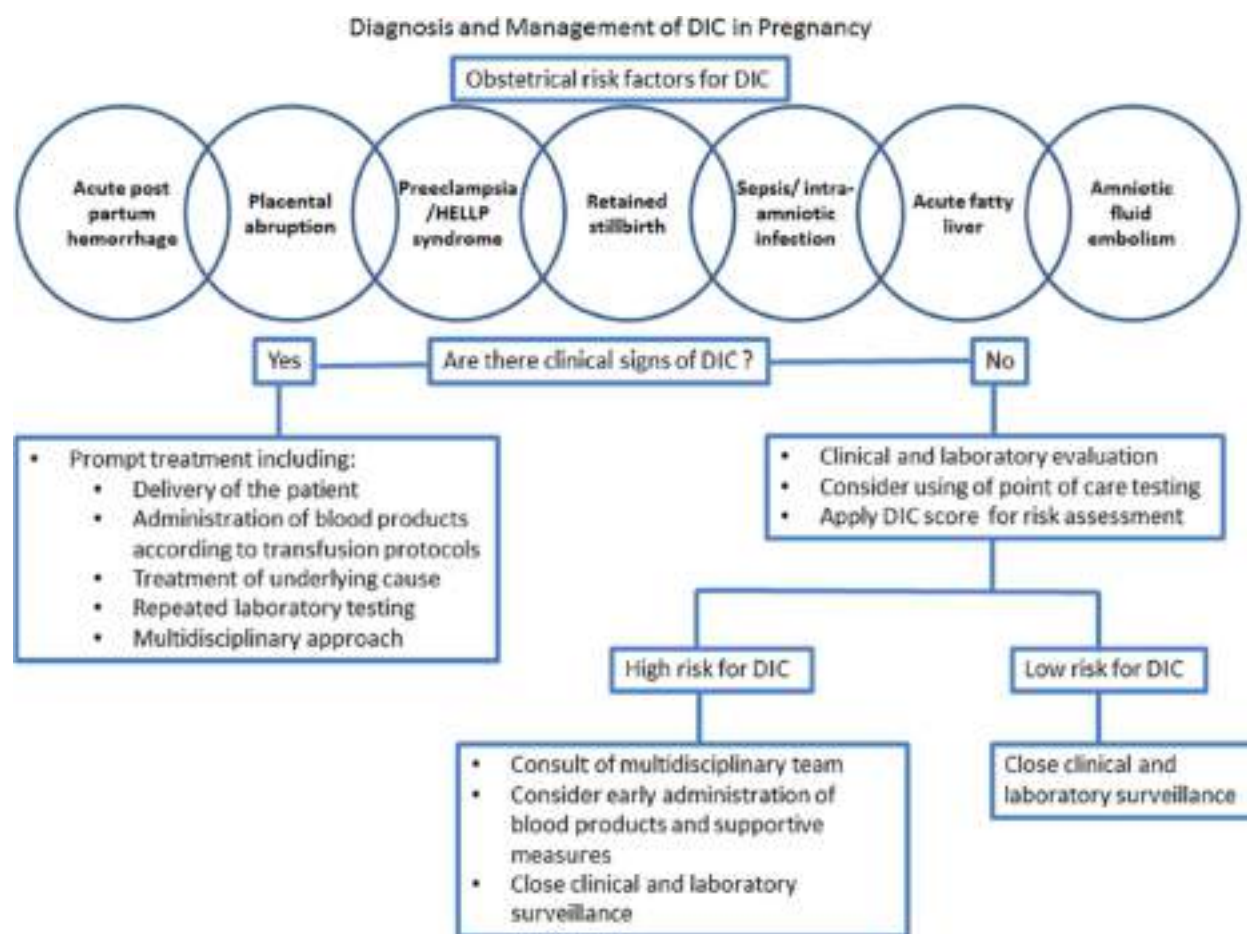


Figure 8 Principles of Diagnosis and management of DIC in pregnancy.

Notes: Reproduced from: Erez O. Disseminated intravascular coagulation in pregnancy - Clinical phenotypes and diagnostic scores. *Thromb Res.* 2017;151203 1:S56-S60.²⁰⁰ With permission. Copyright © 2017 Elsevier Ltd. All rights reserved.

transfusion needed; hence, they could potentially be used as guidance for administration of blood components. Favorable evidence regarding use of these tests, especially to guide fibrinogen replacement therapy in management of obstetric complications, is accumulating.^{123,147,150,161–166} Further research demonstrated that the use of ROTEM-FIBTEM A5 as a point of care testing for fibrinogen concentration assisted in targeting patients with postpartum hemorrhage who will require blood product transfusion. The rationale for its use was that while Clauss fibrinogen tests results are available within an hour of venipuncture, those of FIBTEM A5 are available within 10 minutes. The authors use a FIBTEM A5 of <15 that can be translated to fibrinogen concentration of 3 grams/Liter or 300mg/dl as a cutoff point that requires obstetrician attention in women with PPH and developed an algorithm for the management of women with PPH based on the FIBTEM A5 results (Figure 9).^{167–169} These

encouraging results in the pregnant population await further studies before widespread clinical application of VHA scan as point of care testing to guide blood product transfusion in women with PPH can be implemented.^{170–173}

Another possible clinical application of VHAs could be the identification of women who show a severe hypercoagulable state and could benefit from antithrombotic prophylaxis. In a recent case-control study, Spiezia et al evaluated ROTEM profiles in women with preeclampsia in comparison to healthy pregnant women.¹⁷⁴ Characteristic hypercoagulable ROTEM profiles in women with preeclampsia were found.

Treatments for Coagulopathy

Based on the pathogenesis of microvascular failure and coagulation activation in DIC, strategies aimed at the inhibition of coagulation activation have been found

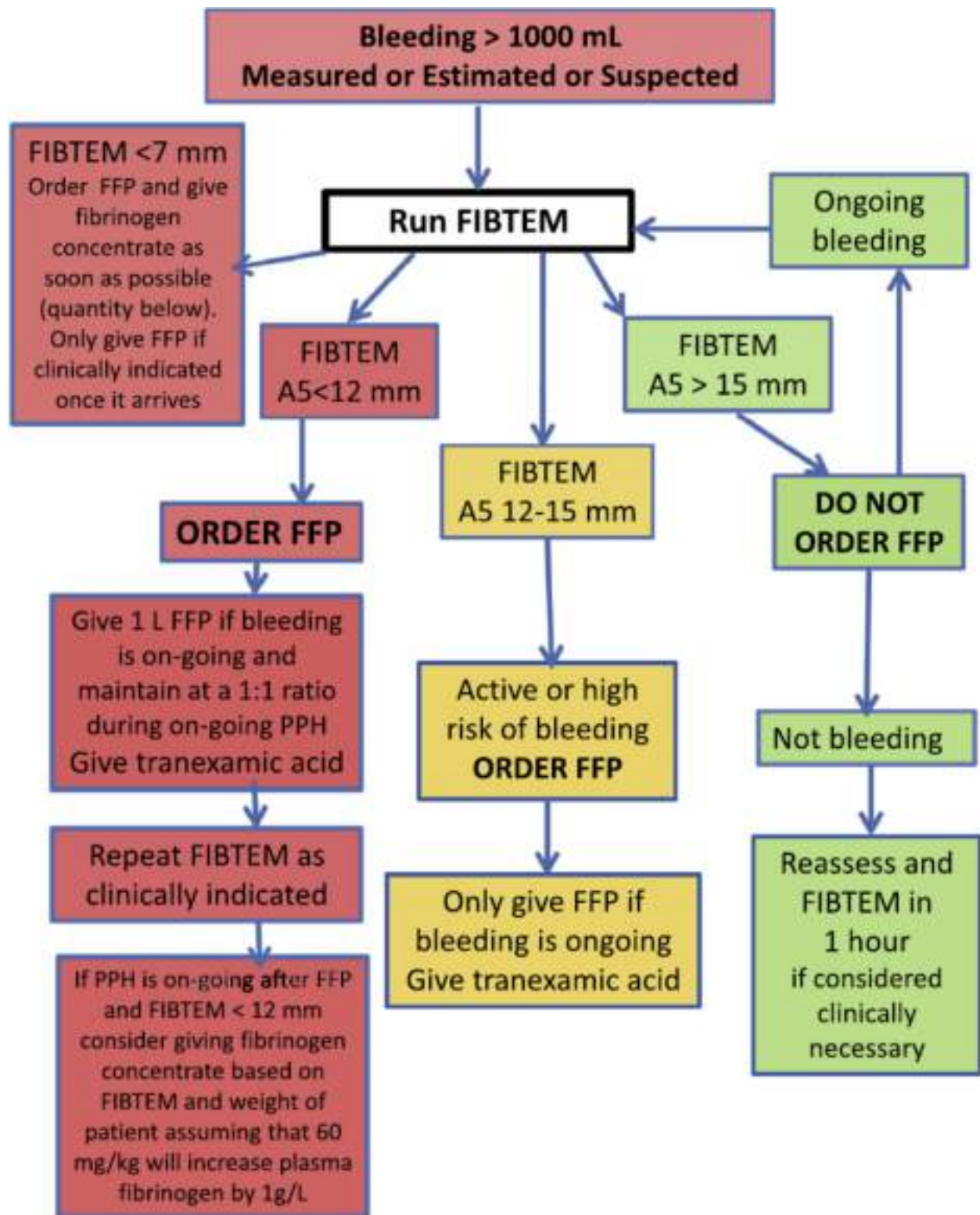


Figure 9 Algorithm for the treatment of obstetrical hemorrhage based on FIBTEM and fibrinogen concentrations.

Notes: Reprinted from: Collins PV, Bell SF, de Lloyd L, Collis RE. Management of postpartum haemorrhage: from research into practice, a narrative review of the literature and the Cardiff experience. *Int J Obstet Anesth.* 2019;37:106–117.¹⁶⁷ With permission Crown Copyright © 2018 Published by Elsevier Ltd. All rights reserved.

favorable in experimental and clinical studies. Most RCTs have been carried out in patients with sepsis, and evidence in obstetric DIC is limited. Tranexamic acid is used as an adjunct to blood product administration and has been studied in PPH. Recombinant activated factor VII (rFVIIa) is used as a second line treatment in women in whom massive transfusion does not halt blood loss. All other treatment modalities are considered experimental at this stage.

Antifibrinolytic Agents

Tranexamic acid (TXA) has been suggested as a treatment for coagulopathy in PPH.¹⁷⁵ TXA prevents the activation of plasminogen by plasmin by blocking its lysine binding sites.¹⁷⁵ Four recent systematic reviews of the use of TXA for reduction of blood loss in PPH came to conflicting results.^{176–179} TXA appears to be a promising drug for the prevention of PPH after cesarean and vaginal

delivery.^{180,181} Patients with the organ failure or non-symptomatic type of DIC may not benefit from antifibrinolytic agents as fibrinolysis is needed for the resolution of widespread fibrin thromboses resulting by DIC.¹⁸² The usual dose is 1gr administered intravenously over 10 minutes up to 4 times daily.¹⁸³

Recombinant Activated Factor VII (rFVIIa)

Guidelines suggest that administration of rFVIIa is warranted in active obstetrical hemorrhage that does not resolve by conventional treatment or to prevent hysterectomy.¹⁶⁰ In such cases, this hemostatic agent decreases maternal mortality due to obstetrical hemorrhage.^{184,185} A review of 99 cases of its use in DIC, of them 32 due to PPH, reported that a median dose of 67.2 mg/kg is successful in controlling the ongoing obstetrical hemorrhage.¹⁸⁶ Optimal use of rFVIIa requires exclusion or correction of metabolic acidosis, hypothermia, hypofibrinogenemia, and thrombocytopenia.¹⁸⁷ A recent RCT, reported

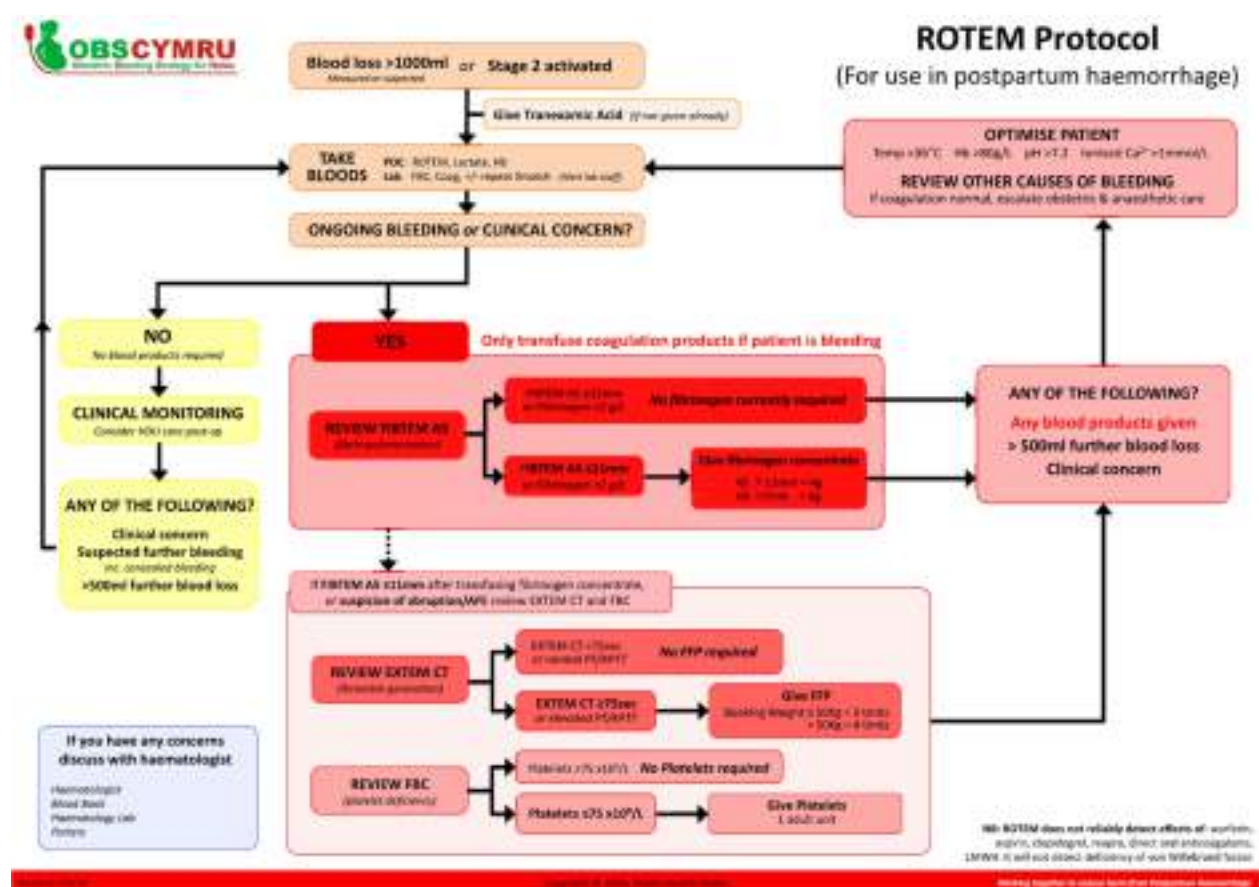


Figure 10 The ROTEM protocol of the OBS Cymru (the Obstetric Bleeding Strategy for Wales) initiative.

Notes: Reproduced with permission from: ROTEM Protocol (For use in postpartum haemorrhage); 2018. Obstetric Bleeding Strategy for Wales. Available from: <https://phw.nhs.wales/services-and-teams/improvement-cymru/improvement-cymru-programmes/maternity-cymru/obs-cymru/obstetric-bleeding-strategy-cymru/rotem-point-of-care-testing>²⁰¹ Copyright © 2016, Public Health Wales.

that rFVIIa reduces by 30% the need for second-line treatments (interventional hemostatic procedures, blood transfusions).¹⁸⁸ However, as the use of rFVIIa increases by two fold the risk for arterial thrombosis, adequate thromboprophylaxis should be administered to these patients following the acute hemorrhagic event. The optimal dose is unclear, however it is preferable to start with a low dose (40–60mcg/kg) to reduce the risk of thrombotic events. Higher doses (eg 90mcg/kg) can be used if the patient is unstable or if blood loss is brisk and ongoing.

Potential Experimental Treatments

Fibrinogen Concentrate

Human fibrinogen concentrates have been used for substitution therapy in cases of hypofibrinogenemia, dysfibrinogenemia, and afibrinogenemia. This product has the potential to administer relative high quantity of fibrinogen in relatively low volume of transfusion in women with obstetrical hemorrhage. Its benefits are that it is readily available, as it can be kept in room temperature or refrigerators, and it can introduce higher quantity of fibrinogen without the need to transfuse large plasma volume as in FFP transfusion.^{167,189} The obstetric bleeding strategy for Wales (OBSCYMRU) initiative has adopted the use for fibrinogen concentrate at the treatment of choice for women with active obstetrical hemorrhage who has fibrinogen concentrations <2g/L or FIBTEM A5<11mm¹⁶⁷ (Figure 10). However, this Fibrinogen concentrate is not readily available worldwide and the current standard of care for the administration of high concentration of fibrinogen in a low volume transfusion is by cryoprecipitate. Each vial contains approximately 1000mg of fibrinogen, doses are repeated according to TEG/ROTEM guidance.

Desmopressin- 1-Deamino-8-D-Arginine Vasopressin (DDAVP)

The synthetic Anti diuretic hormone analog increases factor VIII concentrations and VWF release from endothelial cells, promoting platelet aggregation and adhesion. DDAVP can reduce bleeding resulting from obstetrical hemorrhage with a good safety profile.¹⁹⁰ The current evidence although limited suggest that DDAVP may be beneficial in DIC resulting from abnormal platelet activity.¹⁹¹ However, it is not part of standard treatment protocols.

Recombinant Human Soluble Thrombomodulin (rhTM)

This agent was reported to potentially lower morbidity and mortality of patients with DIC due to sepsis.^{192–194} This anti-

coagulant reduces excessive thrombin activation and regulates the imbalance of the coagulation system. The administration rhTM to patients with obstetric DIC in a single-center, retrospective cohort study, resulted in improved platelet count, D-dimer and fibrinogen concentrations, and prothrombin time, as well as reduction in the need for platelet transfusions; however, these observations were not associated with a change in the rate of organ failure.¹⁹⁵ There is a need for more substantial evidence prior for the inclusion of rhTM in the treatment protocols for obstetrical DIC.

Anticoagulants

In cases of DIC with predominant hypercoagulation and thrombotic phenotype heparin can partly inhibit coagulation in this setting¹⁹⁶ and low molecular weight heparin (LMWH) is superior to unfractionated heparin (UFH) for treating this type of DIC.¹⁹⁷ However, the majority of cases of DIC in pregnancy are associated with a hemorrhagic phenotype of DIC, and heparin may increase bleeding, especially if adequate replacement therapy for consumed clotting factors has not been achieved, hence it is not recommended.

Conclusions

DIC in obstetrics is a life-threatening complication that is secondary to obstetrical and non-obstetrical related complications of pregnancy. Solid clinical and epidemiologic data is still lacking. The SSC Subcommittees on Disseminated Intravascular Coagulation (DIC) and Women's Issues in Thrombosis and Hemostasis of the International Society for Thrombosis and Hemostasis have launched a global registry of DIC in pregnancy to collect this information. We encourage physicians from across the globe to participate using this link <https://redcap.isth.org/surveys/?s=KFC8RN8XWC>. The diagnosis of DIC can be elusive during pregnancy and requires vigilance and knowledge of the physiologic changes during pregnancy. Pregnancy specific DIC scores and adjustment of the cutoff of point of care testing can facilitate the diagnosis and the management of DIC in pregnancy.

Disclosure

The authors report no conflicts of interest for this work.

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First Report from Afghanistan on the Prevalence of Blood-Borne Infections: A Retrospective Cross-Sectional Multicentre Study for an Epidemiological Assessment

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Background: The transfusion of blood and blood components has a significant role in healthcare services. However, it remains a possible risk factor for blood-borne infections. The present study was conducted to assess the prevalence of serological markers of common blood-borne infections among the blood donor population of Afghanistan.

Methodology: This was a cross-sectional study based on retrospectively collected data over a period of six years from 284 blood centres across 34 provinces of Afghanistan. Every blood donor's sample was tested by rapid immunoassays for the serological markers of blood-borne infections namely hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (anti-HCV), anti-human immunodeficiency virus 1/2 (anti-HIV1/2), and anti-Treponema pallidum (anti-TP).

Results: All blood donors during the study period were males. The majority of blood donations were from the family replacement category 56.93% (n = 544,568). The overall pooled prevalence of blood-borne infections was 4.36% with a comparatively higher percentage in family replacement donors 4.88%. The seropositivity for HBsAg, anti-HCV, anti-HIV1/2, and anti-TP was 2.95%, 0.81%, 0.04%, and 0.54%, respectively.

Conclusion: Complete reliance on voluntary blood donors and screening with quality assured highly sensitive assay is recommended to ensure blood safety in the country.

Keywords: blood, screening, Afghanistan, hepatitis, HIV, syphilis

Introduction

The transfusion of blood and blood components has proven to save millions of lives across the globe. However, unsafe transfusion of blood and blood components has a vital role in the spread of blood-borne infections. These infectious agents mainly include but are not limited to, Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), Treponema pallidum (causing syphilis), and Plasmodium species (causing malaria). According to WHO, in middle and lower-income countries, the prevalence of blood-borne infections is on the higher side and quite far from attaining a zero-risk level.¹

In Afghanistan, a country engaged in continuous humanitarian crisis, blood transfusions services are not consistent as per international standards. For the Afghan National Blood Safety and Transfusion Service (ANBSTS), blood safety remains an issue of major concern for factors such as the absence of blood safety

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legislation, inappropriate infrastructure, financial constraints, lack of trained manpower, inadequate data management, and no national donor awareness campaign for donor selection and retention.

There is a dearth of available data from Afghanistan on the prevalence of blood-borne infections in blood donors. The limited data available is from studies and reports estimating the prevalence of these infectious agents in the general population and high-risk groups with small sample sizes.^{2–6}

The assessment of blood donors for blood-borne infections is of utmost significance to assess the burden and risk of blood-borne infections in the general population and provides a shred of scientific evidence for articulating blood safety policies, strategies, and standards. Therefore, the current study was planned to acquire data regarding the prevalence of common blood-borne infections among blood donors from 284 blood centres or blood banks in Afghanistan, covering the whole country. To the best of our understanding, this is the first epidemiological assessment report from Afghanistan on the prevalence of blood-borne infections in the blood donor population.

Materials and Methods

This was a cross-sectional study based on retrospectively collected data from 284 blood centres or blood banks scattered across 34 provinces of Afghanistan. These blood banks include central and regional blood banks of the Afghan National Blood Safety and Transfusion Service (ANBSTS) and numerous hospital-based blood banks working under the respective hospital management. ANBSTS was established in 2009 by the Ministry of Public Health and is solely responsible for all blood banking services across the country.^{7,8}

The study population included prospective blood donors irrespective of occupation, religion, education level, and any ethnic groups, who visited the blood banks over six years between 2015 and 2020.

The blood donors were considered fit for donation if they fulfilled the national criteria, ie weight >50 kg, age 18–60 years, haemoglobin 12.5–16.5 g/dl, blood pressure 120/80 mmHg, no previous history of hepatitis B and C, pregnancy, lactation, and/or menstruation. The blood donors who qualified for donation went through a behavioural screening interview, gave written consent, and donated blood. The behavioural screening interview entails filling out a donor history questionnaire that is made up of questions, grouped into common timeframes and based on principles of

cognitive psychology, to facilitate donor understanding and correct recall of related risk activities. Questions concerning related medications and travel history are also asked to ascertain donor eligibility to donate.

Every blood donor's sample was tested for the serological markers of blood-borne infections namely Hepatitis B surface antigen (HBsAg), anti-Hepatitis C virus (anti-HCV), anti-Human immunodeficiency virus 1/2 (anti-HIV1/2), and anti-Treponema pallidum (anti-TP). Briefly, 5 mL of blood was centrifuged at 5000 rpm for five minutes and plasma separated. The samples were screened for HBsAg, anti-HCV, and anti-HIV by SD Bioline rapid screening devices based on immunochromatographic principle (Standard Diagnostic Inc., South Korea). Screening for anti-TP was also performed through an Alere DetermineTM Syphilis rapid TP assay (Alere North America Inc., USA). The data were entered into Microsoft Excel and later transferred to the IBM Statistical Package for the Social Sciences (SPSS) software, version 24.0. Armonk, New York: IBM Corp. Errors and missing data were adjusted by running cross-tabs and frequency tables. Summary indicators such as frequencies and percentages were calculated. The results were presented using words and tables.

Results

A total of 956,509 individuals donated blood across 284 blood banks in Afghanistan between 2015 and 2020. Nearly 56.93% ($n = 544,568$) of the study participants belonged to family replacement donor's category while remaining were voluntary non-remunerated blood donors (VNRBD) 43.07% ($n = 411,941$). Most of the blood donors 65.36% ($n = 62,518$) were first-time donors.

All blood donations (100%) reported in the 6-year study period were from male donors. The median age of the donors was 24.2 years, with the age range between 18 and 57 years. Most of the donors 42.91% ($n = 410,529$) were of age 18–30 years followed by 35.51% ($n = 339,556$) who were in the age range between 31 and 40 years (Table 1).

The overall pooled prevalence of the four blood-borne infectious markers in the current study was 4.36%. The prevalence in replacement donors was higher at 4.88% compared to voluntary blood donors that was 3.66%. Among the individual infections, the hepatitis B virus was among the most prevalent infection 2.95% ($n = 28,296$). Table 2 shows the different types of blood-borne infections among the blood donor population.

Table 1 Socio-Demographic Characteristics of Blood Donors in Present Study (n = 956,509)

Age Group	Number of Donations	Percentage (%)
18–30 years	410,529	42.92
31–40 years	339,556	35.51
41–50 years	167,379	17.49
> 50 years	39,045	4.08
Total	956,509	100.0
Sex		
Male	956,509	100.0
Female	0	0
Total	956,509	100.0
Types of Donation		
Replacement	544,568	56.93
VNRBD	411,941	43.07
Total	956,509	100.0

Discussion

The prevalence of blood-borne infections among blood donors indirectly echoes the burden of these infections in the population because blood donors represent healthy members of the society.

Afghanistan is facing serious challenges in blood availability and safety due to years of conflict causing a humanitarian crisis. The transfusion of blood and blood components is considered a possible risk factor for the transmission of blood-borne infections posing a threat to the potential recipients. The present study assessed the prevalence of blood-borne infections among blood donors in Afghanistan over six years' period.

A vast majority (42.92%) of the blood donors in the present study were between 18 and 30 years. This finding was consistent with studies from Pakistan (45.6%),⁹ Kenya (42.24%),¹⁰ and Tanzania (44.7%).¹¹ The blood donors in the present study had their mean ages 10–15 years less than those observed in developed western countries.¹²

A possible reason for this difference could be due to the fact that more than 60%¹³ of the general population in Afghanistan is younger than 30 years and demographics are reflected in blood donors also. From the perspective of the voluntary blood donor programme, the emergence of this large cohort of young people is promising.

The current study showed that 43.07% (n = 411,941) of blood donations came from voluntary non-remunerated blood donors (VNRBD) with remaining 56.93% (n = 544,568) being family replacement donors. This is an encouraging finding from a low-income country like Afghanistan. This percentage of voluntary blood donations, although still far from WHO's goal of 100% VNRBD,¹⁴ is better than regional countries including Pakistan,¹⁵ and Bangladesh.¹⁶

There was an absence of female donations and all donors were males. This deficiency of female donations is a source of concern for the national blood service (ANBSTS) as it designates that nearly 48% of the population is not donating. It is believed that lack of female-friendly facilities, nutritional status, prejudices, misconceptions, cultural and social factors contribute to low female donation rates. It is essential to lower the barriers erected by the gatekeepers of family, community, and society, through a public awareness campaign addressing the issue of female donations as one of the principal target areas, among others.

The overall pooled prevalence of infectious markers in our study was 4.36%. Earlier studies from India¹⁷ (4.36%), Ghana¹⁸ (4.06%), Pakistan¹⁹ (4.0%), and Eritrea²⁰ (3.6%) have reported a similar trend. However, the percentage was on the lower side when compared with Nigeria²¹ (28.8%), Bangladesh¹⁶ (7.8%), Ethiopia²² (11.5%), and Tanzania¹¹ (10.1%).

The pooled prevalence of infectious markers significantly dropped in 2020 (2.80%) compared to the years 2015 to 2019 (between 4.12% and 6.26%). This decreased

Table 2 Prevalence of Blood-Borne Infections in Blood Donors 2015–20 (n = 956,509)

Year	Total Blood Donations	HBV	HCV	HIV	Syphilis	Total
2015	64,126	3.59% (n=2306)	0.85% (n=549)	0.06% (n=44)	0.78% (n=504)	5.30% (n=3403)
2016	122,695	3.16% (n=3878)	0.59% (n=733)	0.01% (n=21)	0.36% (n=449)	4.14% (n=5081)
2017	155,715	3.05% (n=4752)	0.60% (n=949)	0.07% (n=111)	0.39% (n=610)	4.12% (n=6422)
2018	179,781	3.74% (n=6733)	1.41% (n=2541)	0.04% (n=89)	1.05% (n=1895)	6.26% (n=11,258)
2019	210,627	2.68% (n=5657)	0.97% (n=2058)	0.04% (n=88)	0.70% (n=1486)	4.41% (n=9289)
2020	223,565	2.22% (n=4970)	0.41% (n=921)	0.03% (n=80)	1.13% (n=294)	2.80% (n=6265)
Total	956,509	2.95% (n=28,296)	0.81% (n=7751)	0.04% (n=433)	0.54% (n=5238)	4.36% (n=41,718)

prevalence may be explained by the capacity development workshops for the donor recruitment staff over the last two years by the ANBSTS. The trainings focused on donor recruitment and retention strategies, donor deferral criteria, donor behavioural screening, and donor haemovigilance.

The present study indicated that HBV (2.95%) is the most prevalent infection among blood donors in Afghanistan. This is comparatively lower than studies from neighboring countries, India²³ (3.44%) and Pakistan²⁴ (3.91%), and those reported from Ethiopia²⁵ (4.2%). This may be attributed to the effective prevention and control mechanisms including mandatory vaccination programmes. However, when compared with a study from Saudi Arabia (0.33%),²⁶ our finding was on the higher side. The infection with HBV results in a permanent deferral of blood donors, hence reducing the pool of potential blood donors in the general population.

The seroprevalence of HCV in the present study was 0.81%, which is high when compared with a study from Bangladesh²⁷ (0.03%). Our finding was on the lower side when compared to previous studies from Sudan²⁸ (1.40%), Pakistan²⁹ (3.26%), Kenya³⁰ (3.21%), and Nigeria²¹ (3.6%). This can be linked to the low prevalence of the HCV among the general Afghan population (0.7%).⁶

In the present study, the seroprevalence of HIV among blood donors was 0.04%. This is comparable to a study from Pakistan³¹ (0.06%). However, it is on the lower side when compared to previous studies conducted in Saudi Arabia²⁶ (0.13%), India³² (0.6%), Nigeria²¹ (4.2%), and Sudan (2.61%).²⁷ A possible reason for this lower rate of HIV infection could be a decrease in the prevalence of HIV at population level and due to enhanced effectiveness of the blood donor awareness programme in ensuring behavioural screening.

The pooled prevalence of syphilis among blood donors was 0.54%. However, throughout the study period, it showed a fluctuating trend, eg, it was 0.78% in 2015, 0.36% in 2016, and 1.13% in 2020.

The overall syphilis prevalence in present study (0.54%) is comparable to earlier studies from Qatar³³ (0.43) and Pakistan³⁴ (0.72%). On the other hand, studies from Bangladesh²⁶ (0.20%), USA³⁵ (0.16%), Saudi Arabia³⁶ (0.04%), and India³² (0.05%) reported a lower prevalence rate for syphilis in blood donors. Conversely, it is lower than 5.72% in Sudan,²⁷ 3.1% in Nigeria,²¹ and 1.9% in Tanzania.¹¹

The difference in the prevalence percentages is dependent on several factors including sample size, study site, society behaviour towards voluntary blood donations,

socioeconomic status, literacy rate, and the quality of screening assay used.

Limitations

The samples were tested through rapid screening devices, and no confirmatory testing such as ELISA, CLIA, or NAT was performed. No associated risk factors and several socio-demographic features of the blood donor were included due to insufficient data/errors in data reporting. All donations were from male donors, the prevalence in the female population was not available.

Conclusion

The blood-borne infections remain a threat to blood safety, thus 100% reliance on voluntary blood donors and screening with quality assured highly sensitive assay is recommended to ensure blood safety in the country. Additional studies are needed to detect the main risk factors and formulate intervention strategies.

Data Sharing Statement

The datasets generated during and/or analysed during the current study are not publicly available due to confidentiality issues but are available from the corresponding author on request.

Ethics Approval and Consent to Participate

The study was approved by the National Research Ethics Committee of the Ministry of Public Health and all study participants, ie, blood donors, provided a written consent. The study was conducted in accordance with the World Medical Association Declaration of Helsinki.

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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.

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Disclosure

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Patterns of Bone Marrow Confirmed Malignant and Non-Malignant Hematological Disorders in Patients with Abnormal Hematological Parameters in Northeast Ethiopia

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Background: Hematological disorders are heterogeneous conditions ranging from malignant to non-malignant disorders. Hematological malignancies comprise a collection of heterogeneous conditions originating from cells of the bone marrow and the lymphatic system. Therefore, this study aimed to determine the pattern of bone marrow confirmed malignant and non-malignant hematological disorders in patients with abnormal hematological parameters.

Methods: Institutional-based cross-sectional study was conducted in Dessie town from April 2020 to June 2021. A total of 228 study participants who had abnormal hematological parameters and referred for bone marrow examination were included consecutively. About 1.5 mL of bone marrow sample and 3 mL of venous blood sample were collected for bone marrow examination, complete blood count analysis and peripheral blood morphology examination. Wright stain, Sudan black B, and Prussian blue stains were used for staining the bone marrow and peripheral blood smears. The result was expressed in mean and standard deviation and presented in texts and tables. Ratio, frequency, and percentage were used to express the magnitude of malignant and non-malignant hematological disorders.

Results: The overall prevalence of hematological malignancies among the study participants was 11.4% with 8.8% in male patients. The prevalence of hematological malignancies were 3.5% CML, 2.6% AML, 1.8% CLL and MM, 0.9% ALL and undifferentiated acute leukemia. On the other hand, 57.0% of the study participants had non-malignant hematological disorders. Regarding non-malignant hematological cases, 24.6% were erythroid hyperplasia, 10.5% aplastic anemia, 8.8% concomitant IDA and MBA, 7.0% MBA, 3.5% leukemoid reaction, 1.8% IDA, and 0.9% visceral leishmaniasis. In patients with HM, 66.7% of AML, 100% of CML and CLL, and 75% of MM patients had increased total WBC count, whereas 66.7% of AML, 62.5% of CML, 75% of CLL, and 50% of MM patients had decreased hemoglobin level. On the other hand, 66.7% of AML, and 50% of CML, ALL, and CLL patients had decreased platelet count.

Conclusion: In this study, 11.4% of the patients had hematological malignant cases, whereas 57% of the patients had non-malignant hematological cases. Therefore, in patients with hematological abnormalities and where conclusive diagnosis could not be made through clinical and other laboratory investigations, bone marrow examination should be done for definitive diagnosis, management and prognosis.

Keywords: hematological malignancies, non-malignant hematological disorders, leukemia, anemia, Ethiopia

Background

Hematological disorders are heterogeneous conditions ranging from malignant to non-malignant disorders.^{1,2} Hematological malignancies comprises a collection of heterogeneous conditions originating from cells of the bone marrow and the lymphatic system.³ The World Health Organization (WHO) classified hematologic malignancies into myeloid neoplasms, lymphoid neoplasms, mast cell disorders, and histiocytic neoplasms primarily based on cell line

involvement in neoplastic transformation.^{4,5} Distinct neoplasms are defined and characterized according to a combination of morphology, immunophenotype, genetic, and clinical features of the patients.⁶ Bone marrow examination (BME) is required as complementary with complete blood count analysis and peripheral blood morphology examination to confirm the diagnosis of hematological disorders.^{1,7}

Hematological malignancies can be classified into different groups that include leukemia, lymphoma, multiple myeloma (MM), myelodysplastic syndrome (MDS), polycythemia vera (PV), myeloproliferative neoplasms (MPNs) and primary myelofibrosis.^{8,9} Based on hematopoietic stem cell involvement and progression of the diseases, leukemia can be grouped into four main groups that include acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL) whereas lymphoma can be grouped into non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma (HL).^{10,11}

Acute leukemia is a proliferation and invasion of immature hematopoietic cells (blasts) in the bone marrow as well as invasion of peripheral blood circulation, solid organs, and lymphatic system. French American British (FAB) has been traditionally set as a minimum of 30% blast cells to diagnose acute leukemia.¹² However, WHO revised the criteria and set as the minimum of 20% of blast cells to diagnose acute leukemia.^{4,5} Acute myelogenous leukemia is the proliferation of leukemic blasts including myeloblasts, monoblasts, megakaryoblasts, erythroblasts, and their precursors in undifferentiated, minimally, or moderately differentiated stages in the bone marrow.¹³ Acute lymphoblastic leukemia is a malignant transformation and proliferation of lymphoid progenitor cells characterized by uncontrolled proliferation of abnormal, immature lymphocytes and their progenitors of B and T-lymphocytes. Moreover, ALL is predominant leukemia that affects children. However, around 75% of adult ALL cases affect the B-cell phenotype, whereas the remaining 25% cases affect the T-cell phenotype.¹⁴

Chronic leukemia is characterized by uncontrolled proliferation and expansion of mature, differentiated cells of the hematopoietic system in the bone marrow as well as in peripheral circulation.⁸ Chronic myeloid leukemia is a group of heterogeneous related conditions that affects the myeloid hematopoietic cell lines including the granulocyte, monocyte, erythrocyte, and megakaryocyte cells.¹⁵ Chronic lymphocytic leukemia relatively affects mature lymphoid cells of the B and T-cell phenotypes.¹⁶ According to the global cancer statistics report by 2018, there was an estimate of a total of 437 thousand new cases and 309 thousand cancer-related deaths from leukemia worldwide. It is the eleventh leading cause of cancer-related mortality.¹⁷ Leukemia accounted for approximately 3.4% of all new cancer cases and 3.8% of all cancer-related deaths worldwide in 2020.¹⁸ Hematological malignancies can affect all age groups but children and older adult individuals are the most affected populations. The incidence rates of all types of leukemia are slightly higher in males as compared to females and it might be affected by geographical and ethnic variations.¹⁹ The causes for HM are not precisely known. Epidemiological studies have been suggested that several risk factors might be associated with the development and the increased risk for HM such as prolonged exposure to ionizing radiation, exposure to benzene, formaldehyde, organic solvents, agricultural pesticides, and herbicides, cigarette smoking, previous cancer treatment with both chemotherapy and radiation therapy, immunological, genetic disorders, and infection with oncogenic retroviruses.^{3,19}

Non-malignant hematological disorders are a group of diverse conditions ranging from inherited conditions like hemophilia and sickle cell disease to common non-hereditary conditions such as venous thromboembolism and different types of anemia.²⁰ Anemia is the condition associated with reduced numbers of red blood cells (RBC) or decreased hemoglobin level (Hgb) and it can be grouped into different categories based on the cause such as nutritional, hemolytic, and aplastic anemia.^{21,22} Nutritional anemia is mainly associated with the deficiency of iron, vitamin B₁₂, folic acid, hormones, other vitamins, and minerals, whereas hemolytic anemia occurs due to premature RBC destruction and aplastic anemia is due to bone marrow failure.^{20,23}

Bone marrow examination has wide application in clinical medicine for the diagnosis and monitoring of the therapy of malignant, non-malignant hematological disorders, and various systemic diseases of unknown origin.^{24,25} There have been few studies conducted to investigate the magnitude of malignant and non-malignant hematological disorders using bone marrow examinations in poor resource setting countries including Ethiopia. Even, there was no similar study conducted in the study area to assess the magnitude of malignant and non-malignant hematological disorders which are difficult to make conclusive differential diagnosis through clinical and other laboratory investigations. Therefore, the

main aim of this study is to determine the magnitude of malignant and non-malignant hematological disorders among patients with abnormal hematological parameters.

Methods and Materials

Study Design, Period, and Area

A prospective institutional-based cross-sectional study was conducted in Dessie town diagnostic medical laboratory pathology department, Northeast Ethiopia from April 2020 to June 2021. The diagnostic laboratory is found in Dessie town about 401 Km far from the North of Addis Ababa, the capital of Ethiopia. The diagnostic laboratory gives both routine and advanced medical laboratory services for patients who came from private and governmental health institutions. It serves as a bone marrow examination and diagnostic center for hematological malignancies referred from both private and government health institutions in the town as well as from the surrounding area.

Study Population, Participants, and Sampling Techniques

The study population comprises all patients who had abnormal hematological parameters and referred to underwent bone marrow examination by their physicians. Thus, a total of 228 study participants who visited the diagnostic laboratory with a request form for bone marrow examination were included in the study consecutively during the study period.

Data Collection and Laboratory Analysis

Socio-demographic characteristics information of study participants were collected using a structured questionnaire. About 1.5 mL of bone marrow sample was collected aseptically by an experienced and licensed internist from the iliac bone after giving local anesthesia of 2% lidocaine hydrochloride and direct bone marrow smears were prepared for examinations. About 3 mL of venous blood sample was collected for complete blood count (CBC) analysis as well as peripheral blood smear preparations. Furthermore, wright stain, Sudan black B, and Prussian blue stains were used for staining the smears and then the preparations were examined by experienced and licensed pathologist.

Data Quality Management

Bone marrow and blood samples were collected and processed according to the standard operating procedures (SOPs). Then, blood samples were properly mixed and homogenized by inverting 8–10 times following strict specimen safety and handling procedures. Bone marrow and peripheral blood smears preparation and examinations were conducted strictly according to SOPs. The performance of the automated hematology analyzer was maintained through the daily running of three-level controls (low level, medium level, and high level) quality control materials. Furthermore, daily background checking was carried out routinely. Automated analyzers and other equipment were cleaned daily before leaving the laboratory as routine day-to-day activities.

Data Management, Statistical Analysis, and Interpretation

Data were coded, entered, and cleaned using Epi Data 3.1 version and then exported to statistical package for social sciences (SPSS) version 23.0 (IBM Corporation, Armonk, NY, USA). The result was expressed in mean and standard deviation and presented in texts and tables. Ratio, frequency, and percentage were used to express the magnitude of malignant and non-malignant hematological disorders.

Results

Magnitude of Hematological Malignancies Among Study Participants

In this study, a total of 228 study participants who had abnormal hematological parameters and were referred for bone marrow examination were included. The mean $\pm$ SD age of the study participants was 35.51 ($\pm$ 14.02) with the minimum and maximum age of the study participants included in the study between 15 and 75 years. Out of 228 study participants, 130 (57%) were males. Furthermore, the majority of the study participants, 128 (56.1%) were grouped within the age between 15 and 35 years. Moreover, the overall prevalence of HM was 11.4% (26/228) with a higher prevalence was

Table 1 Magnitude of Hematological Malignancies Among Study Participants in Northeast Ethiopia from April 2020 to August 2021 (n = 228)

Variables	Category	Hematological Malignancies					
		AML	CML	ALL	CLL	Undifferentiated AL	MM
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age (years)	15–35	6 (2.6)	–	2 (0.9)	2 (0.9)	–	2 (0.9)
	>35	–	8 (3.5)	–	2 (0.9)	2 (0.9)	2 (0.9)
Sex	Male	4 (1.8)	6 (2.6)	2 (0.9)	4 (1.8)	2 (0.9)	2 (0.9)
	Female	2 (0.9)	2 (0.9)	–	–	–	2 (0.9)

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; AL, acute leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma.

among male patients with a prevalence of 8.8% (20/228). The prevalence of HM diagnosed was; 3.5% of CML, 2.6% of AML, 1.8% of CLL, and MM, 0.9% of ALL, and undifferentiated acute leukemia. Besides, 76.9% of hematological malignant cases were dominant among male patients as compared to females (3.3:1 ratio). In addition, 38.5% of HM cases were acute leukemia (AL) and 46.2% cases were chronic leukemia (CL). In this study, 30.8% of cases of AL were found within the younger age range between 15 and 35 years, whereas 38.5% of cases of CL were found within the adult age range between 36 and 55 years. However, 7.7% of cases MM were found within the younger age range and the remaining 7.7% cases of MM were found within the adult age range (Table 1).

Magnitude of Non-Malignant Hematological Disorders Among Study Participants

In this study, 57% (130/228%) of the patients had non-malignant hematological disorders. Out of this, 74 (32.5%) cases of non-malignant hematological disorders belonged to female patients. Non-malignant hematological disorders diagnosed in the patients were 56 (24.6%) cases of erythroid hyperplasia, 24 (10.5%) cases of aplastic anemia, 20 (8.8%) cases of concomitant IDA and MBA, 16 (7.0%) cases of MBA, 8 (3.5%) cases of leukemoid reaction, 4 (1.8%) cases of IDA, and 2 (0.9%) cases of visceral leishmaniasis. In this study, 30 (13.2%) cases of erythroid hyperplasia followed by 18 (7.9%) cases of concomitant IDA and MBA were found between the age of 15 and 35 years. On the other hand, 20 (8.8%) cases of erythroid hyperplasia followed by 10 (4.4%) cases of aplastic anemia were found between the age of 36 and 55 years. The dominant cases diagnosed in male patients were erythroid hyperplasia accounted for 32 (14%) followed by aplastic

Table 2 Magnitude of Non-Malignant Hematological Disorders Among Study Participants in Northeast Ethiopia from April 2020 to August 2021 (n = 228)

Variables	Category	Non-Malignant Hematological Disorders						
		Erythroid Hyperplasia	IDA	MBA	IDA and MBA	Aplastic Anemia	Leukemoid Reaction	Visceral Leishmaniasis
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age (years)	15–35	30 (13.2)	2 (0.9)	12 (5.3)	18 (7.9)	14 (6.1)	4 (1.8)	2 (0.9)
	36–55	20 (8.8)	–	2 (0.9)	2 (0.9)	10 (4.4)	4 (1.8)	–
	>55	6 (2.6)	2 (0.9)	2 (0.9)	–	–	–	–
Sex	Male	32 (14.0)	2 (0.9)	4 (1.8)	–	12 (5.3)	4 (1.8)	2 (0.9)
	Female	24 (10.5)	2 (0.9)	12 (5.3)	20 (8.8)	12 (5.3)	4 (1.8)	–

Abbreviations: IDA, iron deficiency anemia; MBA, megaloblastic anemia.

Table 3 Patterns of Hematological Parameters in Patients with Hematological Malignancies in Northeast Ethiopia from April 2020 to August 2021 (n = 26)

Variables	Category	AML	CML	ALL	CLL	MM
		n (%)	n (%)	n (%)	n (%)	n (%)
WBC count ($10^3/\text{ul}$)	Low	2 (33.3)	–	–	–	1 (25.0)
	High	4 (66.7)	8 (100)	2 (100.0)	4 (100.0)	3 (75.0)
Hemoglobin level (g/dl)	Low	4 (66.7)	5 (62.5)	2 (100.0)	3 (75.0)	2 (50.0)
	Normal	2 (33.3)	2 (25.0)	–	1 (25.0)	2 (50.0)
	High	–	1 (12.5)	–	–	–
Platelet count ($10^6/\text{ul}$)	Low	4 (66.7)	4 (50.0)	1 (50.0)	2 (50.0)	2 (50.0)
	Normal	2 (33.3)	2 (25.0)	1 (50.0)	2 (50.0)	2 (50.0)
	High	–	2 (25.0)	–	–	–
Neutrophil count ($10^3/\text{ul}$)	Low	–	–	–	3 (75.0)	–
	Normal	4 (66.7)	2 (25.0)	2 (100.0)	1 (25.0)	2 (50.0)
	High	2 (33.3)	6 (75.0)	–	–	2 (50.0)
Lymphocyte count ($10^3/\text{ul}$)	Low	2 (33.3)	2 (25.0)	–	–	1 (25.0)
	Normal	4 (66.7)	4 (50.0)	2 (100.0)	–	1 (25.0)
	High	–	2 (25.0)	–	4 (100.0)	2 (50.0)
Monocyte count ($10^3/\text{ul}$)	Low	–	2 (25.0)	–	2 (50.0)	2 (50.0)
	Normal	4 (66.7)	4 (50.0)	–	2 (50.0)	2 (50.0)
	High	2 (33.3)	2 (25.0)	2 (100.0)	–	–

Abbreviation: WBC, white blood cells.

anemia accounted for 12 (5.3%) cases, whereas concomitant IDA and MBA which accounted for 20 (8.8%) of cases followed by MBA accounted for 12 (5.3%) cases were dominant in female patients (Table 2).

Patterns of Hematological Parameters Among Patients with Hematological Malignancies

In this study, hematological parameters in confirmed patients with HM were assessed. The finding showed that leucocytosis, anemia, and thrombocytopenia were common among patients. Among patients with HM, 66.7% of AML, 100% of CML and CLL, and 75% of MM patients had increased total WBC count (leucocytosis), whereas 66.7% of AML, 62.5% of CML, 75% of CLL, and 50% of MM patients had decreased hemoglobin levels (anemia). On the other hand, 66.7% of AML, and 50% of CML, ALL, and CLL patients had decreased platelet count (thrombocytopenia) (Table 3).

Discussion

Hematological malignancies are a group of neoplastic disorders that arise from a malignant transformation of cells of the bone marrow or the lymphatic systems.⁸ A multi-diagnostic approach should be used for the correct diagnosis of hematological malignancies.^{26,27} In this study, the overall prevalence of HM was 11.4% (26/228) in which CML was the dominant case accounted for 3.5% followed by AML (2.6%) then, CLL and MM (1.8%), and ALL and

undifferentiated acute leukemia (0.9%). This result was similar to the findings reported in Morocco (10.9%), and Ethiopia (9.3%).^{26,28} The current prevalence was low compared to the result reported in Eritrea (25.1%), in India (58%), and Togo (31%).^{29–31} The possible reasons for this variation might be the variations in sample size, study population, geographical location, and socio-demographic pattern.

In this study, the finding showed that 38.5% of HM cases were AL out of confirmed cases. This was consistent with the findings reported in India, Saudi Arabia, and Bangladesh.^{32–34} Acute leukemia is a malignant clonal disorder of blood-forming organs involving one or more cell lines in the hematopoietic system characterized by diffuse replacement of bone marrow with abnormal immature and undifferentiated hematopoietic cells (blasts).^{35–37} In the current study, the result showed that 46.2% of HM was chronic leukemia. This result was in line with the findings reported in India (49.5%) and in Togo (46.7%)^{31,32} while low findings were reported in India (28.5%) and Bangladesh (18.2%) compared to the present finding.^{9,30} Chronic leukemia is a broad spectrum of diseases characterized by uncontrolled proliferation and expansion of mature, differentiated cells of the hematopoietic system.^{15,38,39} Furthermore, multiple myeloma (MM) was the lowest case accounted 15.4% out of confirmed HM cases. This result was consistent with the finding in Morocco (12.4%)²⁶ whereas the present result was higher in comparison with other findings done in Bangladesh (10.5%) and Pakistan (9.7%).^{9,40} Multiple myeloma (MM) is a clonal plasma cell neoplastic disorder characterized by proliferation of neoplastic monoclonal plasma cells in the bone marrow that impede hematopoiesis, causes the activation of osteoclasts, suppression of osteoblasts, resulting in bone loss, and triggers production and release of monoclonal antibodies in serum and/or urine of the patient.^{10,41,42}

In this study, the finding showed that 30.8% of cases of acute leukemia (AL) were found within the younger age range between 15 and 35 years, whereas 38.5% cases of chronic leukemia (CL) were found within the adult age range between 36 and 55 years. However, 7.7% of cases of multiple myeloma (MM) were found within the younger age range whereas 7.7% cases of MM were found within the adult age range. This result was in line with the findings reported in Eritrea and Morocco where the higher cases of AL were found in younger age, CL cases in the middle adult age and MM cases in the adult and older age group, in Bangladesh in which AML (28.3%) was dominant within a median age of 35 years and CML (18.2%) was found within the median age of 40 years.^{9,26,29}

In this study, 76.9% of hematological malignant cases were found in male patients as compared to females (3.3:1 ratio). This was in concurrent findings reported in Bangladesh in which HM was dominant in male patients with a male to female ratio of 2.2:1, and leukemia was more prevalent in male patients and male to female ratio was 1.8:1, in India male preponderance with a male to female ratio of 2.3:1, in Pakistan HM is more common in male with the ratio of 2.5:1.^{9,30,40,43} The possible justification might be that male individuals are comparatively more exposed to the occupational and environmental carcinogenic agents which are at higher risk to the incidence of all kinds of leukemia.⁴⁴

In the current study, the myeloid type of HM was dominant and accounted for 53.8% of cases and CML accounted for 30.8% of the cases whereas AML accounted for 23% out of the confirmed cases. This was in line with the findings done in Togo and Bangladesh.^{9,31} Interestingly, AML is fast-growing leukemia characterized by clonal expansion of immature blast cells in the peripheral blood and bone marrow resulting in ineffective erythropoiesis and bone marrow failure. Furthermore, CML is a slow-growing clonal myeloproliferative disorder characterized by the presence of all stages of myeloid development in the peripheral blood believed to be driven by the aberrant protein tyrosine kinase.^{45–47}

Moreover, the lymphoid type of HM was accounted for 38.5% of cases in which CLL accounted for 15.4% followed by MM (15.4%), and ALL accounted for 7.7% out of the confirmed cases. This was in line with the findings done in Bangladesh and Nigeria.^{9,48} Moreover, ALL is a malignant transformation and proliferation of lymphoid progenitor cells characterized by uncontrolled proliferation of abnormal, immature lymphocytes and their progenitors of B and T-lymphocytes. Besides, CLL is an indolent lymphoproliferative disorder manifested by the progressive accumulation of mainly B-lymphocyte in the blood, bone marrow, and lymphatic tissues.^{38,49,50}

In this study, 57.0% study participants had non-malignant hematological disorders. Regarding non-malignant hematological disorders, 56 (24.6%) cases were erythroid hyperplasia, 24 (10.5%) cases aplastic anemia, 20 (8.8%) cases concomitant IDA and MBA, 16 (7.0%) cases MBA, 8 (3.5%) cases leukemoid reaction, 4 (1.8%) cases IDA, and 2 (0.9%) cases visceral leishmaniasis. Concomitant IDA and MBA were dominant and accounted for 8.8% of cases, whereas MBA was dominant and accounted for 5.3% of cases in female patients. The study conducted in Bangladesh

showed that 40.8% cases were non-malignant conditions and the common non-malignant hematological conditions diagnosed in the area were aplastic anemia (15.1%).²⁴

In studies done in Pakistan (10.9%) and India (30%) of the patients had MBA which was higher than the current finding.^{2,51} The different blood cells isolated from patients with MBA are large oval erythrocytes, hyper segmented neutrophils, and abnormal giant platelets.⁵² The causes for MBA might be associated with the deficiencies of vitamin B₁₂, folic acid, nutritional inadequacy, malabsorption, increased demands or utilization of the vitamins, chronic diarrhea, and worm infection.^{53,54} Furthermore, in the studies done in Pakistan, 5.9% and 9.4% of the patients had erythroid hyperplasia.^{24,55} The reason for erythroid hyperplasia may be due to a response to peripheral red blood cell loss or destruction and/or ineffective erythropoiesis associated with hemorrhage, hemolytic anemia, anemia of chronic disease, intrinsic bone marrow disease including aplastic anemia and malignant neoplasms.^{55–57} Moreover, in studies done in Pakistan and India 5.3% and 6.2% of the patients had aplastic anemia, respectively.^{2,58} In studies done in Pakistan, 3.3% and 5.7% and in India 3.2% of the patients had IDA whereas 2.7% and 7.9% had mixed IDA and MBA.^{2,55,59}

In this study, the finding showed that leucocytosis, anemia, and thrombocytopenia were common in confirmed HM patients. Among patients with HM, 66.7% of AML, 100% of CML and CLL, 75% MM patients had high total WBC count (leucocytosis) whereas 66.7% of AML, 62.5% of CML, 75% of CLL, and 50% of MM patients had low hemoglobin level (anemia). In addition, 66.7% of AML, and 50% of CML, ALL, and CLL patients had low platelet count (thrombocytopenia). This was in line with the findings reported in Kenya in which patients with CML had low platelet count and higher bleeding tendency.⁶⁰ On the other hand, a study done in Brazil showed that a high proportion of patients with AML had a high total WBC count and low platelet count⁶¹ and in Sudan high absolute lymphocyte count and thrombocytopenia (39.1%) were reported in CLL patients.⁶² Furthermore, in Pakistan, 80.1% and 96.3 % of AML patients had leucocytosis and thrombocytopenia.⁶³ Another study done in Pakistan showed that total WBC count was high in 52%, 66.6%, 87.5%, and 66.6% of ALL, AML, CML, and CLL cases, respectively, and low hemoglobin level was reported in 82%, 97.4%, 87.5%, and 100% of ALL, AML, CML and CLL cases whereas low platelet count was found in 88%, 92.3%, and 58% ALL, AML and CLL cases, respectively.⁶⁴

Conclusion

The overall prevalence of hematological malignancies in the study was 11.4% and 8.8% of cases were diagnosed in male patients. Generally, the prevalence of hematological malignancies diagnosed were 3.5% of CML, 2.6% of AML, 1.8% of CLL and MM, and 0.9% of ALL and undifferentiated acute leukemia. On the other hand, 57.0% of study participants had non-malignant hematological disorders which includes 56 (24.6%) cases were erythroid hyperplasia, 24 (10.5%) cases aplastic anemia, 20 (8.8%) cases concomitant IDA and MBA, 16 (7.0%) cases MBA, 8 (3.5%) cases leukemoid reaction, 4(1.8%) cases IDA, and 2 (0.9%) cases visceral leishmaniasis. Concomitant IDA and MBA were dominant and accounted for 8.8% of cases followed by MBA accounted for 5.3% of cases in female patients. Therefore, in patients with hematological abnormalities and where conclusive diagnosis could not be made through clinical and other laboratory investigations, bone marrow examination should be done for definitive diagnosis, management and prognosis.

Abbreviations

AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BME, bone marrow examination; CBC, complete blood count; CL, chronic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; FAB, French American British; FNAC, fine-needle aspiration cytology; IDA, iron deficiency anemia; HM, hematological malignancies; MBA, megaloblastic anemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; MPN, myeloproliferative neoplasms; PBM, peripheral blood morphology; RBC, red blood cell; SD, standard deviation; SOP, standard operating procedures; WBC, white blood cells; WHO, World Health Organization.

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 Research and Ethics Review Committee of the College of Medicine and Health Sciences, Wollo University. A support letter was secured from Dessie zonal health office and a permission letter was obtained from the chief executive officer of the diagnostic medical laboratory. Moreover, written informed consent was taken from each study participant before conducting the investigation. This study was conducted in accordance with the declaration of Helsinki.

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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.

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The authors declare that they have no conflicts of interest for this work.

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Effects of Iron Deficiency and Its Indicators on Lymphocyte Subsets: A Study at King Fahd Hospital of the University, Saudi Arabia

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Background: Iron deficiency plays an important role in our body's immunity. Several studies have shown that it is frequently associated with infections.

Objective: This study aimed to discover the effect of iron deficiency on immunity by demonstrating changes occurring in lymphocyte subsets among patients with an established diagnosis of iron deficiency.

Methods: A total of 64 iron-deficient patients and 19 healthy controls were included. Complete blood counts, serum iron, ferritin, and total iron-binding capacity were assessed. Lymphocyte subsets were evaluated by flow cytometry.

Results: Among iron-deficient patients, the anemic ones (Hb ≤ 11 g/dL) showed significantly lower absolute lymphocyte counts ($p=0.013$), lower relative and absolute NK-cell counts ($p=0.025$ and $p=0.003$, respectively), higher relative T-cell and CD4⁺-cell counts ($p=0.026$ and $p=0.002$, respectively). B cells and CD8⁺ T cells were not affected by any iron-deficiency indicators. Iron-deficient anemia patients showed a three- to fourfold increase in risk of having recurrent infections.

Conclusion: Iron deficiency has an obvious effect on lymphocyte subsets. Changes in lymphocyte subsets started mainly in response to decreased hemoglobin, rather than decreased ferritin and/or iron. Synchronously decreased hemoglobin and increased total iron-binding capacity led to absolute decreases in total lymphocytes, mainly NK cells, and relative increases in T cells, mainly the helper ones. Monitoring changes in lymphocyte subsets may be helpful in identifying patients at risk of recurrent infections.

Keywords: iron deficiency, lymphocyte subsets, immunity, flow cytometry

Introduction

Deficiencies in various vitamins and minerals can influence the human body by different mechanisms. Although iron is the most abundant metal on earth, iron deficiency is common clinical condition seen almost daily in clinical practice. It has impacts on both physical and mental development, as well as work performance, especially in children and premenopausal women. Beside its role in red blood-cell formation, iron plays an important role in our defense mechanism in different ways. Worldwide, several observational studies have shown that iron deficiency is frequently associated with increased susceptibility to infection.¹⁻³ Omara and Blakely showed that there was impaired lymphocyte proliferation in rats fed iron-depleted food compared to those fed a regular diet. They also showed that iron deficiency can cause thymic atrophy in animals.⁴ Sejas et al observed that children with iron-deficiency anemia had significantly lower lymphocyte counts than controls.⁵ Other studies have been done on pregnant females with iron-deficiency anemia and found decreased T-cell counts and CD4:CD8 ratios.^{6,7} There is a complex yet not fully understood relationship between iron status and immunofunction, and there have not been enough studies to indicate which indicator of iron deficiency (eg, ferritin level, hemoglobin level, serum-iron level, and total iron-binding capacity [TIBC]) initiates these changes in lymphocyte subsets.⁸ Also, a majority of studies have focused mainly on pediatric age-groups rather than adult cases. The objective of this study was to highlight and expand knowledge about the effect of iron deficiency on

immunity by demonstrating any changes that might occur in patterns of lymphocyte subsets among patients with an established diagnosis of iron-deficiency anemia and discover which indicator(s) initiate these.

Methods

This study was conducted at King Fahd University Hospital, Al-Khobar, Saudi Arabia from July 2018 to July 2019. All inpatients/outpatients of all nationalities presenting during this period and fulfilling our inclusion criteria for possible iron deficiency were included. The study was approved by the institutional review board (IRB-2019-047-Med) and research ethics committee of Imam Abdulrahman University, and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from both patients and controls. For participants aged <18 years, informed consent was obtained from a parent or legal guardian. All data were kept confidential and used for research purposes only.

Inclusion Criteria for Patients

All male and female patients aged >14 years clinically indicated and laboratory-proven to be iron-deficient (ie, with microcytic hypochromic red blood cells, MCV <80 fL, and serum ferritin <20 ng/mL) were included.^{9,10} All patients needed to be free of active ongoing infection or parasitic infestation. Patients had to have no history of autoimmune diseases, malignancies, or other nutritional deficiencies, immunosuppressant drug intake, receipt of radiotherapy or chemotherapy treatment, and no history of hematinic intake in the preceding 3 months. According to the World Health Organization definition of anemia (hemoglobin <12 g/dL for female and <13 g/dL for male patients). Accordingly, our iron-deficient patients were further subdivided into anemic and nonanemic groups where both male and female patients with hemoglobin ≤ 11 g/dL were considered anemic.

Inclusion Criteria for Controls

All apparently healthy asymptomatic persons aged 14–80 years were included as controls provided that their hemoglobin was >12 g/dL, MCV >80 fL, and serum ferritin >20 ng/mL. Also, they had to have no active ongoing infection and no history of hemolytic anemia, chronic diseases, infectious diseases, autoimmune diseases, immunosuppressant-drug intake, or hematinic intake in the preceding 3 months.

Exclusion Criteria for Patients

All patients with known or proven immunodeficiency, autoimmune diseases, chronic systemic illness, active infection or parasitic infestation, possible thalassemia traits, malignancies, protein–energy malnutrition, or other nutritional deficiencies are excluded. Those who had received IV iron or hematinic in the previous 3 months and those with a history of immunosuppressant-drug intake, chemotherapy, or radiotherapy were also excluded.

Blood Sampling

Venous blood samples were collected from patients and controls in EDTA tubes under aseptic conditions for processing. The samples were sent to the laboratory for complete blood counts, serum ferritin, serum iron, TIBC, and flow-cytometry analysis for lymphocyte subsets. All samples of flow cytometry were taken and analyzed within 24 hours of collection. Complete blood counts were performed using an automated blood counter (Beckman Coulter). Serum ferritin was measured using an Architect ferritin-assay kit with chemiluminescent microparticle–immunoassay technology. Other iron profile parameters (serum iron and TIBC) were measured with a Dimension analyzer using spectrophotometry. Lymphocyte-subset enumeration was done using flow-cytometry analysis with a four-color panel FACSCalibur with CellQuest or MultiSet software. Lymphocytes were gated as CD45-positive cells with low side-scatter. B and T lymphocytes were separated using CD3 and CD19 markers. T-helper and cytotoxic T cells were separated using CD4 and CD8, where cells that were CD3/CD4⁺ were considered T-helper cells and those that were CD3/CD8⁺ considered cytotoxic T cells. Natural killer (NK) lymphocytes were identified using CD3–CD16⁺ and/or CD56⁺ cells. Percentage counts were calculated using CD45/side-scatter gating in which lymphocytes were showed bright CD45 and very little side-scatter. Absolute counts were determined by comparing cellular events to bead events automatically.

Table 1 Demographic data

	Iron-deficient patients		Controls	
	n=64	%	n=19	%
Age, years				
14–19	6	9.4	0	0
20–29	14	21.9	1	5.3
30–39	21	32.8	13	68.4
40–49	17	26.6	2	10.5
50–59	4	6.3	3	15.8
>60	2	3.1	0	0
Sex				
Male	1	1.6	4	21.1
Female	63	98.4	15	78.9

Statistical Analysis

The dependent variable was lymphocyte subsets change and the independent variables age, sex, hemoglobin level, serum-ferritin level, serum iron, and TIBC. Data were checked, entered, and analyzed using SPSS 25. Continuous variables are presented as means $\pm$ SD. Categorical variables are presented as numbers and percentages. Unpaired *t*-tests and χ^2 tests were used to test for significant differences in hemoglobin level, MCV, platelet count, serum iron, TIBC, ferritin, and lymphocyte-subset indicators (changes) between the study groups. Logistic regression was used to estimate the risk of recurrent infection in relation to anemia and iron-deficiency indicators. All regression models were adjusted for sex and age. $p < 0.05$ was considered statistically significant.

Results

Demographic Data Analysis

A total of 64 patients with who presented to hematology and family medicine clinics in our hospital with confirmed iron deficiency met the inclusion criteria (Table 1). Mean age was 35.66 ± 12.11 (14–72) years. Patients were divided into anemic and nonanemic groups according to the World Health Organization definition of anemia (hemoglobin < 12 g/dL for female patients and 13 g/dL for male patients). In this study, both male and female patients with hemoglobin ≤ 11 g/dL were considered anemic: 44 (68.8%) met this criterion. Our controls comprised 19 apparently healthy adults fulfilling our inclusion criteria. The mean age of the controls was 38.21 ± 8.33 (25–52) years (Table 1).

Descriptive Analysis

Iron-deficient patients had significantly lower hemoglobin concentrations, MCV, MCH, serum ferritin, and serum iron than controls. On the other hand, iron-deficient patients had significantly higher TIBC than controls (Table 2). There was no statistically significant difference between patients and controls for platelet count ($p = 0.215$, Table 2).

No lymphocyte subsets showed statistically significant differences between iron-deficient patients (low ferritin) and controls (Table 3).

In Table 4, the studied lymphocyte-subset parameters in relation to hemoglobin levels among our iron-deficient patients are shown. Anemic patients tended to have significantly lower absolute lymphocyte counts than nonanemic ones. Also, there were statistically significant differences between anemic and nonanemic iron-deficient patients on relative T-cell counts, relative and absolute NK-cell counts and relative CD4-positive counts (Table 4). There were no statistically

Table 2 Hematological parameters

	Iron-deficient patients, n=64		Controls, n=19		p
	Mean	SD	Mean	SD	
Hb (g/dL)	9.69	1.87	12.34	2.18	<0.001
MCV (fL)	69.9	8.62	82.8	6.83	<0.001
MCH (pg)	22.13	3.56	27.32	2.95	<0.001
Serum iron (µg/dL)	32.34	27.07	59.05	41.71	0.001
TIBC (µg/dL)	405.13	106.94	332.84	73.15	0.007
Ferritin (ng/mL)	13.82	45.87	103.94	109.70	<0.001
Platelets (10 ³ /µL)	286.23	109.58	253.84	47.98	0.215

Note: Bold font indicates statistical significance.

Table 3 Lymphocyte-subset parameters

	Iron-deficient patients (low ferritin), n=64		Controls, n=19		p
	Mean	SD	Mean	SD	
WBCs (10 ³ /µL)	6.37	2.63	6.62	3.18	0.738
Lymphocyte count (10 ³ /µL)	3.205	11.61	2.757	10.21	0.133
T-cell %	74.32	7.46	75.05	4.94	0.690
T-cell absolute count (cells/µL)	1,439.07	483.38	1,352.97	455.17	0.497
B-cell %	13.67	5	12.99	5.599	0.617
B-cell absolute count (cells/µL)	271.06	141.37	236.24	124.20	0.336
NK-cell %	11.44	6.79	11.21	4.73	0.887
NK-cell absolute count (cells/µL)	227.36	166.94	201.45	100.45	0.523
CD4%	44.01	6.80	43.59	6.37	0.812
CD4 absolute count (cells/µL)	858.82	311.48	791.19	302.50	0.405
CD8%	29.56	7.47	30.08	6.69	0.788
CD8 absolute count (cells/µL)	572.43	232	535.71	214.15	0.540
CD4:CD8 ratio	1.62	0.60	1.57	0.69	0.770

significant differences between anemic and nonanemic iron-deficient patients on B-cell counts, CD8-positive cell counts, or CD4:CD8 ratio (Table 4).

Logistic Regression Results

Finally, risk based on history of recurrent infections (two or more severe infections in 1 year or a need for antibiotics for 2 months/year) in relation to iron-deficiency indicators was estimated by logistic regression (Table 5). The results showed that with Hb <11 g/dL the risk of having recurrent infections was significantly elevated (OR 2.55, CI 1.03–6.26;

Table 4 Lymphocyte-subset parameters in relation to hemoglobin level among iron-deficient patients

	Nonanemic (Hb >11 g/dL) iron-deficient patients, n=20		Anemic (Hb ≤11 g/dL) iron-deficient patients, n=44		p
	Mean	SD	Mean	SD	
WBCs (10 ³ /μL)	5.47	1.56	6.78	2.92	0.064
Absolute lymphocyte count (10 ³ /μL)	3.733	12.12	2.965	10.65	0.013
T-cell %	71.28	9.02	75.71	6.26	0.026
T-cell absolute count (cells/μL)	1,498.91	526.031	1,411.88	466.38	0.509
B-cell %	13.80	5.95	13.61	4.58	0.890
B-cell absolute count (cells/μL)	284.17	125.37	256.10	149.06	0.621
NK-cell %	14.24	9.26	10.17	4.94	0.025
NK-cell absolute count (cells/μL)	316.34	231.89	186.92	108.24	0.003
CD4%	40.18	7.15	45.75	5.93	0.002
CD4 absolute count (cells/μL)	830.96	268.01	871.48	331.49	0.633
CD8%	29.86	8.03	29.43	7.30	0.836
CD8 absolute count (cells/μL)	632.15	270.97	545.28	209.77	0.167
CD4:CD8 ratio	1.48	0.64	1.68	0.58	0.213

Note: Bold font indicates statistical significance.

Table 5 Risk based on history of recurrent infections in relation to iron-deficiency indicators on logistic regression

	History of recurrent infections		
	OR	CI	p
Hb (g/dL)	2.55	1.03–6.26	0.041
Serum ferritin (ng/mL)	3.70	1.26–10.83	0.01
Serum iron (μg/dL)	0.38	0.14–1.03	0.059
TIBC (μg/dL)	0.912	0.32–2.59	0.86

Notes: Bold font indicates statistical significance.

$p=0.041$), as well as for ferritin <20 ng/mL (OR 3.70, CI 1.26–10.83; $p=0.01$). However, there were no significantly elevated risks associated with history of recurrent infections with decreased serum-iron or increased TIBC levels.

Discussion

It is well known that women of reproductive age and children are more prone to iron-deficiency anemia. Iron is considered an important element not only for DNA synthesis and RBC formation but also for immune-system integrity.^{11–13} As we know, peripheral lymphocytes play an important role in the body's defenses against infections. The mechanism by which iron deficiency affects these lymphocytes is not well understood. To investigate the important role of iron deficiency and its effects on immunity, this study analyzed the distribution of lymphocyte subsets and their correlations with iron-deficiency indicators, ie, hemoglobin, serum ferritin, serum iron, and TIBC.

There were statistically significant differences in absolute lymphocyte, relative T-cell, relative CD4⁺, relative NK-cell, and absolute counts between anemic (Hb ≤ 11 g/dL) and nonanemic iron-deficient patients. Absolute lymphocyte counts in anemic patients were significantly lower than nonanemic ones ($p=0.013$). This is similar to the results of previous studies.^{14–17} Also, relative T-cell counts were significantly higher in anemic than nonanemic patients ($p=0.026$). This finding is consistent with the results of Sejas et al, Attia et al, and Berger et al.^{5,18,19} van Heerden et al discovered that T-cell proliferation was higher among iron-deficient patients.²⁰ This finding was confirmed by Attia et al, who found that there was an increase in immature T-cell marker CD1a in iron-deficient patients.¹⁹ In our results, relative CD4-positive cell counts showed statistically significant differences between anemic and nonanemic patients ($p=0.002$). Both relative NK-cell and absolute counts were significantly lower in anemic patients than nonanemic ones. To the best of our knowledge, no study has highlighted changes in NK cells in relation to Hb-level changes in humans. Sherman et al and Spear et al studied this relationship only in rats fed iron-depleted food. Both studies highlighted impairment of NK-cell function (cytotoxicity killing power), rather than count.^{21,22}

Differences in means of lymphocyte-subset parameter between patients and controls were not statistically significant. These results are consistent with Ekiz et al, Mohri et al, and Thibault et al who reported that no statistically significant differences in lymphocyte subsets between patients with low ferritin and controls.^{6,23,24} Our results showed no statistically significant difference in lymphocyte subsets between patients with low serum iron and patients with normal serum iron. There were no statistically significant differences between anemic and nonanemic iron-deficient patients in relative or absolute B-cell counts ($p=0.89$ and $p=0.621$, respectively). These results matched those of Beard et al, who concluded that B cells (arm of humoral immunity) seemed less likely to be affected by iron deficiency than T cells (arm of cell-mediated immunity).²⁵

In this study, it was quite obvious that the changes in lymphocyte subsets started mainly in response to decreased hemoglobin levels, rather than decreased ferritin and/or iron levels. The decreased hemoglobin led to absolute decreases in total lymphocytes and NK cells and a relative increase in CD4⁺ T-helper cells. Synchronously, increased TIBC levels leads to an absolute decrease in NK cells and relative increase in T cells. B cells were not affected by any of the iron-deficiency indicators. To our knowledge, no studies have highlighted the risk of recurrent infections in iron-deficiency anemia in adults. Therefore, it was interesting to investigate the relationships between the risk associated with a history of recurrent infections and iron-deficiency indicators in this study. Our results showed that there was a statistically significant increase in risk associated with a history of recurrent infections among iron-deficient patients with decreased Hb and/or ferritin levels. In addition, upper respiratory tract and urinary tract infections were the most common infections reported by the iron-deficient patients.

Recommendations

1. Future studies with larger samples and higher proportion of male sex would be more informative.
2. Studying the effect of iron deficiency on lymphocyte subsets in pediatric age-groups would be extremely beneficial.
3. For all iron-deficiency anemia patients, monitoring lymphocyte-subset changes may predict the risk of recurrent infections.
4. Further similar studies are recommended to explore changes in lymphocyte subsets in relation to anemia other than iron deficiency, particularly for those patients with anemia of chronic diseases.

Conclusion

Iron deficiency has an obvious effect on lymphocyte subsets. Among iron-deficiency indicators, decreased hemoglobin level seems to be the driving force that initiates changes in lymphocyte subsets, rather than decreased ferritin and/or iron levels. Generally, synchronously decreased hemoglobin levels led to absolute decreases in total lymphocytes, mainly NK cells, and relative increases in T cells, mainly the helper ones. Meanwhile, B cells were not affected by any of the iron-deficiency indicators. Furthermore, there was a three- to fourfold increase in risk of recurrent infections among patients with iron-deficiency anemia.

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Disclosure

The authors report no conflicts of interest in this work.

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Bone Marrow Suppression Secondary to the COVID-19 Booster Vaccine: A Case Report

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Abstract: As of September 2021, SARS-CoV-2 booster shots became widely available in the US to ensure continued protection against the virus. A temporal relationship has been previously reported between the first or second dose of the COVID-19 vaccine and the development of thrombocytopenia. However, adverse events related to the third COVID-19 vaccine are still being reported and studied. We report a 74-year-old male who developed bone marrow suppression and pancytopenia recorded seven days after receiving the Pfizer SARS-CoV-2 vaccine. During his hospital stay, the patient's hemoglobin, white blood cell, and platelet levels continued to trend downwards. However, all three levels showed improvement one week after discharge without robust intervention. Global vaccination is of utmost importance, as is understanding and documenting post-vaccination reactions including bone marrow suppression. Prompt evaluation and patient education are imperative to improve patient outcomes and combat hesitancy against vaccine administration.

Keywords: COVID, pancytopenia, vaccination, Pfizer, bone marrow suppression

Introduction

Since its emergence in December of 2019, the rapid spread of severe acute respiratory syndrome coronavirus (SARS-CoV-2) has quickly affected millions of lives across every continent.¹ This highly transmittable and pathogenic viral infection has led to massive mitigation efforts and allocation of resources to prevent the spread of transmission and high mortality related to complications.² The establishment of higher levels of community (herd) immunity and protection against SARS-CoV-2 via the widespread deployment of effective vaccines has become a global effort.³ In December of 2020, the FDA issued an Emergency use Authorization for the Pfizer-BioNTech and Moderna COVID-19 Vaccine as a two-dose series.⁴ In September 2021, booster vaccines became widely administered in the US due to waning immunity of the COVID-19 vaccines against variants of SARS-CoV-2 along with ensuring continued protection against the virus.⁵

Serious adverse events such as anaphylaxis, Guillain-Barre Syndrome, myocarditis, pericarditis, thrombocytopenia, and death have been previously reported following the first and/or second dose of vaccine.⁶ To our knowledge, no cases have been reported regarding bone marrow suppression related to the third COVID-19 vaccine. Adverse events reported between August 12-September 19, 2021 from the COVID-19 booster vaccine supported similar reactions to those after dose two.⁷ According to the Centers for Disease Control and Prevention (CDC), these initial findings indicate no unexpected patterns of adverse reactions after an additional dose of COVID-19 vaccination.⁷ However, adverse events related to the COVID-19 booster are still being reported and studied.⁶ This report presents a case of bone marrow suppression occurring after the third COVID-19 vaccine without a similar reaction after the first or second dose.

Case Report

A 74-year-old male with a history of polychondritis and hypothyroidism presented to the hospital after falling out of his chair and inability to ambulate. The patient was found to be mildly confused upon arrival to the emergency room, limiting our ability to obtain a full verbal history. Chart review revealed the patient had received his third Pfizer Covid

vaccine shot seven days before admission followed by fatigue, decreased appetite, fever, and chills. The patient had received the second Pfizer Covid-19 shot nine months before the booster. No reactions to the previous two shots were noted.

Upon initial evaluation, vital signs were within normal limits and a physical exam revealed significant tenderness in the upper arm and no gross bleeding (Figure 1). Computed tomography (CT) imaging (Figure 2) was significant for enhancement of the left axillary lymph node. The patient's initial complete blood count (CBC) was remarkable for a hemoglobin count of 9.9 g/dl and platelet count of $84 \times 10^9/L$; both values lower than his prior hemoglobin count of 13.7 g/dl and platelet count of $180 \times 10^9/L$ from December of 2020. His mean corpuscular volume (MCV) was elevated at 101.3 femtolitres from his prior MCV value of 95.8 femtolitres in December of 2020. His white blood cell (WBC) count was recorded at $7.6 \times 10^9/L$.



Figure 1 The patient's upper arm showed erythema with no gross bleeding near the injection site

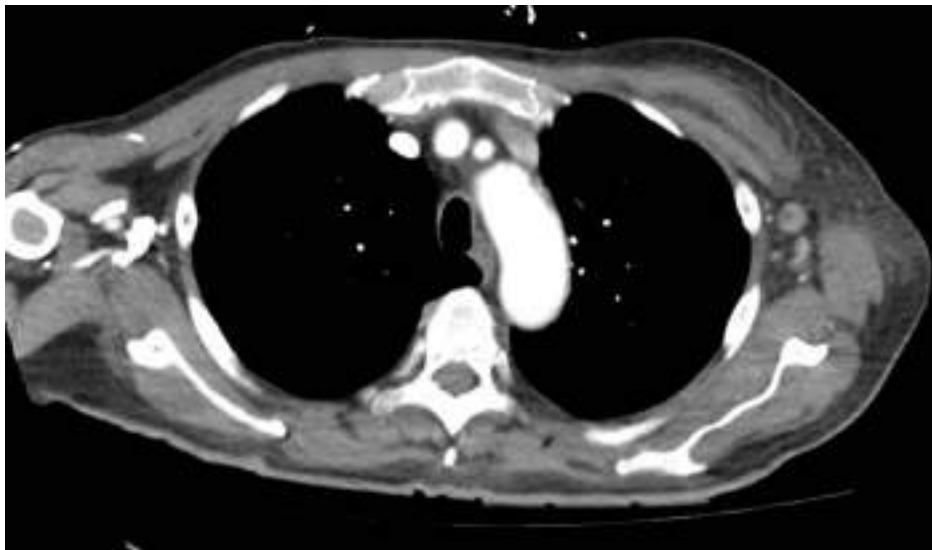


Figure 2 The patient's CT imaging of the thoracic region showed enhancement of the left axillary lymph node.

The hemoglobin, WBC, and platelet count further down trended from his baseline (Figures 3–5). Anemia labs including ferritin levels (554 ng/mL), vitamin B12 (253 pg/mL), total bilirubin (0.5 mg/dL), and reticulocyte count (0.8%) were nonsignificant during the patient's hospital stay. The patient's left shoulder presented with extensive bruising, erythema, papular rash, warmth, and tenderness on palpation during the hospitalization. An improvement in WBC and platelet levels was noted on day 4 of hospitalization.

Before discharge, the patient was fully alert and oriented and reported improvement in his symptoms. Examination of his lateral left arm showed decreased erythema and bruising with slight petechiae. The patient was discharged due to stabilization of labs and encouraged to take oral vitamin B12 supplements. During his outpatient follow-up six days after hospitalization, his hemoglobin increased to 10.5 g/dl, WBC count increased to $4.9 \times 10^9/L$, and platelets increased to $101 \times 10^9/L$.

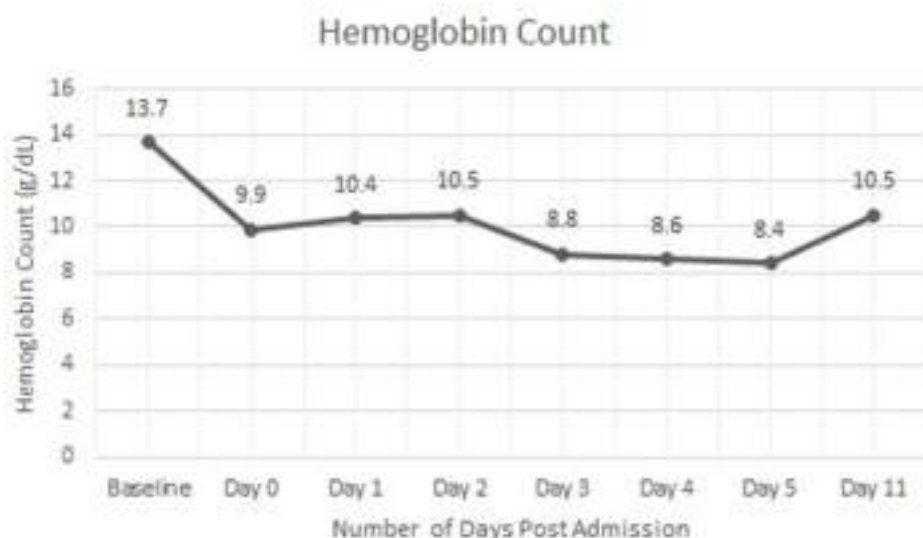


Figure 3 The patient's hemoglobin count throughout his hospital course and 6 days after discharge.

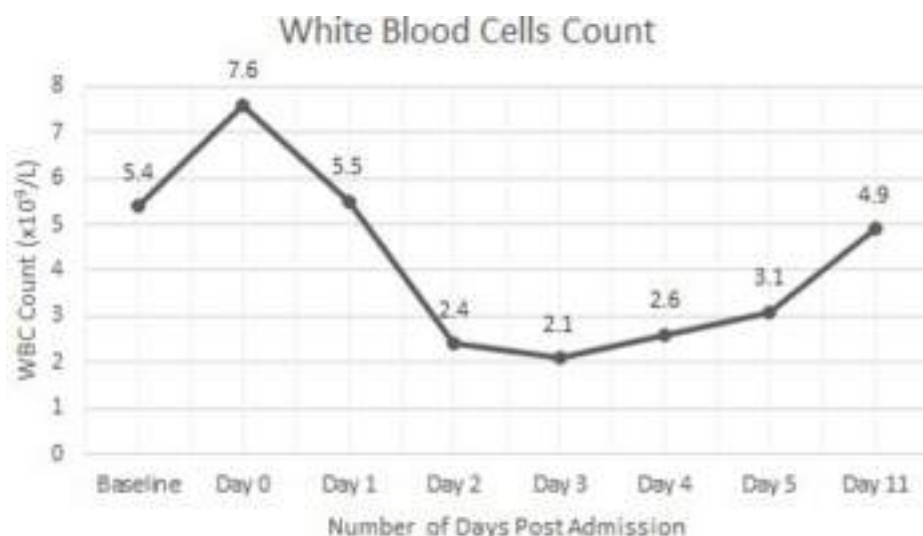


Figure 4 The patient's WBC count throughout his hospital course and 6 days after discharge.

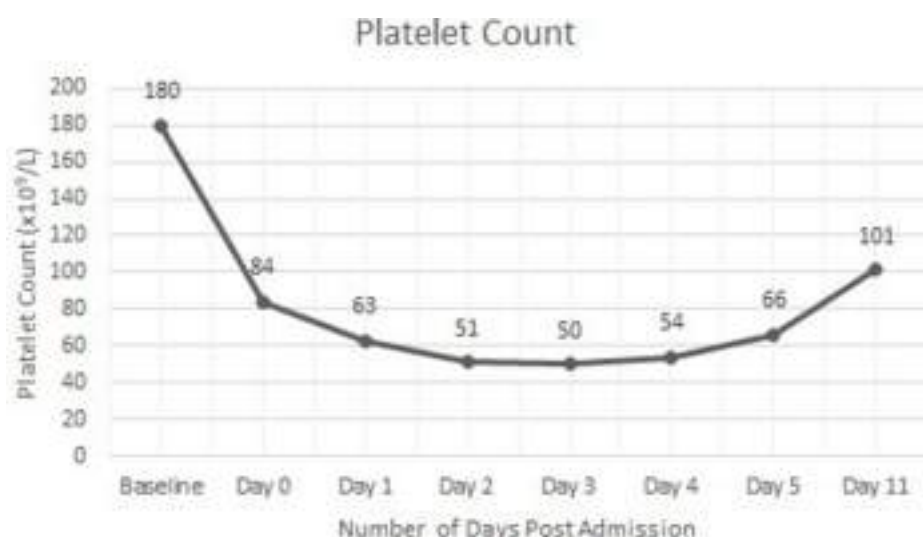


Figure 5 The patient's platelet count throughout his hospital course and 6 days after discharge.

Discussion

This paper presents a patient with pancytopenia recorded seven days after receiving the Pfizer booster vaccine. Interestingly, this patient did not report any reactions after the first or second dose of the Pfizer vaccine against SARS-CoV-2. Pancytopenia refers to a decrease in all peripheral bloodlines and is present when all three cell lines are below the normal reference range.⁸ The patient's physical exam showed no signs of active bleeding along with his labs indicating no evidence of hemolysis. The patient's hemoglobin, platelet, and white blood cell count presented below baseline followed by a decrease and slight improvement during his hospital stay. Six days after hospitalization, all three cell lines showed improvement. The temporal association with the booster vaccine and negative infectious disease workup raised suspicion for vaccine-induced bone marrow suppression. In addition, the patient's reticulocyte count and lactate dehydrogenase value were consistent with hypoproliferation within the bone marrow.

Currently, there is a gap in knowledge of adverse events specific to the third vaccine against SARS-CoV-2 due to the recent initiation of administration and ongoing reporting of events.⁶ To our knowledge, bone marrow suppression after

any dose of vaccine against SARS-CoV-2 has not been previously reported. However, a prior case of pancytopenia after the third vaccination with a recombinant hepatitis B vaccine has previously been reported.⁹ The patient's bone marrow biopsy within this case displayed a paucity of late myeloid elements and CD8+ T cells.⁹ It was believed the patient's CD8+ T cells were causing excessive production of IFN- γ ; a stimulant of negative regulators of hematopoiesis such as tumor necrosis factor and lymphotoxin.¹⁰ IFN- γ has also previously been reported to create immunological effects comprising an upregulation of histocompatibility gene transcription and alteration in class I and II antigen expression at the cell surface.¹¹ It was predicted these changes resulted in an autoimmune reaction causing suppression of maturation of hematopoietic progenitor cells and pancytopenia.⁹ Via a similar mechanism, we believe that our patient's pancytopenia was immune-mediated, potentially triggered by the vaccination.

Vaccines against SARS-CoV-2 (first or second dose) and the induction of Idiopathic Thrombocytopenic Purpura (ITP) have also been recently acknowledged in multiple cases.¹² Our patient presented with low platelet levels and associated petechiae and purpura at the site of the vaccination. However, the patient's presentation of low hemoglobin and white blood cells along with normal reticulocyte levels was more indicative of pancytopenia secondary to bone marrow suppression. In patients presenting with pancytopenia, the history and the physical exam should help assess the severity of the pancytopenia and comorbid illnesses that may complicate the disorder.¹³ In addition, suspicious medications and exposure to toxic agents should be ruled out.¹³ Initial screening laboratory evaluation should include the patient's complete blood count, peripheral blood smear examination, reticulocyte count, complete metabolic panel, prothrombin time/partial thromboplastin time, and blood type and screen. Common interventions to alleviate bone marrow suppression and pancytopenia include treating the underlying cause and utilizing supplements to boost red blood cell production if indicated.

Vaccines against SARS-CoV-2 undergo continuous safety monitoring; adverse events are very rare.¹⁴ However, vaccine hesitancy remains a barrier towards full population inoculation against SARS-CoV-2 and is influenced by misinformation regarding vaccine safety.¹⁵ One study using an anonymous online questionnaire found a person's trust in the effectiveness of the vaccine was a major facilitative factor affecting willingness to vaccinate.¹⁶ The same study also found that 66.7% of unvaccinated participants thought the vaccine's safety was not enough, making it the main reason for reluctance or hesitance to be vaccinated.¹⁶ Therefore, education of adverse events and available interventions post-vaccination is imperative to prevent the spread of misinformation and combat hesitancy towards vaccination.¹⁵

As of September 19, 2021, about 2.2 million people in the United States received a third vaccine against SARS-CoV-2.¹⁷ Among those who received the vaccine, 22,000 people reported the effects of the vaccine with no unexpected patterns of adverse reactions.¹⁷ Our patient demonstrates abnormal pancytopenia first recorded seven days after receiving the booster vaccine, possibly indicating a rare adverse event from the vaccination given the temporal relationship. While additional studies and observations are indicated to verify bone marrow suppression as an adverse reaction, this case report provides an opportunity for patient education and treatment planning before symptoms arise.

Conclusion

Our case report highlights pancytopenia secondary to bone marrow suppression following Pfizer vaccination against SARS-CoV-2. It is important to consider the possibility of bone marrow suppression following the third vaccine against SARS-CoV-2. Although additional studies are indicated to determine the risk factors and pathogenesis of vaccine-induced bone marrow suppression, prompt evaluation and initiation of interventions can improve patient outcomes.

Consent for Publication

Institutional approval was not required to publish the case details. The publication of this study has been consented to by the patient.

Disclosure

The authors report no conflicts of interest in this work.

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Total Knee Arthroplasty: Superiority of Intra-Articular Tranexamic Acid Over Intravenous and Cell Salvage as Blood Sparing Strategy – A Retrospective Study

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Total Knee Arthroplasty: Superiority of Intra-Articular Tranexamic Acid Over Intravenous and Cell Salvage as Blood Sparing Strategy – A Retrospective Study

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Purpose: Total knee arthroplasty is associated with considerable perioperative hemorrhage. The decrease in hemoglobin concentration and the need for allogenic blood transfusion are related to increased morbidity and mortality. Strategies for minimizing perioperative bleeding are used, such as tranexamic acid and cell salvage. The study aimed to compare intravenous, intra-articular tranexamic acid and cell salvage protocols regarding perioperative hemoglobin variation. Secondary outcomes included blood loss; allogenic transfusions; complications and in-hospital stay.

Patients and Methods: Patients submitted to unilateral total knee arthroplasty between January and December 2018 were retrospectively evaluated. After excluding 62 patients, 204 were subdivided into 3 groups according to the protocol used. Statistical analysis was performed with SPSS version 26.0. One-way ANOVA and Kruskal–Wallis tests were used. Considered a p-value of <0.05 for statistical significance.

Results: Variation of hemoglobin in the intra-articular tranexamic acid group was significantly lower than that of intravenous ($p < 0.001$) and cell salvage ($p = 0.001$) groups. Blood loss, variation of hematocrit, need for blood transfusion and in-hospital stay were also statistically significantly lower in the intra-articular tranexamic acid group. The only related complications were in the intravenous tranexamic acid group. No thromboembolic complications were identified which further solidifies the safety of tranexamic acid administration.

Conclusion: This data shows superiority of the intra-articular administration of tranexamic acid over the other techniques in total knee arthroplasty. We propose this protocol as an efficient, low-risk blood-sparing strategy.

Keywords: arthroplasty, knee, acid tranexamic, salvage, transfusion

Introduction

Total knee arthroplasty (TKA) is the procedure of choice for the treatment of degenerative joint disease of the knee and is associated with considerable perioperative hemorrhage.¹ Decrease in postoperative hemoglobin (Hb) concentration (<7 g/dL or $>50\%$ of baseline) is associated with ischemic events and increased morbidity and mortality.² Previously, more than 20% of TKA required transfusion of blood products in the postoperative period.^{1,3} With the improvement of surgical technique, the transfusion rate is currently 1–12%.^{4,5} However, allogenic blood transfusion is associated with allergic reactions,⁶ increased risk of infection of the surgical incision^{7,8} or prosthetic joint infection,⁸ transmission of infectious diseases,^{6,7} immune modulation/suppression,⁹ and increased hospitalization time, morbidity, mortality and costs.^{6–8,10} Strategies focused on minimizing perioperative hemorrhage and the need for transfusion (controlled hypotension, self-transfusion, tourniquet, antifibrinolytics and cell salvage) have been used in orthopedic surgery.¹⁰

Tranexamic acid (TXA) is a synthetic lysine analogue that acts as an inhibitor of fibrin degradation through plasminogen.¹¹ It can be administered orally, intravenously or topically.¹¹ It decreases hemorrhage in the perioperative

Graphical Abstract



period and transfusion rates in cardiac,^{11,12} pelvic,^{11,13} spinal^{11,14} and orthopedic surgery.⁷ However, studies differ in doses, methods and routes of administration, use of tourniquet and cut-off values for transfusion. Cell salvage consists of filtering and reinfusion of the blood losses collected by surgical drains.¹⁵ Currently, there is no consensus on whether it reduces the transfusion need in TKA.^{15,16}

At our institution, we use protocols for either intravenous TXA (TXA-iv), intra-articular TXA (TXA-art), or cell salvage usage in TKA. The primary outcome of our study is the comparison of these 3 methods in terms of perioperative Hb variation. Secondary outcomes are the comparison of drainage blood losses; number of allogenic transfusions; variation in hematocrit (Htc) and platelets (PLT) values; complications and duration of hospital stay.

Materials and Methods

After obtaining approval from the Institutional Ethics Committee, the medical records of patients undergoing TKA between January and December 2018 were retrospectively evaluated. We analyzed both computer and physical registries to collect clinical and demographic data, perioperative laboratory results and records on postoperative complications. A total of 266 patients were identified, all classified as ASA 2 or 3, submitted to unilateral TKA. We excluded a total of 62 cases, resulting in 204 admitted patients subdivided into 3 groups according to the transfusion-sparing technique used: 74 patients in the Cell salvage group, 53 in the TXA-iv group and 77 in the TXA-art group (Figure 1). Exclusion criteria involved previous surgery on the same limb; incomplete registries; pre-operative hemoglobin <10 g/dL; and history of ischemic cardiac disease.

- Cell salvage protocol: the contents of the surgical drain were collected and filtered in the CellTransTM system. Re-administration occurred during the 24-hour post-operative period.

- TXA-iv protocol: 7.5 mg/kg of TXA diluted in 100 mL of NaCl 0.9%. The full dose was slowly administered twice: firstly, 15 minutes before inflating the tourniquet and, secondly, immediately before deflation (total: 15 mg/kg in 200mL of NaCl 0.9%).

- TXA-art protocol: after tourniquet deflation, the surgeon injected 1500 mg of TXA diluted in 20 mL of NaCl 0.9% through the surgical drain, which remained clamped for 30 minutes.

All patients included were operated by different surgeons of the same Orthopedic Department, using the same technique and protocols. All procedures were performed under tourniquet. A classic medial parapatellar approach and a conventional mechanical alignment technique were used in all patients. The same primary prosthesis was applied to all patients. The drainage in the postoperative period was recorded during 24 hours by the nursing team.

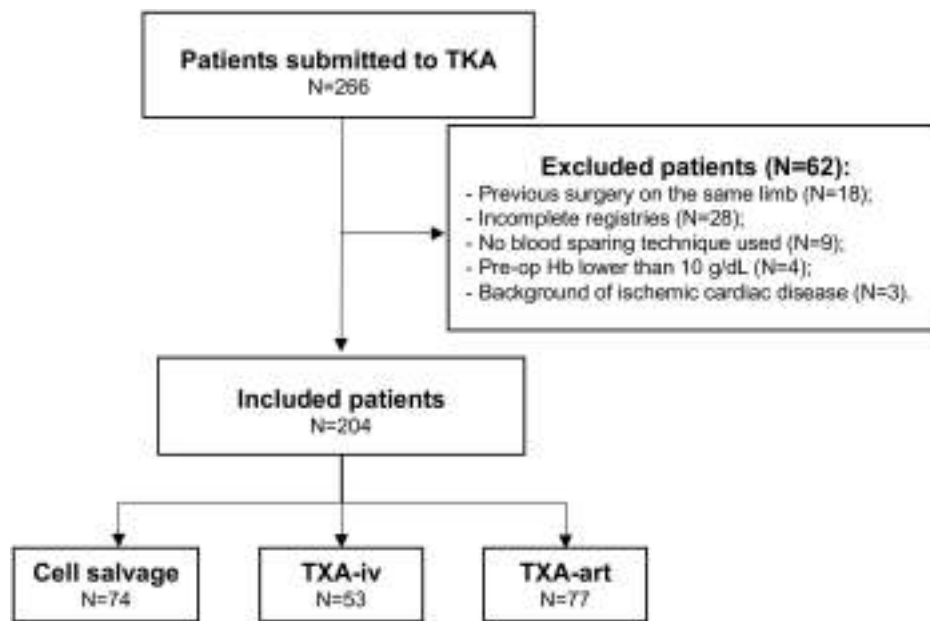


Figure 1 Exclusion criteria and subdivision of patients included in the study.

Blood sampling for complete blood count (CBC) was drawn 24 hours post operatively.

Our hospital uses a Hb transfusion threshold between 8 and 10 g/dL.

The same immediate post-operative care and rehabilitation was applied to all patients included. This consisted of a Fast-Track Protocol which included: passive assisted range-of-movement isometric exercises at 8h to 12h after surgery, followed by active range of movement exercises and walking-assisted gait. Patients were discharged at 3 to 4 days after surgery depending on in-patient rehabilitation results and absence of complications.

Statistical analysis was performed with SPSS version 26.0. The normality of variables was tested with Kolmogorov–Smirnov. For variables with normal distribution, we used the One-Way ANOVA test and the Bonferroni or Games–Howell post-hoc tests, according to homogeneity of variances. For variables without normal distribution, we used the Kruskal–Wallis test. We considered statistical significance with p-value of <0.05.

Results

We started by analyzing the baseline differences between the 3 study groups (Table 1) and did not find statistically significant differences between the means of age ($p = 0.757$), gender ($p = 0.184$), BMI ($p = 0.219$), preoperative Hb ($p = 0.522$), preoperative Htc ($p = 0.512$) and preoperative PLT count ($p = 0.301$). About 24% of patients had preoperative anemia, defined as Hb <13 g/dL. No difference was found between the timing of CBC sampling ($p = 0.588$).

Regarding the primary outcome (Table 2), we found that the mean Hb variation (vHb) of TXA-art was significantly lower than that of TXA-iv ($p < 0.001$) and Cell salvage ($p = 0.001$). On the other hand, the mean vHb in Cell salvage and TXA-iv groups were not significantly different ($p = 0.988$). Postoperative anemia was found in 91% of patients.

Regarding secondary outcomes (Table 2), hematic losses in the TXA-art group in the first postoperative 24 hours were significantly lower than those in the TXA-iv ($p < 0.001$) and Cell salvage ($p < 0.001$) groups. We did not find any difference between the TXA-iv and Cell salvage groups ($p = 0.150$) for this variable. A total of 39 blood units were transfused in 25 patients (23 blood units in the cell salvage group, 14 blood units in the TXA-iv group and 2 blood units in the TXA-art group). The number of transfusions administered did not differ when comparing TXA-art with TXA-iv ($p = 0.724$) groups. TXA-art did have fewer transfusion needs than Cell Trans ($p = 0.044$), as did TXA-iv ($p = 0.009$). The mean variation of Htc (vHtc) in the TXA-art group was significantly lower than that in the TXA-iv ($p < 0.001$) and Cell salvage groups ($p = 0.030$). On the contrary, the vHtc of the Cell salvage and TXA-iv groups were not significantly different ($p = 0.919$).

Table I Demographic Characterization and Comparison Between the Cell Salvage, TXA-iv and TXA-art Groups

	Total (N = 204)	Cell Salvage (N = 74)	TXA-iv (N = 53)	TXA-art (N = 77)	p value
Age (years) (mean ± SD)	70.0 ± 6.5	70.4 ± 6.1	70.1 ± 7.0		1.000
			70.1 ± 7.0	69.6 ± 6.6	1.000
		70.4 ± 6.1		69.6 ± 6.6	1.000
BMI (kg/m ²) (mean ± SD)	26.5 ± 3.3	26.0 ± 3.2	26.5 ± 3.6		1.000
			26.5 ± 3.6	27.0 ± 3.0	1.000
		26.0 ± 3.2		27.0 ± 3.0	0.246
Pre-op Hb (g/dL) (mean ± SD)	13.7 ± 1.1	13.6 ± 1.1	13.7 ± 1.0		1.000
			13.7 ± 1.0	13.8 ± 1.3	1.000
		13.6 ± 1.1		13.8 ± 1.3	0.805
Pre-op Htc (%) (mean ± SD)	41.3 ± 3.3	41.3 ± 3.1	41.0 ± 3.2		1.000
			41.0 ± 3.2	41.7 ± 3.6	0.760
		41.3 ± 3.1		41.7 ± 3.6	1.000
Pre-op PLT (10 ³ /μL) (mean ± SD)	219.9 ± 52.1	216.5 ± 48.6	214.3 ± 56.6		1.000
			214.3 ± 56.6	227.1 ± 52.0	0.509
		216.5 ± 48.6		227.1 ± 52.0	0.629
Post-op CBC timing (hours) (mean ± SD)	23.4 ± 2.5	23.7 ± 2.4	23.4 ± 2.2		1.000
			23.4 ± 2.2	23.2 ± 2.9	1.000
		23.7 ± 2.4		23.2 ± 2.9	0.943
Gender, masculine (%)	50 (25%)	23 (31%)	9 (17%)	18 (23%)	0.184
ASA 2 (%)	138 (68%)	45 (61%)	41 (77%)	52 (67%)	0.146
Pre-op anemia ^a (%)	49 (24%)	21 (28%)	9 (17%)	19 (24%)	NA

Note: ^aAnemia was defined as Hb < 13 g/dL.

Abbreviations: ASA, American Society of Anesthesiologists; BMI, body mass index; CBC, complete blood count; TXA-art, intra-articular tranexamic acid; TXA-iv, intravenous tranexamic acid; NA, non-applicable; SD, standard deviation; Hb, hemoglobin; Htc, hematocrit; PLT, platelets; Pre-op, preoperative.

The mean perioperative variation of PLT (vPLT) was not significantly different between the Cell salvage and TXA-iv groups ($p = 0.794$), nor between TXA-art and TXA-iv ($p = 0.792$). vPLT in the TXA-art group was significantly lower than in the Cell salvage group ($p = 0.044$).

The duration of in-hospital stay was significantly shorter in TXA-art group than in the Cell salvage ($p < 0.001$) and in TXA-iv groups ($p = 0.001$). We found no statistically significant difference between the duration of in-hospital stay in the TXA-iv and the cell salvage groups ($p = 0.853$). We found 2 complications in the TXA-iv group, namely seizures and infection of the surgical wound.

Discussion

We did not find any published studies that directly compared the three methods depicted here in TKA. The use of intra-articular injection of TXA was associated with less decrease in Hb in TKA compared to other two techniques. We established the same superiority regarding blood loss through drainage, vHtc and in-hospital stay time. Injection through the surgical drain allows better exposure to TXA in the surgical site, which may be the reason for decreased blood loss.¹⁷ The clamping of the drain further prolongs this contact, which is especially important in TKA in which the operative

Table 2 Comparison of Primary and Secondary Outcomes

	Total (N = 204)	Cell Salvage (N = 74)	TXA-iv (N = 53)	TXA-art (N = 77)	p value
vHb (g/dL) (mean ± SD)	2.6 ± 1.0	2.8 ± 0.9	2.9 ± 1.2		0.988
			2.9 ± 1.2	2.1 ± 0.8	0.001
		2.8 ± 0.9		2.1 ± 0.8	0.000
24 hr post-op drainage blood loss (mL) (mean ± SD)	574.7 ± 260.0	705.1 ± 262.1	591.5 ± 188.9		0.015
			591.5 ± 188.9	437.9 ± 233.3	0.000
		705.1 ± 262.1		437.9 ± 233.3	0.000
Total number of blood units transfused	39	23	14		0.009
			14	2	0.724
		23		2	0.044
vHtc (%) (mean ± S)	7.7 ± 3.3	8.5 ± 2.7	8.2 ± 4.2		0.919
			8.2 ± 4.2	6.5 ± 2.7	0.030
		8.5 ± 2.7		6.5 ± 2.7	0.000
vPLT (10 ³ /μL) (mean ± SD)	47.6 ± 29.4	53.6 ± 30.5	47.7 ± 30.3		0.794
			47.7 ± 30.3	41.9 ± 26.7	0.792
		53.6 ± 30.5		41.9 ± 26.7	0.044
In-hospital stay (days) (Median /IQR)	5.0 /1.0	6.0 /2.0	6.0 /2.0		0.853
			6.0 /2.0	5.0 /1.0	0.001
		6.0 /2.0		5.0 /1.0	0.000
Complications	2	0	2	0	NA
Post-op anemia [†] (%)	186 (91%)	71 (96%)	51 (96%)	64 (83%)	NA

Note: [†]Anemia was defined as Hb < 13 g/dL.

Abbreviations: TXA-art, intra-articular tranexamic acid; TXA-iv, intravenous tranexamic acid; IQR, interquartile range; NA, non-applicable; SD, standard deviation; vHb, hemoglobin variation; vHtc, hematocrit variation; vPLT, platelets variation; Post-op, postoperative.

tourniquet makes the blood loss more evident in the postoperative period.^{18,19} Recent studies have analyzed the efficacy of the topical/intra-articular route and found a significant decrease in hemorrhage when compared to placebo,²⁰ but not when compared to its intravenous administration.^{17,20,21} Some studies report that the topical route promotes fewer losses and lower transfusion needs compared to the intravenous route.¹⁸

Allogenic transfusions occurred in about 12% of patients, a value similar to that found in the current literature.⁵ The TXA-art group had reduced need of allogenic transfusions (only 2 patients). Although not statistically significant (p = 0.724), TXA-art seems to be better than TXA-iv in this regard. It has been shown that the benefit of cell salvage decreases when a more restrictive transfusion cut off (<8 g/dL) is used.¹⁶

It has been demonstrated that the use of TXA is not associated with increased risk of thromboembolic events in arthroplastic surgery, regardless of personal history^{7,22–24} and the route of administration.^{17,18,20,21} However, studies still advise caution for this potential risk.^{7,24,25} In our study, we did not identify any thromboembolic complications in any group, which further solidifies the safety of TXA administration. In the TXA-iv group, we identified a case of generalized seizures and one case of surgical wound infection. High dose (100 mg/kg) of TXA is associated with perioperative seizures,²⁶ but a single bolus of the usual dose used in orthopedic surgery is considered to be safe in this respect.²⁶

Topical administration of TXA results in higher local levels, lower plasma levels and, theoretically, lower risk of associated complications.^{17,27}

The 24% of preoperative anemia (Hb <13 g/dL) identified is similar to the 12–33% already described in patients undergoing TKA.^{28,29} Preoperative anemia is the most important independent risk factor for transfusion and perioperative morbidity and mortality.³⁰ Postoperative anemia was 91%, higher than the 82% observed in another study.²⁹

Our study presents some limitations: It is a retrospective study; the choice of blood sparing strategy was made according to surgeon's preference, which can cause some group biases not analysed by this study; even though the ideal dose of tranexamic acid remains a subject of discussion, the difference between the dose of tranexamic acid in the two protocols (15mg/kg in the TXA-iv group vs 1500mg TXA-art) can contribute as bias of analysis considering patient's weight. We tried to minimize this by analysing differences in groups' BMI; the wide interval of transfusion threshold (Hb 8–10g/dL) was based on a subjective clinical evaluation which can translate as a limitation on the interpretation of the presented findings. Nevertheless, as it worked as the same bias in all the 3 groups and the results were of significance, we chose to present them.

Conclusion

Based on the results obtained in this study, our institution assumed the preference for the use of intra-articular tranexamic acid in TKA. It seems to be a low-risk, highly efficient blood-sparing strategy. Prospective studies are needed, with a larger sample that allows a conclusive answer to the differences between intravenous and intra-articular administration of TXA and to evaluate possible associated thromboembolic complications.

Abbreviations

CBC, complete blood count; Hb, hemoglobin; Htc, hematocrit; PLT, platelets; TKA, total knee arthroplasty; TXA, tranexamic acid; TXA-art, intra-articular tranexamic acid; TXA-iv, intravenous tranexamic acid; vHb, hemoglobin variation; vHtc, hematocrit variation; vPLT, platelets variation.

Ethics Approval and Informed Consent

The *Comissão de Ética* (Institutional Review Board) of the Centro Hospitalar do Baixo Vouga (Aveiro), approved this research study. The review board has waived the need for informed consent as it is a retrospective study, all patients had signed consent for the surgical and anesthetic procedure and blood transfusions and no extraordinary interventions were performed. The data was accessed only by the authors and confidentiality of the patients was maintained at all times, in compliance with the Declaration of Helsinki.

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Author Contributions

All authors had an important role in designing the study, presenting it to the ethical committee, gathering information, treating the data, writing, and reviewing the manuscript. All revisions and alterations were agreed upon by all authors. All authors decided together to which journal the study should be sent and are accountable for the contents of the article.

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Disclosure

The authors report no conflicts of interest in this work.

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Circulating miR-126-3p and miR-423-5p Expression in de novo Adult Acute Myeloid Leukemia: Correlations with Response to Induction Therapy and the 2-Year Overall Survival

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Background: Acute myeloid leukemia (AML) results from sequential genetic alterations in a normal hematopoietic stem cell or its progenitors giving rise to an autonomous clone that dominates the bone marrow leading to marrow failure. MicroRNAs are short non-coding nucleic acid sequences that regulate post-transcriptional gene expression by base-pairing with their target mRNAs. MiRNAs can be secreted into extracellular fluids and carried to target cells by vesicles or bound to proteins. Intracellular and circulating miRNAs are believed to be useful markers in the diagnosis, prognosis, and treatment of various cancers. Practically, circulating miRNAs are more stable at room temperatures and extreme conditions.

Purpose: This study aimed to compare the expression of miR-126-3p and miR-423-5p in patients and normal subjects and correlate their expression with response to induction therapy and with their 2-year overall survival rate.

Patients and Methods: Circulating miR-126-3p and miR-423-5p was measured in the plasma of 43 adult AML patients and 35 age- and sex-matched controls by quantitative reverse transcriptase PCR. The fold change in differential expression for each gene was calculated using the comparative cycle threshold method.

Results: There was an increase in the expression of the studied miRNAs in patients compared to the control group. The average expression fold change of miR-126-3p was 3.02 ($p = 0.010$). The average expression fold change of miR-423-5p was 4.09 ($p = 0.003$). No significant correlation was found between the expression of miR-126-3p and miR-423-5p in the studied AML patients ($r = 0.094$, $p = 0.22$). Furthermore, no relationship was found between the expression of the studied miRNAs and response to induction therapy or the 2-year survival rate.

Conclusion: Although further studies are needed, our findings highlight the studied circulating miRNAs as possible diagnostic markers for AML.

Keywords: acute myeloid leukemia, miR-126-3p, miR-423-5p, response to induction therapy, overall survival, polymerase chain reaction

Introduction

Acute myeloid leukemia (AML) is known to be associated with a poor prognosis. In the USA, the 5-year survival rate of AML is 29.5%, it is estimated that 20,240 patients will be diagnosed with AML in the year 2021 and 11,400 patients will die from this disease. AML is caused by the stepwise acquisition of genetic mutations that ultimately lead to the autonomous uncontrolled proliferation and restriction of differentiation of a clone of cells which bypasses the normal physiological checkpoints, eventually leading to bone marrow failure with a consequent loss of the function of the cellular elements of blood.^{1,2}

MicroRNAs (miRNAs) are a group of short (21–23 nucleotides) non-coding RNAs that finely regulate gene expression. A single miRNA may regulate the expression of 100 genes. Various human diseases including cancers are

associated with disturbances in the miRNA expression.^{3,4} Most miRNAs are transcribed from their parent DNA sequences first into primary miRNAs which will then be processed into precursor miRNAs, and finally mature miRNAs. MiRNAs regulate gene expression by inducing mRNA degradation and translational repression, thereby the overexpression or the repression of miRNAs will positively or negatively regulate the degree of expression of their target genes.⁵

Circulating miRNAs were detected in body fluids, such as plasma and serum, urine, CSF, breast milk, colostrum, seminal fluid, tears, bronchial lavage, and peritoneal fluid. Unlike intracellular miRNA which is degraded in the extracellular environment within a few seconds, extracellular miRNAs are highly stable at room temperature for up to four days and in extreme conditions such as boiling, multiple freeze-thaw cycles, and high or low pH. This relative stability of extracellular miRNAs may be attributed to their containment within vesicles or other proteins.⁵⁻⁷ The unique characteristics of miRNAs like stability, tissue specificity, and testing simplicity make miRNAs encouraging targets for individualized cancer therapy. Circulating miRNAs are especially promising as rapidly emerging tools for early diagnosis, prognosis, and treatment of various cancers.

MicroRNA-126-3p (miR-126, mir-126, miRNA-126) is expressed in many human cells, such as cardiomyocytes, endothelial, and lung cells.⁸ The important role of miR-126-3p comes from its effect on various signaling pathways, such as CPK⁹ TOM1, PI3K/AKT/mTOR, and MAPK.¹⁰ MiR-126 overexpression has been associated with an increased leukemic stem cell (LSC) population and decreased normal hematopoietic stem cell counterpart.¹⁰ It was found that miR-126-3p overexpression in AML was associated with poor survival, a higher chance of relapse, and expression of genes present in AML LSC/hematopoietic stem cells (HSC) compartments.¹¹

MicroRNA 423 was found to be upregulated in most solid tumors including gastric cancer, hepatocellular carcinoma, glioblastoma, laryngeal carcinoma, lung adenocarcinoma, breast cancer, endometrial cancer, and prostate cancer. On the other hand, miR-423-5p expression was found to be downregulated in several other tumors like ovarian cancer, osteosarcoma, and cervical cancer. The cause of this heterogeneous expression of miR-423-5p in various tumors is not fully understood and might be due to the different functional mechanisms of miR-423-5p in different tumors. The carcinogenic effect of miR-423-5p is attributed to its positive effect on cell proliferation, invasion, metastasis, and chemoresistance and its negative effect on apoptosis.¹²

The change in the expression of circulating miR-126-3p and miR-423-5p in AML patients and their impact on response to induction therapy were not fully reported. Here, we aimed to find the relationship between the expression of plasma circulating miR-126-3p and miR-423-5p in adult *de novo* AML patients and healthy subjects and to correlate their expression with response to induction therapy, also to find the relationship between their expression and the 2-year overall survival (OS) rate.

Patients and Methods

This prospective cohort study included 43 patients with newly diagnosed *de novo* AML patients who attended Baghdad Teaching Hospital, Medical City from December 2018 to September 2019. This research was approved by the Research Ethics Committee at the College of Medicine, University of Baghdad, Iraq. Signed informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Based on preliminary clinical and laboratory findings supportive of an AML diagnosis, further investigations were done to patients' peripheral blood and bone marrow specimens including (as appropriate) bone marrow aspirate and biopsy morphology, immunocytochemistry, immunohistochemistry, and flow cytometry to confirm the diagnosis and decide on the treatment strategy according to clinical and laboratory findings.

Patients were treated by a 7+3 (induction of remission) regimen. Patients were assessed for response to induction therapy on day 28. Patients were classified according to their response to induction therapy to complete remission (CR) and non-remission (NR). Patients were followed up for two years to find the 2-year OS.

Patients less than 14 years old, FAB M3 patients, patients treated by a regimen other than 7+3 regimen, treatment-related and MDS-related AML patients were excluded from this study. Thirty-five apparently healthy age- and sex-matched individuals were enrolled in this study as a control group for comparison with miRNA expression.

Platelet-poor plasma (PPP) was prepared and kept refrigerated at -80°C . PPP samples were transferred with dry ice to si-Paradigm Diagnostic Informatics Laboratory located in New Jersey, USA, for RNA extraction, reverse transcription, and real-time PCR.

Total RNA (including miRNA) was extracted from the plasma of normal and AML patients using miRNeasy Serum/Plasma Advanced Kit (Qiagen cat#217204) and reverse transcribed with miRCURY LNA RT Kit (Qiagen cat# 339340) according to the manufacturer's instructions. The quality of miRNA isolation, cDNA synthesis, and real-time PCR was assessed by using miRCURY LNA RNA Spike-in kit (synthetic short RNA sequences that resemble miRNAs in structure but lack close sequence similarities to known miRNAs) (Qiagen cat# 339390). The levels of expression of miR-16, miR-126-3p, and miR-423-5p were measured by quantitative RT-PCR with a miRCURY LNA SYBR Green PCR Kit (Qiagen cat# 339345) in a Qiagen Rotor-Gene Q. MiR-16-5p was used as a normalization control. The primers used for the detection of miR-126-3p, miR-423-5p, miR-16-5p, Spike in control Uni-SP2, spike-in control Uni-SP4, spike-in control Uni-SP5 and cel-miR-39-3p were the hsa-miR-126-3p miRCURY LNA miRNA PCR Assay (Qiagen cat#339306 product# YP00204227), hsa-miR-423-5p miRCURY LNA miRNA PCR Assay (Qiagen cat#339306 product# YP00205624), hsa-miR-16-5p miRCURY LNA miRNA PCR Assay (Qiagen cat#339306 product# YP00205702), Synthetic UniSP2 miRNA PCR Assay (Qiagen cat#339306 product# YP00203950), Synthetic UniSP4 miRNA PCR Assay (Qiagen cat#339306 product# YP00203953), Synthetic UniSP5 miRNA PCR Assay (Qiagen cat#339306 product# YP00203955) and miRNA cel-miR-39-3p PCR Assay (Qiagen cat#339306 product# YP00203952), respectively. All reactions were run in duplicates.

MiR-16-5p was chosen as a normalization control because it is stably expressed across normal subjects in many diseases including AML. Also, it is equal to the size of our target miRNAs, and has similar extraction efficiency and stability, as well as having expression levels within a similar range of the target miRNAs.^{13–15}

Statistical Analyses

Regarding PCR experiments, data were acquired and analyzed by Rotor-Gene Q 2.3.5 software. The collected data were admitted into Microsoft Excel sheet 2016 and loaded into Statistical Package for the Social Sciences (SPSS) version 28 statistical program. Descriptive statistics were presented as mean $\pm$ standard deviation (SD), frequency, and percentages using tables and graphs. The Mann–Whitney *U*-test was used to test the statistical significance of the nonparametric variables. *P* values equal to or less than 0.05 were considered significant. The relative expression level was calculated by using the delta delta cycle threshold (Livak method, DDCT, or $2^{-\Delta\Delta\text{Ct}}$) analysis method.¹⁶ Pearson correlation was used for correlating miR-126-3p and miR-423-5p expression.

Results

Clinical and Laboratory Characteristics

Some clinical and laboratory characteristics of the studied patients are summarized in Table 1.

MicroRNA Expression

The expressions of miR-126-3p and miR-423-5p were assayed in 43 AML patients and 35 normal controls using qRT-PCR. The mean relative Ct (ΔCt), $\Delta\Delta\text{Ct}$ and fold change of miR-126-3p and miR-423-5p in patients and controls are shown in Figure 1 and Table 2.

Correlation Between miR-126-3p and miR-423-5p Expression

Using Pearson correlation for linear regression analysis, no significant correlation was found between the expression of miR-126-3p and miR-423-5p in the studied AML patients, with a correlation coefficient *r* of 0.094 and *p*-value of 0.22 (Figure 2).

Table I Distribution of Gender, WBC Count, Hemoglobin Concentration, Platelet Count and FAB Subtype in the Studied Patients

	Variable	No. of Patients	%	Range	Mean $\pm$ SD	Median
Gender	Male	21	48.80	N/A*	N/A*	N/A*
	Female	22	51.20			
WBC count ($\times 10^9/L$)	<4	7	16.28	0.8–168	35.73 $\pm$ 43.19	18.10
	4–11	9	20.93			
	>11	27	62.79			
Hemoglobin (g/dL)	Low**	43	100	2.76–11.90	7.90 $\pm$ 2.09	7.90
	Normal	0	0			
Platelet count ($\times 10^9/L$)	Low***	41	95.35	5.00–287	72.36 $\pm$ 52.46	55.00
	Normal	2	4.65			
FAB Subtype	M0	4	9.30	N/A*	N/A*	N/A*
	M1	6	13.95			
	M2	9	20.93			
	M4	15	34.69			
	M5	9	20.93			
	M6	0	0			
	M7	0	0			

Notes: *N/A not applicable. **Below 13.5 g/dL for male patients and below 11.5 g/dL for female patients. ***Below $150 \times 10^9/L$.

Relationship Between miR-126-3p and miR-423-5p Expression and Response to Induction Therapy

The mean relative expression (fold change) of miR-126-3p in the CR group was 2.11 ± 2.03 (range 0.29–7.90, median = 1.54), whereas the mean relative expression in the NR group was 3.91 ± 4.16 (range 0.11–19.73, median = 3.49), p value (Mann Whitney test) was 0.151, showing no significant statistical difference.

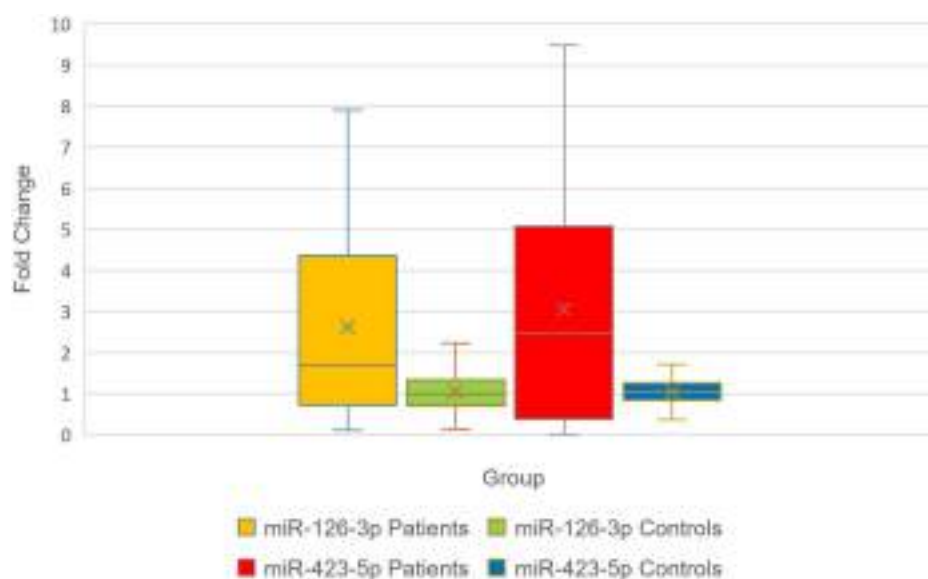


Figure 1 Comparison between the average fold change ($2^{-\Delta\Delta C_t}$) of miR-126-3p and miR-423-5p in patients and control groups ($p=0.010$ and 0.003 , respectively).

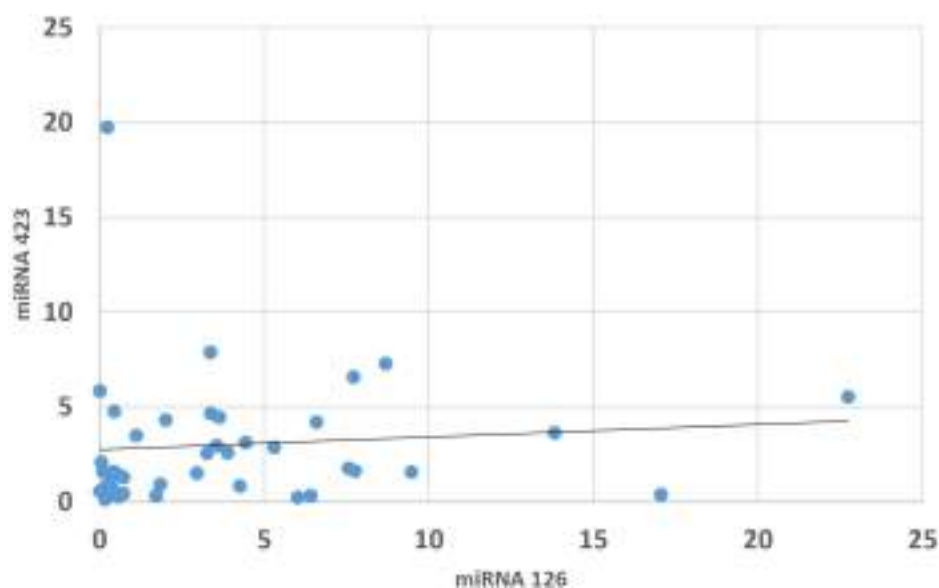
Table 2 Mean, Median and Standard Deviations (SD) of the Studied miRNA PCR Values (Ct, Δ Ct, $\Delta\Delta$ Ct and Fold Change)

		miR-126-3p		miR-423-5p	
		Patients	Controls	Patients	Controls
Ct	Mean	22.42	23.83	23.48	25.18
	Median	22.12	23.75	23.59	24.96
	SD	2.08	1.49	2.21	1.69
Δ Ct	Mean	4.69	5.49	5.74	6.84
	Median	4.68	5.36	5.99	6.69
	SD	1.66	1.00	1.72	0.56
$\Delta\Delta$ Ct	Mean	−0.80		−1.09	
	Median	−0.81		−0.85	
	SD	1.66		1.72	
Fold change ($2^{-\Delta\Delta$ CT)	Mean	3.02		4.09	
	Median	1.76		3.27	
	SD	3.40		4.86	
P value		0.010		0.003	

The mean fold change of miR-423-5p in the CR group was 3.26 ± 2.27 (range 0.00–9.47, median = 3.56), whereas the mean fold change in the NR group was 4.04 ± 4.36 (range 0.00–13.82, median = 2.03), *p* value (Mann Whitney test) was 0.621 showing no significant statistical difference (Figure 3).

Relationship Between miR-126-3p and miR-423-5p Expression and the 2-Year OS

Out of 37 patients who could be tracked up to 2 years, only five could survive 2 years or more after diagnosis. The mean relative expression (fold change) of miR-126-3p in the 2-year survivor group was 2.36 ± 2.56 (range 0.28–6.55, median =

**Figure 2** Correlation between miR-126-3p and miR-423-3p expression in the studied patients represented as pairs.

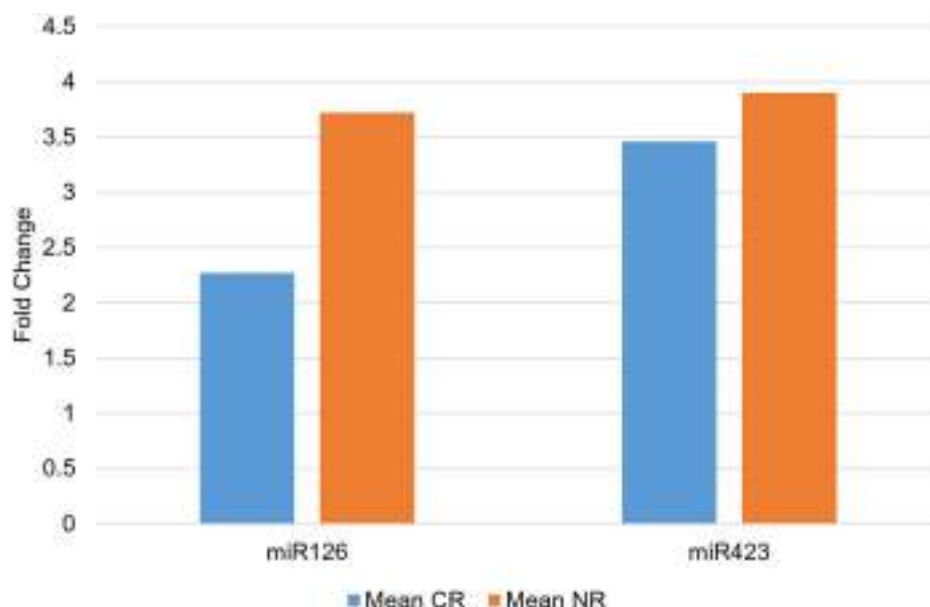


Figure 3 MiR-126-3p and miR-423-3p expression in the CR and NR groups.

1.43), whereas the mean relative expression in the 2-year non-survivor group was 3.22 ± 3.68 (range 0.08–19.73, median = 2.32), p value (Mann Whitney test) was 0.560, showing no significant statistical difference. The mean relative expression (fold change) of miR-423-5p in the 2-year survivor group was 2.72 ± 3.10 (range 0.00–7.71, median = 1.72), whereas the mean relative expression in the 2-year non-survivor group was 4.28 ± 5.22 (range 0.00–22.7, median = 3.30), p value (Mann Whitney test) was 0.589, showing no significant statistical difference (Figure 4).

Discussion

Despite decades of research in the various aspects of AML, including new therapeutic approaches and prognostic markers, most patients do not survive more than 5 years. To improve this outcome, more in-depth study and utilization

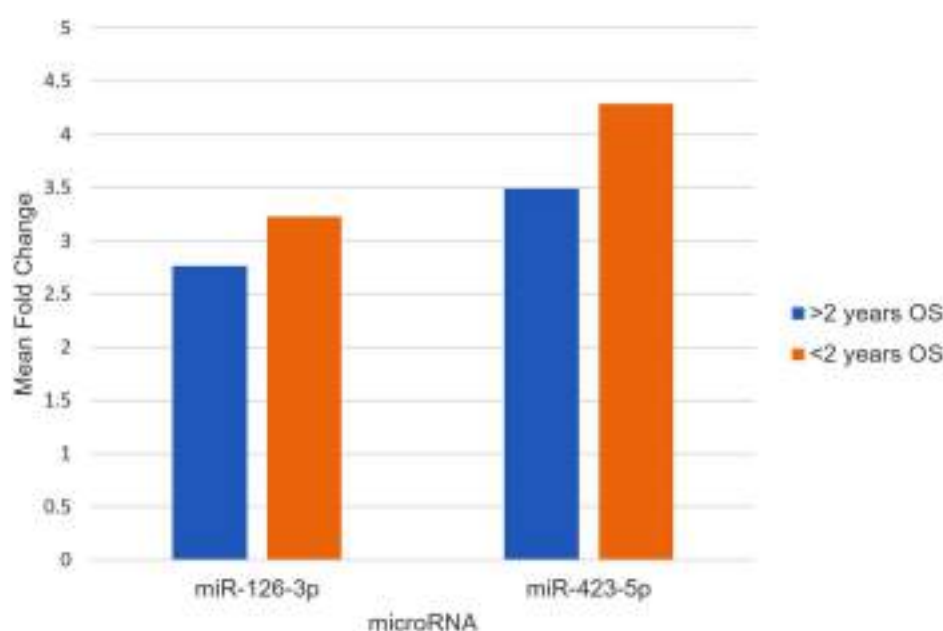


Figure 4 Comparison between miR-126-3p and miR-423-5p mean fold change of 2-years survivors (blue) and non-survivors (red).

of the currently available and newly discovered markers should be done. MicroRNAs are relatively novel players that have been dysregulated in many diseases including cancers. In addition, they have been proposed as possible therapeutic targets and markers for the prediction of outcome. Circulating miRNAs can be secreted by many cancer cells including leukemic cells and can be promising markers in the diagnosis and prognosis of AML. To our knowledge, the study of circulating miRNA-126-3p and miR-423-5p in AML patients' plasma was not done by any researcher before.

In this study, a significant increase in miR-126-3p expression in the studied AML patients was observed. Although the expression level of plasma miR-126-3p was higher in the NR group than in the CR group, this was not statistically significant.

MiR-126-3p is a short non-coding RNA with a wide range of effects, through its regulatory role of several signaling molecules. MiR-126-3p down-regulates CT10 regulator of kinase (CRK), an intracellular signal transduction molecule important in regulating cell adhesion, proliferation, migration, and invasion.⁹ Dysregulation of the CRK pathway is associated with increased susceptibility to cancer.¹⁷ MiR-126-3p upregulates CXCL12 expression.¹⁸ Interaction between CXCL12 expressed on stromal cells and its receptor CXCR4 (CD184) on leukemic blasts is important to keep leukemia homed to its microenvironment protected from chemotherapy, also it counteracts apoptosis and recruits progenitor cells.¹⁹ High CXCR4/CXCL12 expression was associated with adverse prognosis and has been a potential target for anti-leukemia drugs.^{20,21} MiR-126-3p downregulates PU.1, a transcription factor involved in the differentiation of myeloid cells and compromised in several AML subtypes. Minimal reduction of PU.1 was linked to leukemia initiation and the formation of preleukemic stem cells in AML.²² It has been found that miRNA-126-3p is downregulated in several solid tumors.²³ In AML, Li et al found that miRNA-126-3p was upregulated in AML with inv(16)/CBFβ-MYH11 or t(8;21)/AML1-ETO.²⁴

MiR-126-3p is also proposed to exhibit its leukemogenic properties by targeting the PI3K/AKT/mTOR signaling pathway to promote quiescence, increase self-renewal, and promote chemotherapy resistance of LSC.²⁵ PI3K/AKT/mTOR signaling is overactive in many cancers including AML, thus reducing apoptosis, and allowing proliferation, giving a proliferation advantage over differentiation.^{26–28} Therapeutic agents to target PI3K/AKT/mTOR signaling pathway have been studied. Dual PI3K/mTOR inhibition was cytotoxic not only to leukemic blasts' bulk but also to leukemia-initiating cells.²⁹

MiRNA-126 has been studied as a potential therapeutic target in AML. Targeting of miRNA-126 LSCs by nanoparticles containing antagomiR-126 resulted in a reduction of LSCs by depletion of the quiescent cell subpopulation.³⁰ In addition, therapeutic depletion of miR-126 in inv(16) AML xenograft models with a specifically designed inhibitor of miR-126 called miRisten inhibited the survival of AML cells, reduced leukemia burden and LSC activity.³¹

In this study, circulating miR-423-5p was significantly overexpressed in AML; however, there was no statistically significant relationship between plasma miR-423-5p expression and response to induction therapy in the studied AML patients.

The study of miR-423 in a normal state and different solid tumors revealed its effects on various processes inside the cells and its role in the biology of solid tumors. However, its role in leukemias has not been fully elucidated. MiR-423 negatively regulates colony-stimulating factor 1 (CSF1), a cytokine that controls the production, differentiation, and function of macrophages, and may affect CDK expression and induce cell arrest in the G0/G1 phase, by upregulating Cyclin-dependent kinase inhibitor 1A (CDKN1A), a negative regulator of the cell cycle, keeping the cell in a quiescent state, a requirement for LSCs to escape the effect of chemotherapeutic drugs and to keep a reserve of leukemia-initiating cells.³²

Overexpression of miR-423-3p indicated poor prognosis and promoted cell proliferation, migration, and invasion in lung cancer.³³ Metastasis suppressor 1 (MTSS1), a multifunctional molecular effector with an important role in cancer development, carcinogenesis, and metastasis, was a direct miR-423-5p target.^{34,35} It was observed that miR-423-5p mimics promoted the proliferation and inhibited apoptosis of bone marrow mesenchymal stem cells from orofacial bone (OMMSCs), while antisense inhibitors of this miRNA had the opposite effect.³⁶ In the setting of leukemia, very few studies relevant to miR-423 were found. A study of bone marrow circulating miRNAs in AML found that miR-423-5p

was overexpressed in AML bone marrow samples.³⁷ Moreover, MiR-423 (among other miRNAs) was overexpressed in two AML cell lines HL-60 (human promyelocytic leukemia cell line) and THP-1 (human monocytic leukemia cell line).³⁸ Merkerova et al studied the miRNA expression profile of 1145 miRNAs in CD34+ cells of MDS patients and found that hsa-miR-423-5p was downregulated in those patients.³⁹

Conclusion

There was an increase in the expression of circulating miR-126-3p and miR-423-5p in AML patients as compared to normal age- and sex-matched controls. This might highlight the role of both miRNAs in the initiation of AML and the possibility of being diagnostic and therapeutic targets. This is the first report of the significance of the plasma circulating form of both miRNAs in AML patients, which might pave the way for further studies with a larger cohort of patients to compare the plasma levels of the studied miRNAs with other diagnostic and prognostic indicators in AML, given that plasma miRNAs are stable at extreme conditions and can be measured with relative ease compared to intracellular miRNAs.

Data Sharing Statement

Data related to this research paper including raw data, materials and methods, PCR curves, Spike-in control data, software and algorithms will be provided upon reasonable request.

Ethics and Consent

This research was approved by the Research Ethics Committee at the College of Medicine, University of Baghdad, Iraq. Signed informed consent was obtained from each patient in accordance with the Declaration of Helsinki. There is no material reproduced from other sources to need special permission from the copyright owner. This research does not include any clinical trial.

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Disclosure

The authors report no conflicts of interest in this work.

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“Bloodless” Major Pulmonary Resection in Two Jehovah’s Witnesses Patients with Non-Small Cell Lung Carcinoma

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“Bloodless” Major Pulmonary Resection in Two Jehovah’s Witnesses Patients with Non-Small Cell Lung Carcinoma

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Background: Blood transfusion rates during surgery and hospitalization for thoracic surgery vary from 16% to 55%. The religious beliefs of Jehovah’s Witnesses (JW) permit medical and surgical procedures but exclude the use of blood and blood products. Performing major pulmonary resection without the possibility of compensating for blood loss is a daunting challenge that few surgeons are willing to meet.

Methods: The clinical and surgical data on two JW patients who underwent major pulmonary resections for non-small cell lung carcinoma (NSCLC) in Tel Aviv Medical Center between the years 2019 and 2020 were retrieved from the departmental databases and analyzed for the requirement and consumption of blood products. The patients were a 70-year-old female and a 49-year-old man that have underwent a completion right upper lobe lobectomy and a left lower lobe lobectomy, respectively.

Results: None required blood transfusions and the surgeries were successful, demonstrating that it is possible to successfully perform “bloodless” major pulmonary resection while respecting the limitations set by the patient’s religious beliefs.

Conclusion: This concept paves the way for the consideration of major pulmonary resection for patients who are currently denied such procedures due to religious restrictions or to their being high-risk surgical candidates, when resources are limited or lacking.

Keywords: bloodless thoracic surgery, lobectomy, Jehovah’s Witnesses

Introduction

Jehovah’s Witnesses (JW) is a Christian denomination whose followers numbered 8.5 million in 2018, and among whom transfusion of allogeneic red blood cells, platelets, and fresh-frozen plasma is forbidden.¹ Blood transfusion rates during surgery and hospitalization for thoracic surgery vary from 16% to 55%.^{2,3} There are only few reports on pulmonary resections for these patients in the literature.^{4,5} We present our experience in carrying out “bloodless” major pulmonary resection on two JW patients, and describe the steps that were taken in order to respect the limitations set by the patients’ religious beliefs. These cases provide a unique view into the possibilities that bloodless surgery could benefit not only JW patients, but also members of the general population, such as those at high risk for surgery and living in areas of the world where resources are limited. However, “bloodless” surgery in JW patients continues to carry great risk of morbidity and mortality,⁶ requiring a multidisciplinary team to determine the best approach on a case-by-case basis.

Patients and Methods

Patients

The reported patients were referred to the department of thoracic surgery between 2019 and 2020. Each case examined at a tumor board meeting, and surgery was preceded by multidisciplinary discussions that involved surgeons, anesthesiologists,

the blood bank's medical staff, and hospital administration, together with the patient and family members in the presence of a JW congregation's representative and hospital ethics committee members.

Methods

Preparation of these surgical patients included special emphasis upon minimizing the need for blood products that would be required for the various types of surgery. The alternative options for blood and blood components consisted of self-donation, acute normovolemic hemodilution and cell salvage. Intermittent pneumatic compression (IPC) devices used to prevent pulmonary embolism, and hemostatic devices and topical hemostatic sealants, as well as the direct control of bleeding points were applied to reduce blood loss during surgical procedures. The surgeries consisted of a right upper lobe (RUL) completion lobectomy, and a left lower lobe (LLL) lobectomy. The potential operated blood loss was calculated for each patient. A state of controlled hypotension with propofol and remifentanyl was used to reduce blood loss, and a normotensive state was ensured prior to completion of the surgery.

Patients Consent for Publication

The institutional review board approved this study and the patients gave their consent to report the clinical information relating their case for a medical publication.

Case 1

A 70-year-old female (JW member) with a history of hypertension was admitted for a RUL completion lobectomy due to recurrence of a 2.0 cm primary lung adenocarcinoma according to positron-emission tomography-computed tomography (PET-CT). The preoperative discussion with the patient and family members included explanations on alternative options for blood and blood components. The patient and her family refused self-donation and acute normovolemic hemodilution and agreed only to cell salvage as a last resort in the event of massive bleeding despite having been made aware of a greater risk of hemorrhage due to this being a second thoracic surgery. The coagulation test screen and iron levels were normal, and the hemoglobin level was 13.5 gr/dl. The patient received intravenous folic acid and iron preoperatively for 5 days and 1000 mg tranexamic acid intravenously before the surgery. IPC devices used to prevent a pulmonary embolism. The patient underwent a standard right posterolateral serratus-sparing re-thoracotomy, adhesions between the lung and chest wall scrupulously separated, and an anatomic RUL completion lobectomy performed. Controlled hypotension was maintained with propofol and remifentanyl, with return to a normotensive state prior to the completion of surgery. Enhanced hemostasis achieved by a 2.5 cc biological glue EVICEL™ (Ethicon Inc., Bridgewater, NJ). The operative blood loss was 30 mL. The postoperative period was uneventful, and the patient was discharged on the fifth postoperative day with a hemoglobin level >13 gr/dl.

Case 2

A 49-year-old previously healthy male (JW member) was admitted for a LLL lobectomy due to 2.0 cm primary lung adenocarcinoma according to positron-emission tomography-computed tomography (PET-CT). He refused self-donation and acute normovolemic hemodilution and agreed only to cell salvage. The coagulation test screen and iron levels were normal, and the hemoglobin level was 14.0 gr/dl. The patient received 1000 mg tranexamic acid intravenously before the surgery. IPC devices were used to prevent a pulmonary embolism. A standard posterolateral serratus-sparing thoracotomy incision was made and an anatomic LLL lobectomy performed. Controlled hypotension maintained with propofol and remifentanyl, with return to a normotensive state prior to the completion of surgery. The operative blood loss was 10 mL. The postoperative course was uneventful and the patient was discharged on the fourth postoperative day with a hemoglobin level >13 gr/dl.

Both patients were discharged in good physical condition and are under medical follow-up and disease free to date.

Discussion

The role of faith in the ability to heal the body both physically and mentally is well recognized. JW patients are known to refuse surgical interventions that involve the administration of blood and/or blood components. Chand et al reported that

very few of their JW patients (12.5%) would agree to accept blood transfusion or other blood components.⁷ There are only two publications describing the “bloodless” approach during pulmonary resections.^{4,5} The authors concur with our position that the optimal plan of a “bloodless surgery” must include pre-, intra- and postoperative consideration of strategies that take into account the patient’s surgical history, the use of medication potentially causing coagulopathy, and hemoglobin and hematocrit levels, platelet count, and coagulation profile. Minimizing the number of blood samples taken by using lower volume tubes is also important, as well as administration of iron replacement and recombinant human erythropoietin to correct perioperative anemia. On the other hand, “bloodless” surgery in JW patients continues to carry great risk of morbidity and mortality,⁶ despite the thoracic surgeon’s efforts to avoid blood loss.

Trzciński et al recently reported that reducing the need for transfusions by ensuring minimal blood loss during surgery is achievable with minimally invasive or staged procedures.⁸ We suppose that the choice of surgical technique should be chosen according to the surgeon’s experience in contending with intraoperative hemorrhage. Accordingly, we prefer thoracotomy to thoracoscopy, generally due to better control of hemorrhage, even though thoracoscopy is a less invasive technique.^{4,5}

Efforts to reduce operative blood loss improves patient’s outcomes.⁹ Shah et al emphasizes different measures employed by surgeons to reduce intraoperative blood loss: stop use of direct acting anticoagulants 48 hours before surgery, intraoperative use of cell salvage when anticipated blood loss is greater than 500mL, routine administration of tranexamic acid when anticipated blood loss is high, and others.⁹ Direct control of bleeding points and the use of hemostatic devices or topical hemostatic sealants is also useful. The general principles of anesthesia in “bloodless surgery” are starting with continuous hemodynamic monitoring of patient, avoidance of hypothermia during surgery, acute normovolemic hemodilution, regional anesthesia, and the induction of controlled hypotensive anesthesia by maintaining mean arterial pressure of 50–65 mmHg, using a propofol and remifentanyl infusion.

We observed that the special measures that we took to respect the JW beliefs did not require extraordinary efforts in executing successful surgeries, but every surgery must be examined on a case-by-case basis. Our experience with these patients leads us to speculate that the general population could also benefit from a “bloodless” surgical environment and thereby possibly extend the surgical conditions under which some high-risk surgical candidates could become suitable for surgery. Other potentially enormous benefits would be the saving of limited resources by meaningful reductions in these requirements, and the possibility of performing the surgeries in the absence of such resources. Another important point contributing to a successful surgery, in our opinion, although not proven scientifically, is the surgeon’s ability to identify with and respect the patient’s faith. By such consideration, we are able to perform quality surgeries and preserve the patients’ rights.

Finally, communication between the surgeons and the anesthesiologists is essential in preparing for major pulmonary resection, and frank communication between the professional team and the patients and their families is the key to the establishment of trust and cooperation when there are obstacles to the unrestricted use of blood products in major thoracic surgery.

Disclosure

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Distribution of ABO and Rh-D Blood Group Antigens Among Blood Donors in the Amhara Regional State, Ethiopia

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Background: The ABO and Rh blood group systems are the most commonly used blood group systems in clinical settings. They are clinically significant for blood transfusion, organ transplantation, genetic studies, forensic determinations, and medico-legal issues. Therefore, this study aimed to determine the distribution of ABO and Rh-D blood groups among blood donors in the Amhara regional state, Ethiopia.

Methods: A cross-sectional study was conducted from March 2016 to September 2018 among blood donors in the four blood bank districts (Gondar, Bahir Dar, Debre Markos, and Dessie) of the Amhara regional state, Ethiopia. A total of 1040 blood donors aged from 18 years old to 60 years old were included using a simple random sampling technique. A structured questionnaire and a data collection sheet were used to obtain the socio-demographic data and blood group types of study participants. The ABO and Rh-D blood groups were typed using commercially prepared antisera after 1 mL of blood was taken from blood bags. The data was cleared by Epi-Info Version 7 and extracted into SPSS version 25 for analysis. The distribution of data was checked by using the Shapiro-Wilk test. Then, the data was presented in tables and figures.

Results: In this study, out of 1040 study participants, 55.6% were males with a median age of 20 years (IQR = 19–24 years). In this study, the distribution of ABO blood types O, A, B, and AB was 41.6% (433/1040), 28.7% (298/1040), 22.2% (229/1040), and 7.7% (80/1040), respectively. On the other hand, the Rh-D positivity was 92.5% (962/1040).

Conclusion: The overall predominant ABO blood group in the Amhara regional state was O (41.6%), and the least was AB (7.7%). Besides, the overall Rh-D negative blood distribution was 7.5%, which ranged from 4.46% to 9.6%. Therefore, this information would be useful to the blood banks, especially in the planning of blood transfusion programmers.

Keywords: ABO, Rh-D, blood group, Amhara, Ethiopia

Background

Blood is the most essential body fluid, which is responsible for the transportation of nutrients, enzymes, and hormones throughout the body.¹ Primarily, the blood has a fluid part called plasma and blood cells (white blood cells, red blood cells (RBC), and platelets). Of the cells, the RBC membrane is complex and contains numerous antigens that are made from glycoproteins and glycolipids.² Based on the surface of RBC antigens, more than 100 blood group systems have been identified. However, the ABO and Rh blood group antigens are the most commonly used blood group antigens in a clinical setup and are used to avoid transfusion reactions and maternal death.^{3,4}

The ABO blood groups were the first human blood group system that was discovered by Karl Landsteiner. The ABO antigens are controlled by 3 allelic genes placed on the long arm of chromosome 9q and are mainly responsible for the RBC membrane structural integrity and transportation of molecules through membranes.^{5,6} The ABO blood group is commonly tested for transfusion of blood and its components, organ transplantation, genetic studies, and forensic determinations, as well as for medico-legal issues like paternity testing.⁷ The ABO blood group can be

categorized into four main blood groups. These are A, B, AB, and O, which are encoded with N-acetyl D-galactose amine, D-galactose amine, both N-acetyl D-galactose amine and D-galactose amine, and no antigen, respectively. However, the blood plasma contains reverse antibodies, which are targeted against particular antigens.⁴ The ABO blood group antibody occurs without any exposure to RBC by transfusion or pregnancy, called “natural” or “expected” antibodies.⁷

In 1941, Landsteiner and Weiner discovered the Rhesus (Rh) blood grouping system.⁸ The Rh blood group is a multi-antigen system expressed by 3 pairs of closely linked allelic genes located on chromosome 1, which consists of more than 50 antigen expressions. However, the major Rh antigens of medical importance are D, C, E, c, and e.^{5,9} An individual who has the Rh-D antigen is called Rh-D positive, while those lacking the antigen are called Rh-D negative. When a Rh-D negative person is transfused with Rh-D positive blood or a Rh-D negative mother is carrying a Rh-D positive fetus, the antigen will be recognized as a foreign particle by the immune system, which produces an antibody, resulting in a hemolytic transfusion reaction and hemolytic disease in the fetus and newborn.⁷

Knowledge of ABO and Rh blood group distribution will help to reduce maternal mortality rates through effective management of blood bank inventory. Blood bank access will also provide a safe and sufficient supply of blood, reducing the number of preventable disease deaths through effective management of blood bank inventory. Besides, the distribution of ABO and Rh-D blood groups varies in different populations (races, ethnic groups, and socio-economic classes). According to studies on ABO distribution in Ethiopia, the “O” blood group was the most dominant, followed by the “A” and “B” blood groups.^{1,10–15} The proportion of the “O” blood group ranged from 21.67%¹⁶ to 47.04%.¹⁵ On the other hand, the “AB” blood group was the least prevalent blood group type, which ranged from 2.87%¹² to 24.7%.¹⁶ The Rh-D positive blood group was the most common, accounting for 92.77%¹¹ to 60.13% of the population.¹⁶ Even if studies on the ABO distribution have been conducted in Ethiopia, the distribution of ABO in the Amhara regional state at a regional level is not known. Therefore, this study aimed to determine the distribution of ABO and Rh-D blood group antigens among the Amhara regional state population.

Materials and Methods

Study Setting and Study Population

A population-based cross-sectional study was conducted from March 2016 to September 2018 to assess ABO and Rh-D blood group distribution in the Amhara regional state blood bank districts (Gondar, Bahir Dar, Debre Markos, and Dessie). The Amhara region has a land area estimated at 170,000 square kilometers. It is located in the northwestern part of Ethiopia between 8° 45' and 13° 45' North latitude and 36° 20' and 40° 20' East longitude. Based on the 2007 census conducted by the central statistical agency of Ethiopia, the Amhara regional state has a population of more than seventeen million people. Of those, 8,641,580 were men and 8,580,396 were women; urban inhabitants constituted around 12.27% of the population.¹⁷

A total of 1040 study participants aged from 18 years up to 60 years were included. All adult individuals living in the Amhara region were considered our source populations. While the blood donors who were coming to the blood bank center in the Amhara region for blood donation were considered as the study population, to select the study participants, a simple random sampling technique was used, and we used blood bank donor eligibility criteria. The blood banks select blood donors with nationally standardized criteria. According to these nationally harmonized criteria, blood donors' general good health shall be assessed through medical history, physical examination, and hemoglobin determination. Those with known diseases, those taking pharmacologically active substances or any prescription drugs, smokers, individuals who had major surgery in the past year, pregnant and lactating women, individuals who had donated blood in the previous 4 months and those who had received a blood transfusion in the previous year were excluded. Further, the blood donors should be between the ages of 18 and 65, weigh more than 45 kilograms, and have a normal hemoglobin value (13 for males and 12 for females), as well as normal vital signs (37 degrees Celsius, diastolic blood pressure of 70–90 mmHg, and systolic blood pressure of 100–150 mmHg). If the blood donor fulfilled the above-mentioned eligibility criteria, he/she could donate blood, and the donated blood was screened for infectious diseases like human immunodeficiency virus, Hepatitis B virus, Hepatitis C virus, and syphilis using ELISA techniques at each blood bank center. Study

participants who volunteered to donate blood in Amhara Regional State were included in this study. However, study participants whose blood showed auto-control positivity were excluded from the study.

Data Collection and Laboratory Procedures

The structured questionnaires and a data collection sheet were used to obtain socio-demographic and laboratory data from study participants. The data was collected by trained nurses and laboratory technologists. First, training was given to the data collectors about the aim of the study. Then, the data collection procedure was collected under the supervision of a principal investigator.

Socio-Demographic Data Collection

A pre-tested structured questionnaire was used to collect sociodemographic data. To keep the quality of the data, the questionnaire was first written in the English language, then translated into the local language (Amharic), and finally back into the English language. To improve the quality of the data, the questionnaires were pre-tested in the Metema blood bank before the study began.

Blood Sample Collection

We follow the blood bank's procedure for blood collection and ABO and Rh-D blood grouping. First, the study participants were asked to give informed consent and undergo a physical examination for blood donation. Then the blood bank collected one unit of blood (450mL) from eligible donors. A total of 1 mL of blood was collected from each blood unit for the ABO and Rh blood grouping tests. All the procedures were followed according to the Ethiopian National Blood Bank service protocols.

ABO and Rh-D Typing

ABO and Rh-D phenotyping were conducted using the slide method. One drop of whole blood was added to a clean slide and a commercially prepared standard antiserum (anti-A, anti-B, anti-D) with an equal amount was mixed. Then, after 2 minutes of incubation at room temperature, the results were recorded after looking with the naked eye and a microscope. The ABO and Rh-D blood group data were collected using a data collection format. To keep the quality of the result, known A-cell, B-cell, and Rh-D positive and negative cells were used.

Data Entry and Analysis

The data was entered into Epi-Info version 7. Then, it is transported to the SPSS version 25 software for the analysis. The study participants' age distribution was checked by the Shapiro–Wilk test. For normally distributed data, the mean and standard deviation were used, while for skewed data, the median and interquartile range (IQR) were used. Categorical variables were described using frequency and percentages.

Ethics Approval

Ethical clearance was obtained from the institutional review boards of the University of Gondar with a reference number of VP/RCS/05/19/2015. Written informed consent was taken from all study participants. Additionally, the confidentiality of information was assured by using codes for each specimen and the results. This study was conducted strictly following the Declaration of Helsinki.

Results

Sociodemographic Characteristics of Study Participants

In this study, a total of 1040 study participants were included. Of the total study participants, about 55.4% (576/1040) were males, and the median age of the study participants was 20 years (IQR = 19–24 years), with an age range of 18 to 61 years old. The majority of 79.5% (827/1040) and 87.1% (906/1040) of the study participants were students and single in their marital status, respectively (Table 1).

Table 1 Sociodemographic Characteristics and Blood Group Distribution Among Blood Donors in Amhara Regional States

Variables	Categories	Frequency	Percentages (%)
Sex	Male	576	55.4
	Female	464	44.6
Age in years	18–20 years	534	51.3
	21–25 years	323	31.1
	26–30 years	124	11.9
	>30 years	59	5.7
Address	Gondar	250	24.0
	Debre Markos	269	25.9
	Bahir Dar	306	29.4
	Dessie	215	20.7
Occupational status	Student	827	79.5
	Employed	144	13.8
	Farmer	2	0.2
	Merchant	58	5.6
	Daily laborer	6	0.6
	Others	3	0.3
Marital state	Single	906	87.1
	Married	97	9.3
ABO	A	298	28.7
	B	229	22
	AB	80	7.7
	O	433	41.6
RH	Rh positive	962	92.5
	Rh negative	78	7.5

ABO and Rh-D Blood Group Distribution of the Study Participants

The distribution of ABO blood groups among Amhara regional state blood donors was 41.6% (433/1040), 28.7% (298/1040), 22% (229/1040), and 7.7% (80/1040) were O, A, B, and AB blood groups (Table 1).

Further, the combination of ABO and Rh blood group distribution was A Rh-D positive, A Rh-D negative, B Rh-D positive, B Rh-D negative, AB Rh-D positive, AB Rh-D negative, O Rh-D positive, and O Rh-D negative, with 26%, 3%, 20%, 2%, 7%, 0%, 39%, and 3%, respectively. From districts, the predominant ABO blood group was the O blood type, and from the Rh blood group, the Rh-D positive was dominant in all districts (Figure 1).

In the Rh-D blood groups, the Rh-D positivity was 92.5% (962/1040) and the Rh-D negativity was 7.5% (78/1040) (Figure 2). Further, the Rh-D negative blood group in Gondar, Debre-Markos, Bahir Dar, and Dessie was 9.6% (24/250), 4.46% (12/269), 8.59% (26/303), and 7.5% (16/214) (Table 2).

Discussion

All humans have similar ABO and Rh blood group systems; while the difference is in the frequencies and distributions of blood types in different races, ethnic groups, and socio-economic groups or amongst different populations. Therefore, this study aimed to determine the distribution of ABO and Rh-D blood group antigens among blood donors in Amhara regional state blood bank districts.

In the current study, the distribution of O, A, B, and AB blood groups among Amhara regional state blood donors was 41.6% (433/1040), 28.7% (298/1040), 22.2% (229/1040), and 7.7% (80/1040), respectively. This showed that the “O” blood group was the dominant blood group, followed by A and B, and the least common blood group was AB. Also, the distribution of ABO in the blood bank districts is similar to the overall Amhara regional state distribution, where the “O” blood group is the dominant, followed by the “A” blood group, then the “B” blood group, and the “AB” blood group is the least common in all blood bank districts. This study is similar to a study conducted in India,¹⁸ Bangladesh,¹⁹

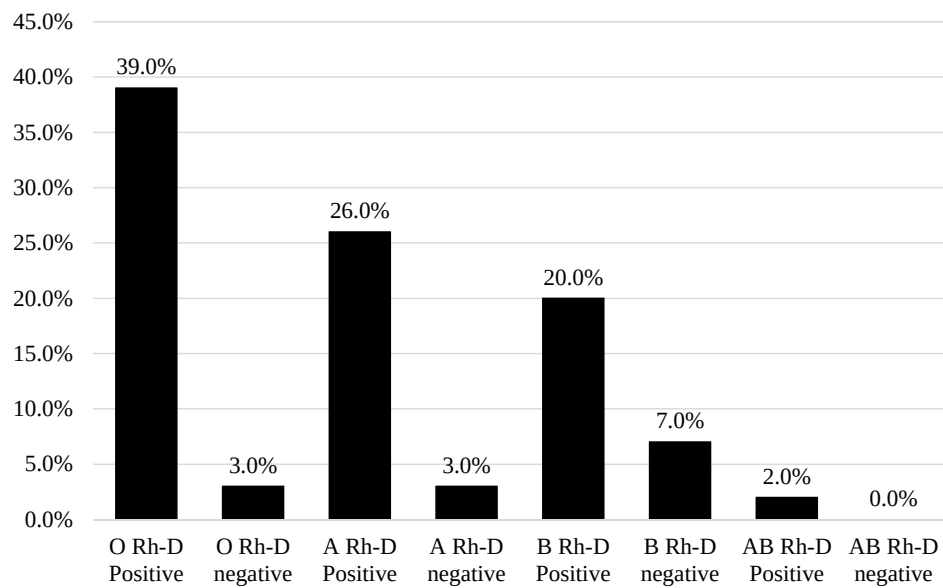


Figure 1 ABO and Rh-D blood group distribution among blood donors in Amhara regional states blood bank districts.

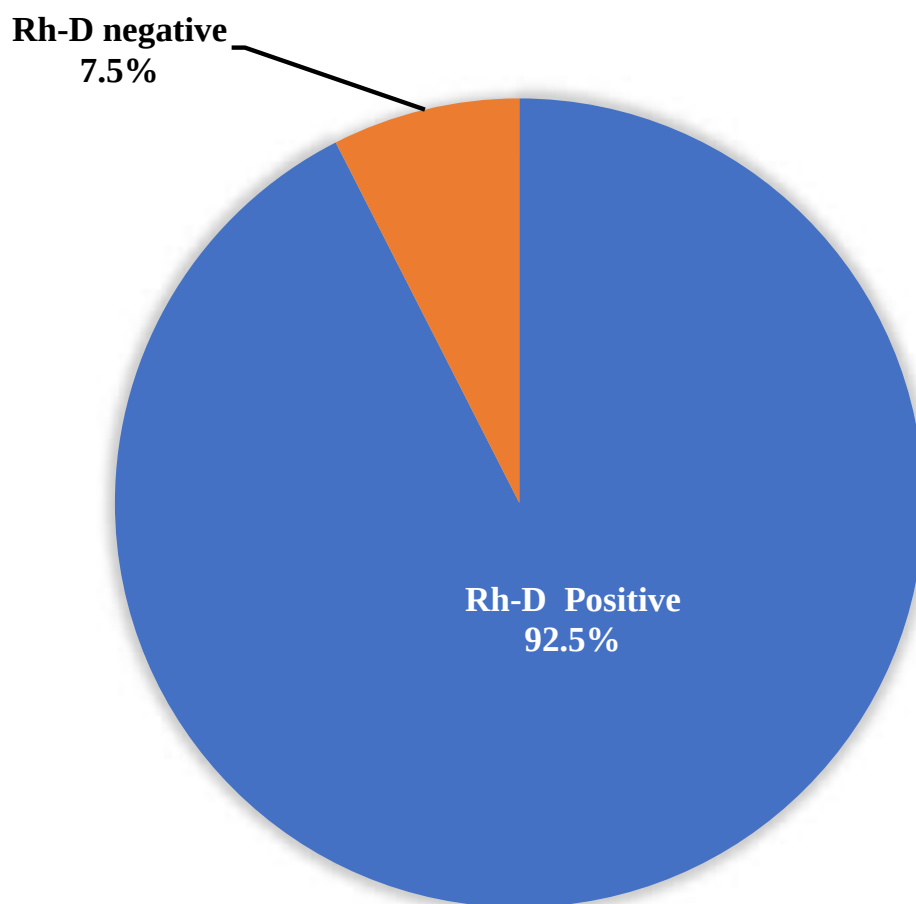


Figure 2 Rh-D blood group distribution among blood donors in Amhara regional states blood bank districts.

Table 2 Sociodemographic Characteristics and Blood Group Distribution Among Blood Donors in Amhara Regional States Districts

Variables	Categories	Blood Donors Categorized by District			
		Gondar (n = 250)	Bahir Dar (n = 306)	Debra-Markos (n = 269)	Dessie (n = 215)
Sex	Male	124 (49.6%)	175 (57.2%)	157 (58.4%)	120 (55.8%)
	Female	126 (50.4%)	131 (42.8%)	112 (36.6%)	95 (44.2%)
Marital Status	Single	240 (26%)	286 (31%)	251 (27.2%)	146 (15.8%)
	Married	10 (8.5%)	20 (17.1%)	18 (15.4%)	69 (59%)
Age	18–20 years	129 (24.2%)	202 (37.8%)	146 (27.3%)	57 (10.7%)
	21–25 years	99 (30.7%)	77 (23.8%)	76 (23.5%)	71 (22%)
	26–30 years	19 (15.3%)	24 (19.4%)	33 (26.6%)	48 (38.7%)
	>30 years	3 (5.1%)	3 (5.1%)	14 (23.7%)	39 (66.1%)
Occupation	Student	230 (27.8%)	269 (32.5%)	240 (29%)	88 (10.6%)
	Employed	9 (6.3%)	34 (23.6%)	29 (20.1%)	72 (50%)
	Farmer	0	1 (50%)	0	1 (50%)
	Merchant	6 (10.3%)	0	0	52 (89.7%)
	Daily laborer	4 (66.7%)	0	0	2 (33.3%)
	Others	1 (33.3%)	2 (66.7%)	0	0
ABO Blood group	A	80 (26.8%)	77 (25.8%)	79 (26.5%)	62 (20.8%)
	B	60 (26.2%)	70 (30.6%)	49 (21.4%)	50 (21.8%)
	AB	18 (22.5%)	25 (31.3%)	25 (31.3%)	12 (15%)
	O	92 (21.2%)	134 (30.9%)	116 (26.8%)	91 (21%)
Rh-D Blood group	Positive	226 (23.5%)	280 (29.1%)	257 (26.7%)	199 (20.7%)
	Negative	24 (30.8%)	26 (33.3%)	12 (15.4%)	16 (20.5%)

Nigeria,^{20–22} Uganda,⁶ and Ethiopia (Jimma,¹³ Gambella,¹⁴ and Bahir-Dar).¹⁰ The studies indicated that the “O” blood group was the most common, and AB was the least common. In contrast to this, a study conducted in India²³ and Pakistan^{24,25} showed that the “B” blood group was dominant, followed by the “O” blood group. Similarly, a study conducted in Woldia, Ethiopia¹⁶ showed that the A blood group was the predominant blood group compared to others. The discrepancy between our findings and those of other studies could be attributable to differences in sample size, sampling technique, and genetic variation across ethnic groups within the Amhara region’s population and populations in other nations.

In the current study, the Rh-D positive blood group was 92.5% (95% CI: 92.49, 92.51) and the Rh-D negative blood group was 7.5% (95% CI: 7.495, 7.505). This showed that nearly ten times more of the Rh-D positive blood group type was collected from blood donors than the Rh-D negative blood group types. This indicated that Rh-D negative was the least common blood group type. This study was nearly similar to the study conducted in Jimma¹³ in which 7.2% of Rh-D negative blood types were identified. In contrast, this study’s finding was higher than a study conducted in Bangladesh,¹⁹ India,²³ Nigeria,^{21,22,26} Uganda,⁶ and Niger²¹ which ranged from 1.2% to 6.55%. In contrast, this study is lower than a study conducted in India,¹⁸ and different parts of Ethiopia (Woldia,¹⁶ Gambella,¹⁴ and Bahirdar¹⁰) showed that 8.9%, 39.86%, 19.37%, and 8.5% were Rh-D negative, respectively. Further, in this study, the Rh-D negative blood group in Gondar, Debre-Markos, Bahir Dar, and Dessie districts was 9.6% (24/250), 4.46% (12/269), 8.59% (26/303), and 7.5% (16/214), respectively. This showed that the Rh-D negative blood group ranges from 4.46% to 9.6%.

In this study, the combination of ABO with the Rh blood group distribution among the study participants was as follows: A Rh-D positive, A Rh-D negative, B Rh-D positive, B Rh-D negative, AB Rh-D positive, AB Rh-D negative, O Rh-D positive, and O Rh-D negative were 26%, 3%, 20%, 2%, 7%, 0%, 39%, and 3%, respectively. This showed that the “O” positive and “A” positive blood groups were the first leading blood types, and the “AB” negative and “O” negative blood groups were the least common blood group. However, the O Rh-D negative blood group was the universal cell donor that can help many patients during emergency transfusion, but of the total donors, only 3% (31/1040) were “O” Rh-D negatives, so the blood bank should be aware of this and the blood should be used wisely. This study is similar to a study conducted in Bahir-Dar.¹⁰ “O” Rh-D positive and “A” were the first two dominant blood groups, while “AB”

Rh-D negative and “O” Rh-D negative were the least dominant blood types among blood donors. In contrast to this, a study conducted in India²³ and Pakistan^{24,25} showed that “B” positive was the dominant blood group followed by the “O” positive blood group. The variation of this study with other studies might be related to the difference in sample size, diagnostic methods, and genetics among the ethnic groups studied populations.

Conclusion and Recommendations

In this study, the predominant blood group type was “O” and the least was AB among blood donors in the Amhara regional state blood bank districts. Besides, the Rh-D negative blood distribution in the Amhara regional state blood bank districts was 7.5%, and its range was from 4.46% to 9.6% in all the blood districts included in this study. Therefore, this information would be beneficial to blood banks, particularly in the development of blood transfusion programs for the most efficient use of blood collected from donors.

Abbreviations

EDTA, ethylene di-amine tetra acetic acid; IQR, interquartile range; RBC, red blood cell; Rh, rhesus; UoG-CSH, University of Gondar Comprehensive Specialized Hospital.

Data Sharing Statement

All data generated and/or analyzed in this study are available within the manuscript.

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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 it to the current journal; gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Disclosure

The authors declare that there is no conflicts of interest regarding the publication of this manuscript.

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Apixaban Use in Patients with Protein C and S Deficiency: A Case Series and Review of Literature

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Apixaban Use in Patients with Protein C and S Deficiency: A Case Series and Review of Literature

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Background: Historically, warfarin was the mainstay anticoagulant agent to manage patients presenting with thrombotic disorders caused by Protein C or S deficiency. Several direct oral anticoagulants (DOACs) were introduced over the past decade. They showed superiority over warfarin in patients with venous thromboembolism in many landmark trials. Insufficient data are available that examine the outcome of utilizing apixaban in patients with protein S deficiency induce thrombosis.

Case(s) Presentation: We reported the clinical outcomes of utilizing apixaban in four patients with systemic thrombosis caused by protein C or S deficiency who presented to a tertiary hospital in Riyadh, Saudi Arabia. Four patients exhibited typical features of thrombotic events. After confirming the diagnosis, one patient was initially started on apixaban, and the other three patients were converted from warfarin to apixaban. Three of the four patients tolerated the apixaban during the follow-up period. Additionally, they did not have any bleeding or thrombotic complications. However, one patient developed recurrent thrombotic events despite switching to different type of DOAC and was ultimately transitioned back to warfarin.

Conclusion: Based on the available emerging evidence and our case series, the use of apixaban could be effective in preventing recurrent thrombotic events in patients with inherited thrombophilia without safety concerns. Further, large studies are warranted to investigate the safety and efficacy of apixaban in these population.

Keywords: apixaban, protein S deficiency, thrombosis, bleeding

Introduction

Several endogenous proteins such as protein S, protein C, and antithrombin are known to maintain the hemostatic equilibrium.¹ The deficiency of any of these proteins could disrupt the hemostatic equilibrium and eventually cause venous thromboembolism (VTE).¹ These deficiencies might be acquired or inherited as autosomal dominant traits.¹ There are other types of thrombophilia, such as Factor V Leiden mutation, homocystinuria, and prothrombin G20210A.² Individuals with hereditary protein C or S deficiency are more prone to develop VTE by approximately 11-folds compared to individuals without protein C or S deficiency.³

VTE manifested as either deep vein thrombosis (DVT), pulmonary embolism (PE), or both. Patients with inherited thrombophilia may require receiving anticoagulation therapy as primary or secondary prevention.^{1,4} Historically, vitamin K antagonists (VKAs) such as warfarin were used to treat inherited thrombophilia's induced VTE.⁵ Over the past decades, several large randomized clinical trials showed that most of direct oral anticoagulants (DOACs) are as effective as warfarin with better safety profile.⁶⁻⁸ These drugs were developed to address some of the primary drawbacks of warfarin, such as inter-individual variability, the prolonged time to attain full therapeutic effect, the narrow therapeutic index, the frequent coagulation monitoring, and the complex medication and food interactions.⁹ Moreover, several

studies showed that DOACs may possess an incremental cost-effectiveness ratio and better quality of life for the treatment and prevention of VTE compared to warfarin therapy.^{10,11}

Most of the studies that evaluated the efficacy and safety of DOACs in this subset of the patient population were small and predominantly included patients with low risk for thrombophilia (Factor V Leiden and prothrombin G20210A). Furthermore, the use of apixaban in hereditary thrombophilia, specifically high-risk thrombophilia (protein S deficiency), is controversial due to the lack of solid evidence. This case series aims to represent the experience of using apixaban in four patients with protein C and S deficiency.

Case #1

In our first patient case, we describe a 41-year-old woman known to have chronic kidney disease (CKD) and peptic ulcer disease (PUD). Protein S deficiency was detected upon presentation with an extensive portal vein and superior mesenteric vein thrombosis and prior to initiating this patient on any anticoagulant. As a result, warfarin was initiated, targeting an International Normalized Ratio (INR) range of 2–3. She continued on warfarin for nine months. Her blood test showed a decreased protein S activity (22.8%). During the duration of treatment with warfarin, she presented to the emergency department four times. She was treated for bleeding and supratherapeutic INR levels. A multidisciplinary decision was made to switch her from warfarin to apixaban 2.5 mg BID. She remained on apixaban for approximately three years with no recurrent thrombotic or bleeding events up until her most recent follow-up (Tables 1 and 2).

Case #2

In our second case, we describe a 49-year-old man with a body mass index of 33.5 kg/m² who was medically free. However, he presented to the emergency department with shortness of breath, pain in the lower extremity, and coughing blood-streaked sputum. He was diagnosed with an unprovoked DVT and PE. His protein S activity was 44.5%, and he was initiated on apixaban with a loading dose of 10 mg BID for seven days and continued on a maintenance dose of 5 mg BID. He had no thrombotic or bleeding events for over five years and up until the date of this paper (Tables 1 and 2).

Case #3

In our third case, we describe a 36-year-old man known to have protein S deficiency detected on presentation with a DVT at a young age (17 years). He had extremely decreased protein S activity (20%). He was treated with warfarin for over 14 years until another DVT occurred and was switched to rivaroxaban 20 mg once daily. He remained on rivaroxaban for approximately four years until he developed a PE. Then he was switched to apixaban 2.5 mg BID, later his dose was increased to 5 mg BID. Two years later, he developed a massive PE requiring intensive care unit admission, and he was switched back to warfarin at that time. Despite the patient adherence, no reason was identified for his recurrent VTE events while being on apixaban therapy. (Tables 1 and 2).

Case #4

Our last case is a 51-year-old woman known to have a low protein C activity (49%) and protein S activity (25%) with a prior history of cerebral venous thrombosis on lifelong anticoagulation using warfarin. She was on warfarin before the cerebral thrombosis event and remained on it after the event. However, a decision was made to switch her to apixaban 5 mg BID after developing subarachnoid hemorrhage and having reports of supratherapeutic and fluctuating INR levels with warfarin. For approximately five years period and until her most recent follow-up she had no thrombotic or bleeding events while on apixaban (Tables 1 and 2).

Discussion

In this case series, we presented the data for four patients with protein C or S deficiency complicated by VTE or cerebral venous thrombosis maintained on apixaban. Three of the presented cases remained free from thrombotic or bleeding events for several years after apixaban initiation. However, one of the cases developed a new thrombotic event that required an alternative anticoagulant.

Table 1 Baseline Characteristics

Demographic Data	Case #1	Case #2	Case #3	Case #4
Age (years)	41	49	36	51
BMI (kg/m ²)	32.47	33.51	32.4	34.13
Gender	Female	Male	Male	Female
Weight (kg)	77	98	112	82
Blood group	O+	NA	B-	NA
Co-existing illness				
Hypertension (HTN)	No	No	Yes	Yes
Asthma	No	No	No	Yes
Chronic obstructive pulmonary disease (COPD)	No	No	No	No
Diabetes mellitus (DM)	No	No	No	Yes
Chronic kidney disease (CKD)	Yes	No	No	No
Ischemic heart disease (IHD)	No	No	No	No
Atrial fibrillation (AFib or AF)	No	No	No	No
Heart failure (HF)	No	No	No	No
Acute coronary syndrome (ACS)	No	No	No	No
Dyslipidemia (DLP)	No	No	No	Yes
CVA (Stroke)	No	No	No	Yes
Hypothyroidism	No	No	No	No
Valvular disease	No	No	No	No
Venous thromboembolism (VTE)	No	Yes	Yes (PE)	No
CABG	No	No	No	No
Liver disease (any)	No	No	No	No
Peptic ulcer disease	Yes	No	No	No
Systemic lupus erythematosus (SLE)	No	No	No	No
Antiphospholipid antibody syndrome (APLS)	No	No	No	No
Cancer	No	No	No	No
History of major bleeding	No	No	No	No
Baseline Labs				
Protein S activity	22.8%	45.5%	20%	25.1%
Protein C activity	NA	NA	NA	49%
Serum creatinine (μmol/l)	90	88	90	55
Blood urea nitrogen (BUN) (mmol/l)	6.7	4.9	NA	3

(Continued)

Table 1 (Continued).

Demographic Data	Case #1	Case #2	Case #3	Case #4
Total bilirubin ($\mu\text{mol/L}$)	5	NA	10.2	5.7
INR	1.97	1.03	1.75	5.09
Platelets count ($10^9/\text{L}$)	317	239	259	415
Activated partial thromboplastin time (aPTT) (seconds)	NA	NA	37.1	57.4
Alanine aminotransferase (ALT) (U/L)	13	29	41	43
Aspartate aminotransferase (AST) (U/L)	17	24	108	24
Albumin (gm/L)	31	41	44	40
White blood cells (WBCs) ($10^9/\text{L}$)	7.44	6.4	4.85	14.16
Blood glucose level (mmol/l)	5.9	4.8	NA	8
Hematocrit (Hct) (L/L)	0.33	0.45	0.47	0.37
Fibrinogen (gm/L)	3.17	NA	NA	NA
D-dimer (mg/l)	1.79	6.47	NA	NA
Apixaban use	Yes	Yes	Yes	Yes
Date of initiation	28/06/2018	24/11/2016	2018	05/10/2016
Dose of apixaban	5 mg	10 mg	5 mg (initially) then increased to 10 mg	10 mg
Concomitant antiplatelets use (ie, aspirin, clopidogrel, ticagrelor)	No	No	No	No
GI prophylaxis	Yes (PPI)	Yes (PPI)	No	Yes

Table 2 Outcomes after Apixaban initiation

Outcomes	Case #1	Case #2	Case #3	Case #4
Major bleeding	No	No	No	No
Minor bleeding	No	No	No	No
Hospital admission within 6 months of apixaban initiation	Yes	Yes	No	Yes
Hospital admission within 12 months of apixaban initiation	Yes	Yes	No	Yes
New stroke within 6 months of apixaban initiation	No	No	No	No
Any new stroke after apixaban initiation	No	No	No	No
New VTE within 6 months of Apixaban initiation	No	No	No	No
Any new VTE after Apixaban initiation	No	No	Yes (PE)	No
Any new thrombosis after apixaban initiation	No	No	Yes (PE)	No
Switched to other anticoagulants (eg, warfarin)	No	No	Yes (Warfarin)	No

Published guidelines did not make a firm statement regarding the anticoagulation modality and duration in patients with thrombophilia due to limited evidence. The American Society of Hematology (ASH) has provided a conditional recommendation for a shorter course (3–6 months) for the primary treatment of unprovoked VTE in patients with more than one nonenvironmental risk factor for recurrent VTE. These risk factors included inherited thrombophilia, older age, and male gender. Following the primary treatment course, the ASH suggested indefinite antithrombotic therapy over stopping anticoagulation. Moreover, they recommended an annual visit to review the clinical indication for indefinite antithrombotic therapy as well as any complications for the secondary prevention of recurrent VTE.⁴

Secondary prevention of VTE with VKA could be challenging due to frequent laboratory testing, narrow therapeutic index, complex dosage regimens, and drug–drug or drug–food interactions. Apixaban has several advantages over warfarin as having similar efficacy profile with minimal risk of bleeding.¹² It could be an appealing option for patients with protein C or S deficiency requiring prolonged anticoagulation therapy. Protein S deficiency patients are known to have complex anticoagulation management that further complicate the appropriate choice of anticoagulants agent. Several reports evaluated the use of DOACs in patients with thrombophilia and showed relatively positive efficacy and safety outcomes. However, the majority of the available evidence in this field are originating from case studies and retrospective cohorts with numerous confounding factors.

Skelley et al conducted a systematic review to evaluate the use of DOACs in patients with hypercoagulable states related to thrombophilia.¹³ Six case studies were included in which dabigatran was used in one case, and rivaroxaban was used in the remaining five cases. Overall, all patients did not develop recurrent VTE episodes except one patient who developed a thrombosis in the popliteal and tibial veins. The only clinical trial included in that review was a post-hoc, sub-group analysis conducted on the data from the RE-MEDY trial. The authors concluded that the use of dabigatran in patients with thrombophilia demonstrated non-inferiority compared to warfarin in terms of recurrent VTE or VTE-related death.¹³ Consistent with our case series, a follow-up study conducted on 45 patients with severe inherited thrombophilia, found that DOACs were safe and effective for treating 18 patients with protein S deficiency with VTE.¹⁴ In these 45 patients, both rivaroxaban and apixaban showed no thromboembolism, stroke or major bleeding during the 29-month follow-up period. In agreement with this study, we reported no stroke events for at least six months of apixaban initiation in the four cases.

Additionally, none of our cases developed minor or major bleeding after apixaban use. In line with our report, a recent meta-analysis showed no difference in major bleeding between patients treated with DOACs compared to VKAs, including those with protein S deficiencies.¹⁵ This meta-analysis reviewed studies involving rivaroxaban, dabigatran, and edoxaban but did not include any study related to apixaban use in protein S deficiency.

On the other hand, a single-center cohort study was conducted on 56 VTE patients that included 12 patients with protein S deficiency in which apixaban was used in 25% of them.¹⁶ This study reported one non-fatal VTE that was observed in one patient on apixaban. Additionally, this study showed that major bleeding and clinically relevant non-major bleeding occurred in only six patients (10.7%), in which two of them were on apixaban.¹⁶ Similar to our conclusion, this study concluded that full-dose apixaban is effective and safe in patients with severe inherited thrombophilia including protein S deficiency. However, the degree of protein S deficiency needs to be assessed prior to apixaban use in order to accurately link it with the clinical outcomes. Further, large studies are warranted to explicitly investigate the safety and efficacy of apixaban in this subset of patients. Our case series is the first to describe the use of these agents in a Saudi individuals. Based on our reported cases, the use of apixaban as alternative to warfarin is feasible and might be safe and effective.

Conclusion

Based on the available emerging evidence and our case series, the use of apixaban could be effective in preventing recurrent thrombotic events in patients with inherited thrombophilia without safety concerns. Larger randomized clinical trials are warranted to confirm our finding.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

The study was approved by King Abdullah International Medical Research Center Institutional Review Board, Riyadh, Saudi Arabia (Reference No: NRC21R/550/12). Participants' confidentiality was strictly observed throughout the study by using anonymous unique serial numbers for each subject and restricting data only to the investigators. Written informed consent was obtained from the four patients for publication of this case series and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

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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.

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Mask Protection Against Lead Exposure and Its Correlation with Erythropoiesis in Automotive Body Painters at Ligu District, Semarang, Indonesia

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Mask Protection Against Lead Exposure and Its Correlation with Erythropoiesis in Automotive Body Painters at Ligu District, Semarang, Indonesia

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Introduction: Automotive body painters are at risk of excessive lead exposure that may compromise their health. This study examined the protective effects of masks against lead exposure and its correlation with blood profile of automotive body painters at Ligu district, Semarang, Indonesia.

Material and Methods: This cross-sectional study included 61 painters at Ligu district, Semarang, Indonesia (mean age 45 years, 100% male). Mask use was categorized into “frequent” and “infrequent”. Blood samples were taken to measure lead exposure and haematological parameters (haemoglobin/HGB, erythrocytes/RBC, haematocrit/HCT, mean corpuscular haemoglobin/MCH, mean corpuscular volume/MCV, mean corpuscular haemoglobin concentration/MCHC). Effectivity of mask use against lead exposure was analysed with Fisher’s exact test, while lead exposure and haematopoietic parameters were evaluated with Pearson’s correlation tests.

Results: There were 26 automotive painters (42.6%) classified as unfrequent mask users, and all of them had high blood lead levels. Most haematopoiesis parameters were in normal range. Workers with infrequent mask use were 1.269 more likely to be exposed to lead compared to those with frequent mask use (CI 95%, 1.082–1.552). Significant associations were found between lead exposure and RBC ($p=0.0$, $r=-0.53$), HGB ($p=0$, $r=-0.61$), and HCT ($p=0.00$, $r=-0.61$). No significant correlations were observed between lead exposure and MCV, MCH, MCHC.

Conclusion: Lead exposure was significantly associated with haematological parameters RBC, HGB, and HCT, confirming the effects of prolonged exposure on blood profile. However, mask use provided significant protection against lead exposure in automotive body painters and should be an obligatory gear for workers.

Keywords: mask, lead, haematopoiesis, automotive painters

Introduction

Lead is a common material found in various industries. Automotive painting, for instance, uses lead to improve paint quality in terms of brightness, solubility, and drying time.¹ As a highly toxic non-essential metal, lead comes with a number of environmental risks and health hazards.^{2–6} Regular exposure to lead may cause anaemia, kidney and liver damage.^{7,8}

The impact of lead exposure depends on the route, duration, and dose. Lead can enter the human body through ingestion, absorption, and most predominantly, inhalation (80%).⁹ Approximately half of lead exposure in children is obtained through ingestion, while it contributes to only 10–15% in adults due to more advanced hygiene practices.^{10–12}

Car painters are exposed to lead during the entire painting process, starting from sanding, priming with epoxy, mixing paint with thinner, paint spraying until finishing with varnish. Lead may enter the body through inhalation, absorption

through the skin, or ingestion by unhygienic hand-to-mouth transmissions. Lead is mostly absorbed through skin after 12 hours of constant exposure.¹³

In the body, lead is metabolized and circulated in blood, tissues, and bones. As a result, biomarkers for lead exposure include blood, soft tissues, bones, hair, urine, sweat, teeth, nails, and bile.¹⁴ The majority (95%) of absorbed lead is carried by the erythrocytes, making it the best marker to measure lead exposure.² Lead interferes with erythropoietin, a hormone that promotes erythropoiesis, thus causing hampered erythrocyte production.¹⁵ Lead also inhibits delta-aminolevulinate dehydratase (ALAD) enzyme which is involved in the formation of heme, consequently reducing haemoglobin levels and altering erythrocyte volume as seen from decreased haematocrit levels. Those changes can be observed from erythrocyte indices, particularly mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC). Erythrocyte indices are useful to identify anaemia.^{16–18}

For automotive body painters, mask usage may play a big role in the amount of lead exposure. Masks serve as a protective item against lead inhalation, but its use vary between individuals from regular, occasional, to never. The objective of this study is to examine the degree of protection from wearing masks against lead exposure and its correlation with haematopoietic systems in automotive body painters in Ligu district, Semarang, Indonesia. Ligu district is in the northern area of Semarang which is a heavily populated with automotive body painting as the main home industry of the region.

Materials and Methods

Design and Subjects

In this cross-sectional study, data was collected in automotive body painting workshops at Ligu district, Semarang, from December 2020 to March 2021. Subjects were randomly chosen according to the following inclusion criteria: (i) provided written consent, (ii) currently works as an auto body painter in workshops at Ligu district, Semarang. Exclusion criteria were (i) has worked in the workshop for less than 5 years (because they might get lead exposure from other places), (ii) has working hours less than 8 hours in total daily work load, and (iii) is consuming multivitamin supplements. Based on the inclusion and exclusion criteria, then 61 research subjects were obtained.

Ethical Consideration

As a scientific study involving humans, this study received ethical approval prior to data collection. In order to avoid conflicts of interest, ethical approval was obtained from the Health research ethics committee from other university, namely the Health Research Ethics Committee, Faculty of Medicine, Universitas Sultan Agung Semarang, with the number: 183/VII/2121/Komisi Bioetik. The ethical approval was given based on the results of an evaluation conducted by the ethics examiner team, which showed that this study had applied ethical principles of medical and health research, especially the Declaration of Helsinki which upholds autonomy, does not harm, maintains justice and benefits for the participants, such as: counseling based on the results of related laboratory examinations. All participants also agreed to be involved as research subjects by signing an informed consent, having previously been clearly informed about the purpose and procedures of this study.

Measurements

General Characteristics of Subjects

The general characteristics of subjects (age, sex, work period, working hours/day, room ventilation, exercise habit) were obtained using a questionnaire.

Mask Use

Mask use of automotive body painters at workshops in Ligu district were observed daily for a week. Afterwards, subjects were interviewed about their average mask use during work: always, sometimes, or never. The result from the interview is later categorized as “frequent” and “infrequent” (for workers who sometimes or never wear masks). Observations and interviews were conducted simultaneously as a cross check. This was done in order to minimize bias in data collection.

Lead Levels, Haemoglobin (HGB), Erythrocytes/Red Blood Cell (RBC), Haematocrit (HCT), MCV, MCH, MCHC

Blood samples were taken from the superficial veins of antecubital fossa and then stored in purple evacuated tubes (with K2EDTA). The samples were stored in a plastic transport box and sent to the BioMedical Laboratory Universitas Muhammadiyah Semarang for analysis using a haematology analyzer by photometry and electrical impedance. The results of laboratory tests were based on the following normal limits: Lead levels ($<40 \mu\text{g/dL}$), HGB ($13.3\text{--}16.7 \text{ g/dL}$), RBC ($4.5\text{--}5.7 \times 10^{12} /\text{L}$), HCT ($35\text{--}53\%$), MCV ($77\text{--}98 \text{ fL}$), MCH ($26\text{--}33 \text{ pg}$), MCHC ($33\text{--}37 \text{ g/dL}$). The measurement of blood lead was done at GAKI Laboratory Universitas Diponegoro using atomic absorption spectrophotometer (AAS).

Other Data

Additional data that were collected consisted of age, gender, work period, working hours/day, ventilation, and exercise habits. Ventilation was measured based on room area and window width which should be around 10–25%. Exercise habits were categorized as “active” when done twice a week and “not active” when only done once or not at all in a week.

Statistical Analysis

Categorical variables (age, gender, work period, working hours/day, ventilation, exercise habits) were described in numbers and percentage. The degree of protection from masks were analysed using Fisher’s exact test, while the association between lead exposure and haematopoietic system were evaluated with Pearson’s correlation tests.

Results

The characteristics of subjects are presented in Table 1. Long-term work period (>5 years) and long working hours (>8 hours) were specifically used as an inclusion and exclusion criteria to determine subjects. The 61 subjects were chosen as they fulfilled the inclusion criteria while the remaining four were excluded. We found that subjects with infrequent mask use had higher blood lead levels (100%, $n=26$) than those with frequent mask use (Table 2). In this case, the limit for high blood lead levels is 40 micrograms per deciliter.¹⁹ The risk of lead exposure was 1.296 times higher for infrequent mask users in comparison to frequent users ($p=0.016$).

Table 1 Characteristics of Subjects

Mean Age (Years)	44.23±10.33
Gender	
Male	61 (100%)
Female	0 (0%)
Work period	
≤5 years	4 (6.2%)
>5 years	61 (93.8%)
Working hours/day	
≤8 hours	4 (6.2%)
>8 hours	61 (93.4%)
Room ventilation	
Inadequate	44 (72.1%)
Adequate	17 (27.9%)
Exercise habit	
Not active	37 (60.7%)
Active	24 (39.3%)

Table 2 Relation of Blood Lead Concentrations and Mask Use in Automotive Body Painters

Mask Use	Blood Lead Levels				Total		p	RR	95% CI
	Normal (<40 µg/dL)		High (≥40 µg/dL)						
	n	%	n	%	n	%			
Infrequent	0	0	26	100	26	100	0.016	1.296	1.082–1.552
Frequent	8	22.9	27	77.1	35	100			

Most haematological parameters for all subjects were still within normal range, including MCHC (Table 3). Average blood lead levels were above normal values although it was still generally acceptable (Table 4). This refers to Decharat et al that the blood lead level that can still be tolerated is <60 micrograms per deciliter.¹⁹

Blood lead levels were significantly and strongly correlated with HGB, RBC, and HCT (−0.61, −0.53, −0.61), suggesting that higher lead exposure caused decreased levels of HGB, RBC, and HCT. On the contrary, no significant correlation was observed between blood lead levels and MCV, MCH, and MCHC.

Discussion

Results from this study affirmed that mask use largely affected the extent of lead exposure. Automotive body painters at Ligu district Semarang with infrequent mask use were 1.296 times more likely to be exposed to lead compared to those

Table 3 Haematological Parameters of Automotive Body Painters in Ligu District, Semarang, Indonesia

Parameters				Normal Limit
	Low	Normal	High	
HGB	5 (8.2%)	53 (86.9%)	3 (4.9%)	13.3–16.7 g/dL
RBC	20 (32.8%)	30 (49.2%)	11 (18.0)	4.5–5.7 × 10 ¹² /L
HCT	18 (29.5%)	43 (70.5%)	0 (0%)	35–53%
MCV	14 (23%)	41 (67.2%)	6 (9.8%)	77–98 fL
MCH	19 (31.1%)	34 (55.7%)	8 (13.1)%	26–33 pg
MCHC	0 (0%)	61 (100%)	0 (0%)	33–37 pg/dL

Table 4 Relation of Haematological Parameters with Blood Lead Levels

	Normal Limit	Average	Max	Min	Analysis		
					Correlation	p	r
Blood Pb	<40 µg/dL	42.99±4.53	52.3	27.4			
HGB	13.3–16.7 g/dL	14.01±1.33	16.1	11.3	Lead-HGB	0.00	−0.61
RBC	4.5–5.7 × 10 ¹² /L	4.94±0.61	5.98	3.99	Lead-RBC	0.00	−0.53
HCT	35–53%	42.14±4.11	48.6	34	Lead-HCT	0.00	−0.61
MCV	77–98 fL	28.53±2.42	33.3	22.7	Lead-MCV	0.63	0.06
MCH	26–33 pg	85.77±7.28	100.90	68.4	Lead-MCH	0.58	0.01
MCHC	33–37 pg/dL	33.25±0.39	35.9	32.5	Lead-MCHC	0.46	0.09

who frequently wore masks. This study also found a significant difference ($p=0.016$) between employees with frequent mask use and employees with infrequent use against lead exposure, further proving the importance of masks as a protective gear against pollutants including lead that can be absorbed through inhalation. Kuruvilla et al also reported that the use of activated carbon fabric (ASF) masks that successfully reduced lead exposure.²⁰

Our study had several limitations, particularly the type of masks. There is a wide variety of mask types, including disposable face masks, bandana masks, pre-shaped face masks, and full-face respirators.^{21,22} Each type provides different levels of protection against airborne contaminants, thus longitudinal studies with specified type of masks are required. The findings in this study are independent of our previous work where mask use had no significant correlation with cognitive deficits from lead exposure. Cognitive decline is considerably harder to measure as detectable signs may only appear after a long period of time even though lead exposure may exceed the tolerable level at present.²³

We found a strong negative correlation between blood lead concentrations with HGB, RBC, HCT. Inhalation is a major exposure route for lead since more than 40% of lead is absorbed into the respiratory system before getting distributed in blood. Approximately 95% of lead is attached to red blood cells and the rest is dissolved in plasma. Some lead is stored in tissues and bones while the excretion is primarily through kidney and digestive system.²⁴ Lead has been proven to disrupt heme synthesis and shorten the lifespan of erythrocytes, consequently decreasing HGB, RBC, and HCT concentrations. Lead interferes with the enzymatic activity in the beginning, middle, and end of heme biosynthesis. At the initial steps, lead inhibits δ ALAD enzyme, an enzyme that triggers heme biosynthesis.²⁵ As a result, the transformation of δ ALA into porphobilinogen (PBG) is blocked²⁶ and the concentration of δ ALA is increased in blood and urine. Lead also hinders coproporphyrinogen oxidase in the midway process of heme synthesis, leading to elevated coproporphyrinogen levels. The last enzyme affected by lead is ferrochelatase²⁷ which resulted in increased protoporphyrin concentration in red blood cells or known as free erythrocyte protoporphyrin.²⁸ In addition, lead has pro-oxidant activity that may incite oxidative stress in vulnerable erythrocyte membranes, thus reducing erythrocytes lifespan and eventually causing hemolytic anaemia.^{27,29}

No significant association was found between lead exposure with MCV, MCH, and MCHC. Blood lead levels in automotive body painters were generally high but still within tolerable range (40 $\mu\text{g/dl}$ -60 $\mu\text{g/dl}$). Value threshold for blood lead range 40 $\mu\text{g/dl}$. Similarly, all haematological parameters were also within normal range. Erythrocyte indices (MCV, MCH, MCHC) can be calculated from HGB, RBC, and HCT. The calculation of erythrocyte indices revealed the mean volume of erythrocytes and mean haemoglobin concentration per erythrocyte. Clinically, erythrocyte indices are used to diagnose and identify anaemia.

The results of this study are expected to provide justification that the use of masks as personal protective equipment for automotive painters is very important, because it has been proven to have an effect on lead levels in the blood which in turn can have an impact on HGB, RBC, HCT levels. Indeed, there were also changes in MCV, MCH, and MCHC levels; but still within a tolerable level. Logically, if the concentration of lead exposure studied was higher, it is possible that changes in MCV, MCH, and MCHC levels would exceed tolerable limits. Of course, to prove this, further research is needed involving automotive paint workers with a high duration and frequency of work or who are too ignorant to use masks as personal protective equipment.

Conclusion

In conclusion, the results of this study showed that automotive body painters who occasionally or never wear masks while working had higher risks for lead exposure than workers who always wear masks. We also affirmed that lead exposure significantly affected blood profile, particularly HGB, RBC, and HCT. Further longitudinal studies with specified mask types are required to be able to make recommendations of protective gears for automotive body painters.

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Disclosure

The authors declare no potential conflict of interest regarding the study and the publication of this paper.

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Packed Red Blood Cell Supernatants Do Not Promote Growth or Cisplatin Resistance of Myeloid Leukemia K-562 Cells

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Background: A decreased immune surveillance as a consequence of packed red blood cell (PRBC) transfusions has been linked to cancer recurrence and progression, but a causal mechanism remains unclear. During processing and storage of PRBC, numerous bioactive substances accumulate in the acellular fraction (supernatant) of PRBC.

Aim: The study aimed to determine whether the supernatant of leukocyte-reduced (LR) and non-leukocyte-reduced (NLR) long-stored PRBC can modulate the survival and proliferation of myeloid leukemia K-562 cells, and the influence of cisplatin (cisPt) on these processes.

Methods: Viability, proliferation, DNA damage, intracellular reactive oxygen species (ROS), caspase-3/7 and caspase-9 levels were determined in response to the LR or NLR, fresh (day 1) and long-stored (day 42) PRBCs.

Results: The supernatants of fresh (day 1) and stored (day 42) PRBC, in the absence and presence of cisPt, promoted apoptosis of K-562 cells via the increased production of reactive oxygen species (ROS) and increased level of DNA damage, which was manifested by the viability reduction and inhibition of K-562 cell proliferation. No significant influence of the pre-storage leukocyte-filtration and storage time of PRBC units on their anti-proliferative effect was demonstrated.

Conclusion: The findings may suggest that the PRBC acellular fraction does not affect chronic myeloid leukemia (CML) progression. However, these issues are pioneering and require further study.

Keywords: red blood cell, transfusion, myeloid leukemia, K-562 cells, cisplatin

Introduction

Anemia (defined as hemoglobin (Hb) levels below the normal value) is a common cancer-related complication, which can be caused by cancer itself, as occurs in blood malignancies as a result of the bone marrow infiltration by the malignant cells, and/or by a side effect of a high-dose chemotherapy.¹ Therefore, patients with some hematologic malignancies, such as acute myeloid leukemia (AML) require frequent packed red blood cell (PRBC) transfusions.² Although essentially safe, PRBC transfusion is often associated with the significant risks of severe post-transfusion reactions such as, among others, transfusion-related immune modulation (TRIM).³

A decreased immune surveillance as a consequence of blood transfusions has been linked to cancer recurrence and progression. Moreover, soluble factors present in PRBC can directly stimulate tumor growth and spread.⁴ An association between perioperative transfusion of allogeneic blood products and risk for recurrence has been proved in colorectal⁵⁻⁷ and a few other cancers,⁸ but the mechanism of this phenomenon has not been clarified yet. There are also some controversial studies not confirming such relationships in many types of cancer.⁸

Upon processing and routine storage, red blood cells undergo numerous biochemical and physiological changes with the concomitant release of numerous, potentially hazardous, bioactive substances (ie, extracellular Hb, heme, iron, proteolytic enzymes, pro-inflammatory cytokines, chemokines, immunomodulating and vasoactive mediators, lipids and microparticles (MPs), mainly released from erythrocytes) into the acellular fraction of the PRBC unit.⁹ Pre-storage leukoreduction reduces 3 logs (99.9%) leukocyte numbers¹⁰ which significantly decreases the accumulation of metabolites and cellular components into the PRBC unit.¹¹

K-562 is the human erythroleukemia cell line of CML in the blast phase having the Philadelphia chromosome (Ph), caused by the t (9:22) chromosomal reciprocal translocation which produces the *BCR-ABL1* fusion gene. The product of this gene, the chimeric BCR-ABL1 protein (p210^{BCR-ABL}) is characterized by deregulated, constitutively activated tyrosine kinase and plays an important role in the induction of CML.¹² Cisplatin, PtCl₂(NH₃)₂, (cisPt) is a cytostatic drug, commonly applied in laboratory research that has also been clinically used with some success in the chemotherapy of several types of malignant tumors.¹³ Cisplatin has been shown to induce apoptosis in CML cells.¹⁴

To the best of our knowledge, only a few previous reports have evaluated the response of cancer cells to PRBC.^{15,16} Moreover, no previous study has addressed its influence on the anti-proliferative effects of anti-neoplastics. This in vitro study was designed in a classic model system applying K-562 cells (widely used as a “workhorse” cell line) and cisPt (one of the most known anti-cancer drugs) which have commonly been used in biomedical research. The aim of the study was to determine whether the progressive changes occurring in long-stored PRBC (42 days established as an acceptable norm) can modulate the survival and proliferation of K-562 cells, and the influence of leukoreduction on these processes. To achieve this goal, we evaluated the effect of the acellular fraction (supernatant) of the fresh (day 1) and stored (day 42) PRBC, that had been pre-storage leukoreduced or the non-leukoreduced, on the viability and proliferation of K-562 cells, as well as on DNA damage, ROS levels, and apoptosis, in the absence and presence of cisPt.

Materials and Methods

Reagents

Cell Counting Kit-8 (CCK-8), cisplatin (cisPt), Cell Proliferation ELISA - BrdU colorimetric Kit (Roche), DAPI (4',6-diamidino-2-phenylindole dihydrochloride), 2',7'-dichlorofluorescein diacetate (H₂DCF-DA), penicillin-streptomycin solution, normal melting-point agarose (NMP), RPMI-1640 medium with L-glutamine, Triton™ X-100, hydrogen peroxide, and low melting-point agarose (LMP) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS), Hanks BSS (HBSS) without phenol red with calcium and magnesium were purchased from Biological Industries (Cromwell, CT, USA), and heat-inactivated fetal bovine serum (FBS) from Biowest (Nuaillé, France). All other analytical grade and high-quality chemicals were obtained from local commercial suppliers, such as Chempur (Piekary Slaskie, Poland) or POCH S.A. (Gliwice, Poland).

Packed Red Blood Cells

Five SAGM-preserved PRBC transfusion units were purchased from the Regional Blood Donation and Blood Treatment Centre in Lodz (Poland). Each of the SAGM-preserved non-leukoreduced (NLR; leukocytes <10⁹/unit) PRBCs, prepared in accordance with the standard procedures currently applied in blood banks, were divided into four equal aliquots by transferring them to the transfer bags in a closed system. Leukocytes from two transfer bags were removed during the gravity filtration process using a leukocyte depletion filter (BioR Flex, Fresenius Kabi AG, Bad Homburg, Germany) to obtain the pre-storage leukoreduced PRBC (LR; leukocytes <10⁶/unit), the other two transfer bags were left unfiltered. All processes were carried out under sterile conditions in the Regional Blood Donation and Blood Treatment Centre (Lodz). The NLR and LR PRBC had been stored, for 1 day or 42 days, at 4±2°C. This study was approved by the University of Lodz Research Ethics Committee (no. 26/KBBN-UŁ/I/2017).

Supernatants from Stored PRBC

Supernatants from PRBC, both NLR and LR, on day 1 (sNLR1 and sLR1, respectively) and on day 42 of storage (sNLR42 and sLR42) were obtained in accordance with the method described by Westerman et al.¹⁷ Briefly, PRBC units were centrifuged at 2000 g for 10 min at 4°C, the supernatants were removed and spun at 3000 g for 10 min at 4°C. Supernatants received by this method contain erythrocyte-derived microparticles (EMPs). Supernatants from PRBC were filtered through a syringe PES filter membrane with pore size 0.22 µm (TPP Techno Plastic Products AG, Switzerland), aliquoted, and stored at -70°C.

Cell Culture and Treatment

Human chronic myelogenous leukemia cell line (K-562; ATCC® CCL-243™) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin/mL) in a humidified atmosphere with 5% CO₂ at 37°C.

For each experiment, cells were seeded on 96-well tissue culture plates and grown for 24 h in a complete culture medium supplemented with 5% (in some experiments also 10 and 20%) by volume of PRBC supernatants (sNLR1, sLR1, sNLR42, sLR42) or with medium alone as a control, in the presence or absence of cisPt. The optimal concentration of cisPt (10 µM) used in experiments was determined experimentally. First, IC₅₀ was determined for the K-562 cell line, then a 2-fold lower cisPt concentration ($\frac{1}{2}$ IC₅₀) was finally used in each experiment.

Cell Viability

The colorimetric assay, cell counting kit-8 (CCK-8), was conducted to assess cytotoxicity after treatment with the supernatants. K-562 cells (5000 per well) were grown in 96-well plates for 24 h, then the supernatants and cisPt (where needed) were added to fresh culture medium in the plates. After 24 h, cells were washed twice with DPBS, then 100 µL of fresh media and CCK-8 solution (10 µL) were added to each well, followed by incubation for 4 h, at 37°C with 5% CO₂. The absorbance at 450 nm was determined in the microplate reader SPECTROstar® Nano (BMG LABTECH GmbH, Ortenberg, Germany). Cell viability was expressed as a percentage of the control (untreated) cells.

Proliferation Assay

For the quantification of K-562 cell proliferation, the bromodeoxyuridine (BrdU) proliferation assay was performed with a commercially available kit (BrdU colorimetric Kit, Roche) following the manufacturer's instruction. The absorbance at 370 nm and 492 nm (reference wavelength) were measured in the microplate reader SPECTROstar® Nano. Cell proliferation was expressed as a percentage of the untreated cells.

Comet Assay

The genotoxic effect of PRBC supernatants was assessed by the comet assay performed under alkaline conditions according to the procedure of Singh et al.¹⁸ with some modifications.¹⁹ K-562 cells were incubated for 60 min at 37°C with 5 and 20% of the supernatants. Cells suspended in fresh culture medium served as a negative control. Positive control was prepared by treating cells with 10 µM hydrogen peroxide. After incubation, supernatants were removed by centrifugation (180 g, 15 min, ambient temperature). Pellet of cells was suspended in 1.0% LMP agarose and spread onto microscope slides pre-coated with 0.5% NMP agarose. Next, the cells were lysed for 1 h, at 4°C in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10). All remaining steps including electrophoresis, DAPI comet staining, and measuring were performed according to Czubatka et al.²⁰

ROS Level

The intracellular ROS levels were measured using the spectrofluorimetric method with the H₂DCF-DA fluorescent probe.²¹ K-562 cells (5000 per well) were centrifuged (1500 g, 5 min) and then the pellet was suspended in HBSS with 10 µM H₂DCF-DA (30 min, 37°C, 5% CO₂). The fluorescent probe was removed, and cells were washed twice with HBSS. Cells were re-suspended in the washing buffer and seeded on a 96-well black bottom microplate. The PRBC

supernatants and cisPt (where needed) to the final concentration of 10 μM ($\frac{1}{2}$ IC_{50}) were added into wells. Cells treated with 10 μM H_2O_2 were used as positive controls, cells suspended in HBSS (untreated) as negative controls. After incubation (60 min, 37°C), fluorescence intensity was read at $\lambda_{\text{ex}} = 485$ nm excitation and $\lambda_{\text{em}} = 538$ nm emission (Fluoroskan™ Microplate Fluorometer; Thermo Scientific™).

Activity of Caspases 3/7 and 9

Caspase-3/7 and 9 enzymatic activities in K-562 cells were assessed using the commercial fluorescent apoptosis system, Apo-ONE® Homogeneous Caspase-3/7 Assay, and luminescent assay, Caspase-Glo® 9 Assay (Promega; Madison, USA), respectively. The assays were performed according to the manufacturer's instructions. Cells were seeded (20,000 and 40,000 cells/well, respectively) on 96-well black or white bottom microplates, respectively. The culture medium served as the blank ("background"), untreated cells as the negative control. The positive control was also performed using cells supplemented with cisPt (apoptosis inducer). Cells incubated with cisPt and an inhibitor of caspase activity Z-VAD-FMK (final concentration 20 μM) served as the additional control. The fluorescence was measured at $\lambda_{\text{ex}} = 485$ nm excitation and $\lambda_{\text{em}} = 538$ nm emission (Fluoroskan™ Microplate Fluorometer; Thermo Scientific™) after the profluorescent Z-DEVD-R110 substrate addition. Whereas the luminescent signal was read using the Synergy™ HTX Multi-Mode Microplate Reader (BioTek; Winooski, USA) after the addition of the luminogenic substrate containing the LEHD sequence with a proteasome inhibitor (MG-132).

Statistical Analysis

All obtained results are presented as mean values $\pm$ SD. Using Shapiro–Wilk test, the normality of the results was analyzed. Then, the non-parametric Levene's test for homogeneity of variance was performed. Based on the Levene's test (ANOVA) followed by post-hoc Tukey's test the differences between values were evaluated. All obtained data were analysed using StatSoft Inc. "Statistica" v. 13.1. The values of $p < 0.05$ are considered to be statistically significant. All presented figures were prepared using GraphPad Prism 5 Software.

Results

As shown in Figure 1A and B, a significant reduction of the percentage of viable K-562 cells after 24-h culture with the supernatants has been noted, at doses 10 and 20% v/v, compared to untreated control cells (by 60–67%; $p < 0.001$). Moreover, these supernatants' doses significantly augmented the cytotoxic effect of cisPt against K-562 cells. Exposure to 5% sNLR1 and sNLR42 resulted in an approximately 2-fold slighter cytotoxic effect compared to the control (Figure 1C). Since experiments carried out with 2% supernatants did not prove statistically significant changes in cell survival (data not shown), the dose of 5% was finally taken as optimal for further experiments.

Subsequently, the supernatants (sNLR1, sLR1, sLR42) have been found to significantly inhibit (by above 25%; $p < 0.05$) cell proliferation when assessed by BrdU incorporation (Figure 2). Cells cultured in the presence of cisPt and supernatants isolated from unfiltered units of PRBC showed more than 2-fold reduced proliferative capacity ($p < 0.01$) compared to cells treated with cisPt. Cisplatin alone inhibited the proliferation of K-562 cells by about 50% ($p < 0.001$) compared to control.

The amount of intracellular ROS was determined using the $\text{H}_2\text{DCF-DA}$ fluorescent probe. K-562 cells exposed to sNLR42, sLR1 and sLR42 showed an increased ROS level by 55% ($p < 0.01$), 67% ($p < 0.01$) and 42% ($p < 0.05$), respectively, compared to untreated cells (Figure 3). In the experimental system in which cells were treated with cisPt, also an increase in ROS levels (by above 30%; $p < 0.05$) was observed in the presence of sNLR42 and sLR1. Cisplatin-induced ROS generation in K-562 cells was to a degree approximately 50% greater than that in control.

The degree of DNA damage (ie single and double-strand breaks and alkali-labile sites) can be detected by the alkaline comet assay. As shown in Figure 4, supernatants from the filtered PRBC units (5% v/v), especially sLR1, caused above 5-fold ($p < 0.01$) increase of the percentage of "tail" DNA compared to control, while higher concentrations (20% v/v) of sNLR1, sLR1, and sLR42 turned out to be more genotoxic for cells (a 6–8-fold increase of the total DNA damage levels).

DNA damage, mostly double-strand breaks, if not repaired, is known to trigger apoptotic cell death. Activities of caspase-3/7 in K-562 cells treated with sNLR1, sNLR42, and sLR1, as well as caspase 9 in cells exposed to sNLR1 and

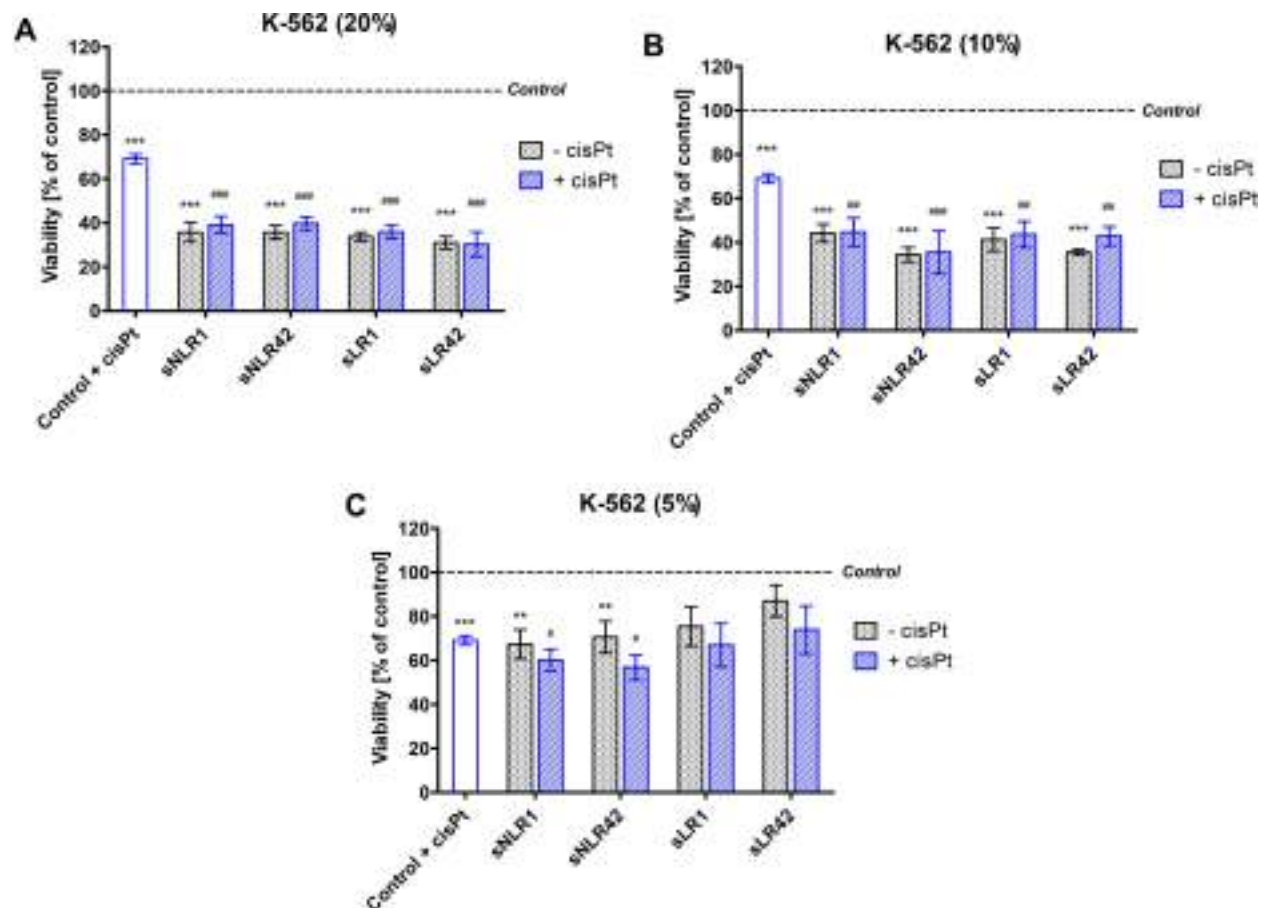


Figure 1 Viability of K-562 cells after incubation (24 h) with different concentrations of the PRBC supernatants in the absence or presence of cisPt (10 µM) evaluated using the CCK-8 assay. The figure shows mean values from 5 independent experiments (A and B) and 10 independent measurements for (C). All data is presented as a percentage of control (untreated cells - assumed as 100%). Error bars denote $\pm$ SD; # $p < 0.05$ vs control with cisPt, ** $p < 0.01$ vs control (untreated cells), *** $p < 0.01$ vs control with cisPt, **** $p < 0.001$ compared to the appropriate control.

sLR1 were found to be elevated (by approximately 36%; $p < 0.05$ and above 25%; $p < 0.001$), respectively (Figure 5A and B). The anti-cancer drug cisPt significantly activated caspase-3/7 (by approx. 48%; $p < 0.05$) and caspase-9 (by approx. 40%; $p < 0.001$), while the caspase inhibitor Z-VAD-FMK inhibited their activity. In the presence of cisPt, supernatants from PRBC units on day 1 of storage also increased the activity of both caspase 3/7 and 9, compared to cells cultured with cisPt alone.

The effect of supernatants on the survival of K-562 cells in the presence of Z-VAD-FMK was determined (CCK-8 assay). The viability of cells cultured in the presence of supernatants and the inhibitor, both with and without cisPt, was similar to that of untreated cells.

Discussion

Numerous biological mediators (eg as cytokines, eicosanoids, growth factors) that have pro-tumoral activity have been identified in the acellular fraction of PRBC, proposing that it may have tumor-promoting effect,¹⁰ therefore it was reasonable to hypothesize that PRBC would increase growth and proliferation of cultured cancer cells. Unexpectedly, our studies demonstrate that PRBC supernatants substantially decreased the survival of K-562 cells in a commercial viability assay (Figure 1), and they also inhibited the proliferative capacity of K-562 cells (Figure 2). These cytotoxic effects against the cancer cells were found to be independent on the PRBC ex vivo storage duration and leukocyte depletion. Similar cytotoxic effects have also been noted in the presence of the anti-cancer drug (cisPt). Further, intracellular ROS levels were determined, to clarify whether these could be involved in inhibiting the growth and proliferation of cancer

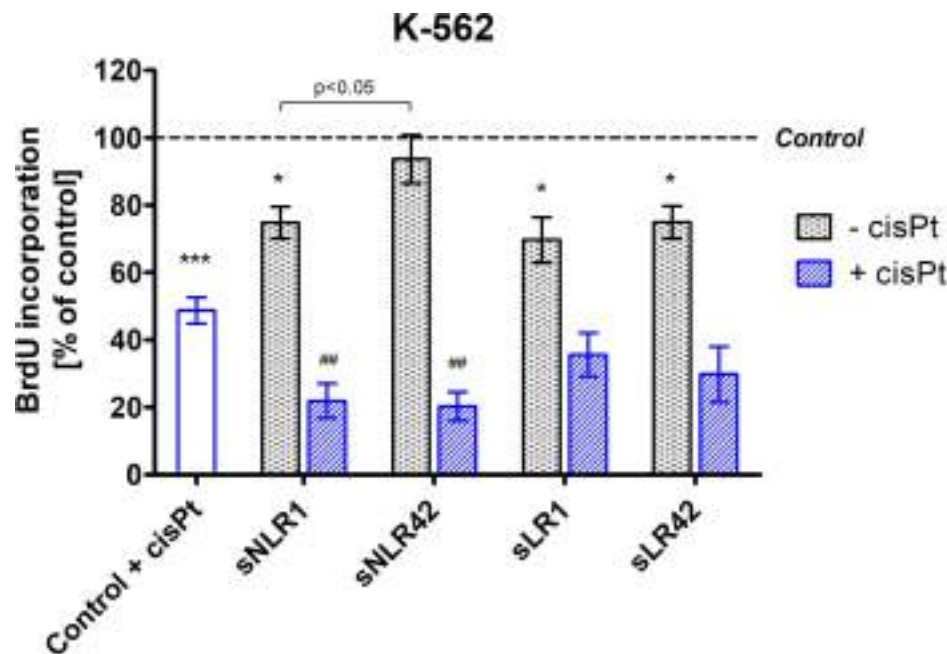


Figure 2 Proliferation of K-562 cells, assessed by the BrdU incorporation during DNA synthesis, after incubation (24 h) with the PRBC supernatants (5%) in the absence or presence of cisPt (10 μ M). All data is presented as a percentage of control (untreated cells - assumed as 100%). Error bars denote $\pm$ SD, n = 3 (independent experiments); *p<0.05 vs control (untreated cells), ##p<0.01 vs control with cisPt, ***p<0.001 vs control (untreated cells).

cells grown in a medium supplemented with the supernatants (5%), which confirmed that indeed the supernatants induced the excessive ROS generation (Figure 3). The elevated ROS in cells may contribute to the development of DNA damage (and other cellular biomolecules) and hyperactivation of ROS signaling pathways leading to cell death. A tendency to

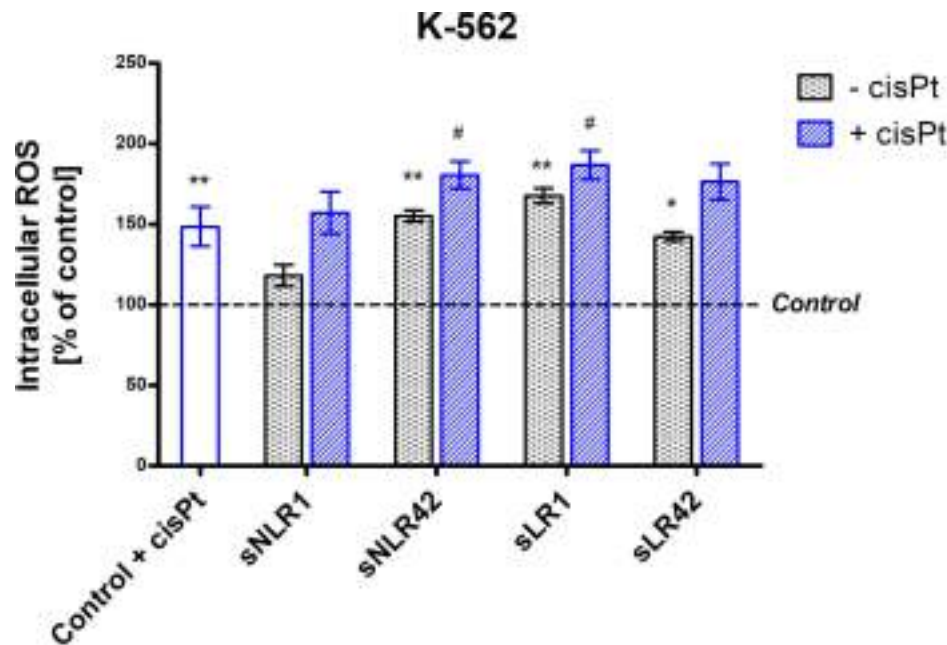


Figure 3 The intracellular ROS level in K-562 cells after incubation (60 min, 37°C) with the PRBC supernatants (5%) in the absence or presence of cisPt (10 μ M). All data is presented as a percentage of control (untreated cells - assumed as 100%). Error bars denote $\pm$ SD, n = 3–5 (independent experiments); */##p<0.05 compared to the appropriate control, **p<0.01 vs control (untreated cells).

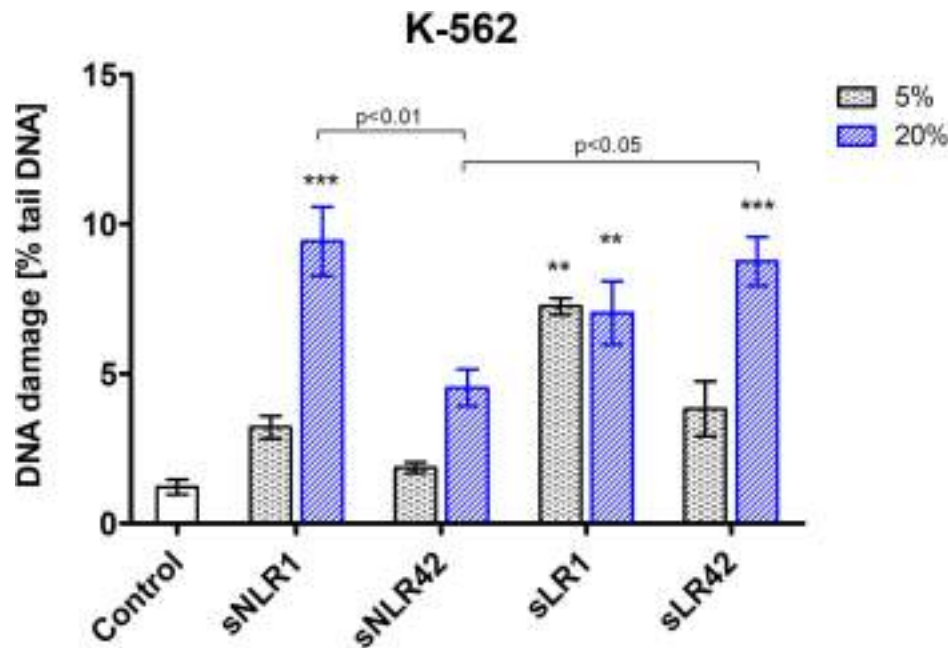


Figure 4 The DNA damage, measured as a percentage of tail DNA in the alkaline comet assay, in K-562 cells pre-incubated for 1 h at 37°C with the PRBC supernatants (5% and 20% v/v). All experiments included a negative (untreated cells) and positive (10 μ M H₂O₂) control. Error bars denote $\pm$ SD, n = 3 (independent experiments); **p<0.01, ***p<0.001 compared to untreated cells.

increases the DNA damage levels after incubation of cells with the lower supernatant concentration (5%), as well as much stronger genotoxic effect of the supernatants at their higher dose (20%), were observed (Figure 4).

Many intra- and extracellular factors can regulate the apoptosis process. The elevated levels of DNA damage (mostly double-strand breaks, if not properly repaired by the cellular DNA repair mechanisms) and/or excessive intracellular ROS, among others, are common factors inducing apoptosis.^{22,23} Caspases (cysteine-aspartic proteases) are a family of protease enzymes essential for the execution of programmed cell death. Caspase-9 is involved in the initiation of the apoptotic process in the endogenous mitochondrial pathway. Its activation mediates the excitation of caspase-3 and 7, which act as effectors in the executive phase of the apoptotic process.^{22,23} Supernatants, regardless of the preparation method, both in the presence and absence of cisPt, were shown to enhance the apoptosis (Figure 5A and B), which was additionally confirmed by inhibiting the activity of caspases (CCK-8 assay performed in the presence of Z-VAD-FMK, an inhibitor of caspases) (Figure 5C).

Nowadays, in the era of the second-generation tyrosine kinase inhibitors (TKI), cisPt is not routinely used in the treatment of CML or AML. However, cisPt and its analogues have been widely used in the treatment of various types of human cancer, both as monotherapy and in combination with other drugs.¹³ Cisplatin is also commonly applied in laboratory research as a cytotoxic drug that kills cancer cells by a direct interaction with DNA through the formation of intra-strand bonds that inhibit replication, leading to cell cycle arrest and then apoptosis.^{13,24} Cisplatin-induced DNA damage activates various signaling pathways to prevent or promote cell death. The mechanism of the anti-tumor activity of cisPt is not fully understood. Only 1% of the cisPt present in a cell has been shown to bind to nuclear DNA. The remaining percentage reacts with membrane and cytoplasm components, which indicates that the drug may also exert a cytotoxic effect through mechanisms independent of nuclear DNA binding,²⁵ eg, by inducing the production of ROS.²⁶ Our data confirmed the involvement of ROS in the mechanism of the drug cytotoxic action on K-562 cells (Figure 3). Moreover, PRBC supernatants had the slight effect (Figure 1) or potentiated (Figures 2, 3, 5A and B) the cytotoxicity of cisPt against K-562 cells.

The results indicate that one of the possible mechanisms responsible for the cytotoxic effect of the supernatants on K-562 cells is the excessive oxidative stress (OS), which can result from the presence of Hb degradation products in the PRBC unit. Due to the innovative nature of the study and the lack of appropriate references, the interpretation of the

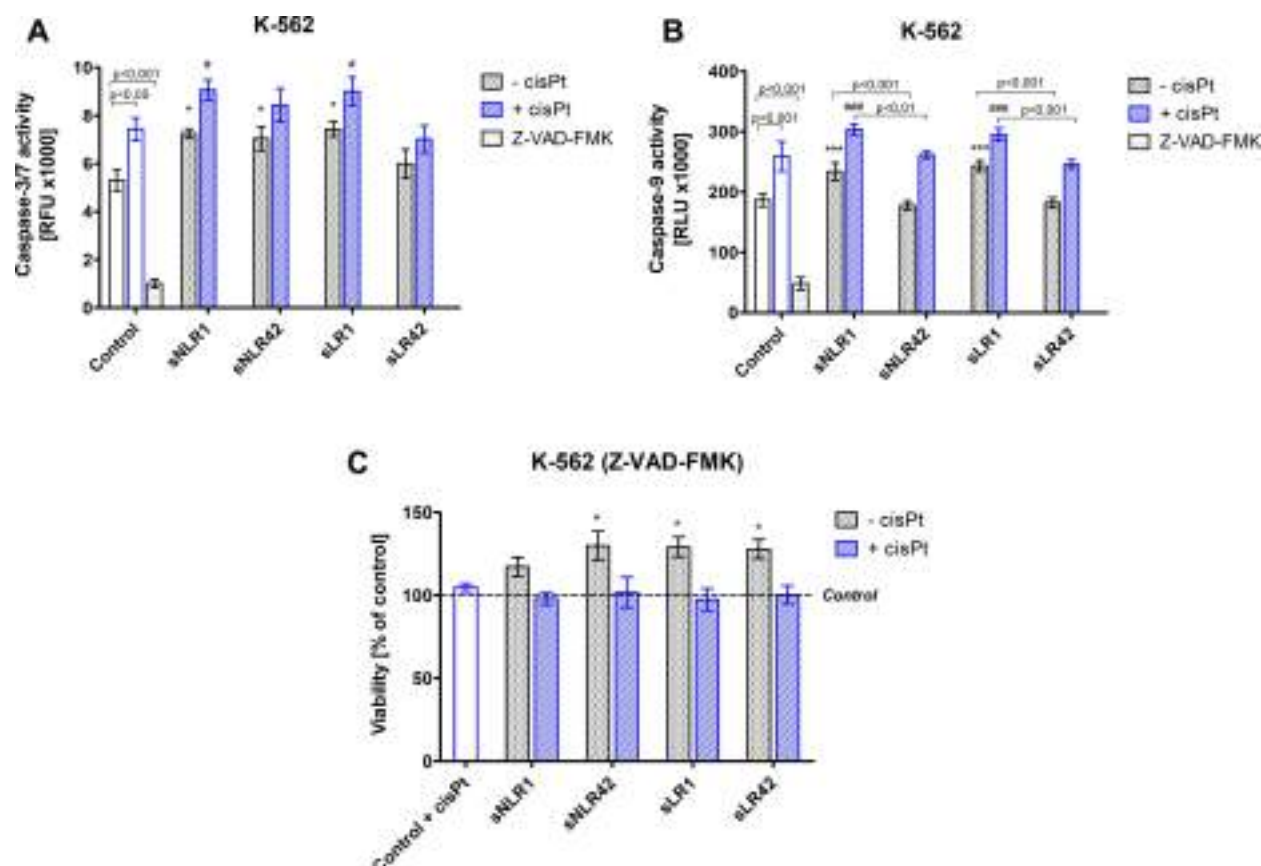


Figure 5 Activities of caspase-3/7 (**A**) and caspase-9 (**B**) in K-562 cells treated (24 h) with the PRBC supernatants (5%) in the absence or presence of cisPt (10 μ M). (**C**) shows K-562 cells viability in the presence of caspase inhibitor Z-VAD-FMK, PRBC supernatants, and cisPt. Data obtained from viability measurements are presented as a percentage of control (untreated cells - assumed as 100%). Error bars denote $\pm$ SD, $n = 3$ (independent experiments); * $p < 0.05$, *** $p < 0.001$ compared to the appropriate control.

results and determination of their clinical relevance is difficult. Free Hb, heme, and iron, delivered to the recipient's circulation with the transfused PRBC, are sources of non-transferrin bound iron (NTBI) and may be toxic,²⁷ eg by participating in ROS generation.^{27,28} Chronic OS and the accompanying inflammation are well-known factors influencing the progression of neoplastic disease.²⁹ Compared to normal cells, cancer cells are characterized by much higher ROS levels, due to, among others, mitochondrial dysfunction and increased metabolism. On the other hand, very high concentrations of intracellular ROS are extremely toxic to cells, which can be used in anti-cancer therapies based on either the inhibition of the cellular antioxidant defense or the enhancement of ROS production.³⁰

The role of OS in the progression of hematopoietic malignancies (including CML, AML) has been well documented but is still not fully understood.³¹ Our results remain inconsistent with the data published by Barnett et al¹⁵ who showed that supernatants of long-stored (unfiltered and filtered) PRBC increased proliferation and migration of mouse pancreatic cancer cells (Pan02), and when administered intravenously contributed to pancreatic cancer progression in mice. The inconsistency with the obtained results may be related to the use of a completely different line of cancer cells, additionally of mouse origin. It is possible that substances present in PRBC supernatants may have different effects in different types of cancer. Moreover, Zhuang et al reported the effect of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), accumulating in PRBC, on the proliferation of human hepatocellular carcinoma cells (HepG2).¹⁶ The authors demonstrated that the supernatants of unfiltered RBCs increased the proliferation of these cells.¹⁶ The discrepancy between their and our results may result from not only different cell lines used but also from other differences in experimental systems (cells were incubated with supernatants for 48 h and no information was provided on the amount of supernatant used in the study). In our studies, the incubation time of cells with supernatants

(24 hours) was selected due to the population doubling time, which in the case of K-562 cells ranged between 18 and 34 hours. This time, however, may have been too short to properly assess the pro-proliferative properties of the supernatants. In all the conducted experiments, a direct influence of SAGM on the tested parameters was excluded. Since we used the syringe filters with a pore size of 0.22 μm before freezing the supernatants used in the study, the participation of microparticles in the effect of the supernatants on cells can also be largely excluded. Tayer et al³² also used 0.22 μm filters in their studies to remove MPs from samples.

Possibly, some antitumorigenic cytokines, ie, interferons, IL-4, IL-6, IL-12, TNF- α , TGF- β ,³³ are also responsible for the cytotoxic effect of the supernatants. These cytokines are pleiotropic and depending on the concentration or stage of the disease, they may have different properties. For example, their low concentrations may initiate the growth of neoplastic cells, and high concentrations inhibit them. In the supernatants of unfiltered RBCs, no effect of storage on the increase of IL-6 concentration was demonstrated.³⁴ The Generally accepted theory of “cytokine fields” in the tumor microenvironment, defining the ratio of the amount of Th2 and Th3 cytokines in relation to Th1, allows to some extent to understand the role of these protein mediators in neoplastic diseases.^{35,36}

The results of the current study indicate that the storage time of PRBC and leukocyte depletion did not significantly influence the response of K-562 cells to the supernatant. In agreement with these in vitro findings, in the study on patients diagnosed with cancer, Kekre et al³⁷ reported no impact of the transfused PRBC storage duration on OS or cancer recurrence. Filtration does not fully prevent the accumulation of some inflammatory agents in clinically relevant concentrations in the supernatant. Therefore, the presence in supernatants of polyunsaturated fatty acids and their oxidation products, demonstrated both in unfiltered and filtered units, on the 42 day of storage³⁸ may be responsible for their cytotoxicity towards K-562 cells. In the fresh PRBC the concentration of 12-HETE (involved in such processes as oxidative stress, inflammation, and neoplastic disease) does not differ between filtered and unfiltered units.³⁹

Conclusion

To sum up, the supernatants in both experimental systems (in the absence and presence of cisPt) promoted apoptosis of K-562 cells via the pro-oxidative effect (increased ROS production and consequently increased genotoxicity), which was manifested by the viability reduction and inhibition of K-562 cell proliferation. No significant influence of the pre-storage leukocyte-filtration and storage time of PRBC units on their anti-proliferative effect was demonstrated. These findings may suggest that the PRBC acellular fraction does not affect the CML progression. However, at the current phase of the research our results may not be of much relevance from a clinical point of view. This issue requires further study. One of the important questions remains whether PRBC supernatants can modulate the anti-proliferative effects of other anti-cancer drugs, approved to treatment of AML/CML either in modern therapies (imatinib and other second-generation TKI) or in traditional cytotoxic chemotherapy (anthracycline, cytarabine, omacetaxine, etoposide, cladribine, or fludarabine).

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Disclosure

The authors report no conflicts of interest in this work.

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Relationship Between Initial Parenteral Iron Therapy Dosing and Treatment Effectiveness: A Real-World Retrospective Analysis

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Purpose: Replacement iron is the main treatment for iron deficiency, but the relationship between initial intravenous (IV) dose and need for additional treatment is unclear. This study explored patterns of IV iron dosing in US clinical practice.

Methods: Patient records were obtained for adults who received IV iron for anemia between 2015 and 2017. Patients were classified into four groups: those who received <1500 mg and ≥1500 mg IV iron and those received ≤1000 mg and >1000 mg within 3 weeks of their first dose. The proportion of patients requiring additional IV iron after 30 days of the initial dose was evaluated.

Results: Data were obtained for 2959 patients receiving iron sucrose (44.2%), ferric carboxymaltose injection (FCM) (25.8%), and ferumoxytol (FM) (14.3%). Overall, 567 patients (19%) received ≥1500 mg of IV iron and 942 (32%) received >1000 mg of IV iron within the first 21 days. Mean (SD) baseline iron deficit was 1001 mg (312). Patients who received ≥1500 mg had a 32% lower probability of receiving additional IV iron than those who received <1500 mg (adjusted hazard ratio [HR]: 0.68 [95% confidence interval (CI): 0.58, 0.81]) and incurred significantly fewer outpatient visits for all causes ($p < 0.001$) and IV iron treatment ($p < 0.001$). Patients who received an initial dose of >1000 mg had a 41% lower probability of receiving additional IV iron than those who received ≤1000 mg (adjusted HR: 0.59 [95% CI: 0.52, 0.67]) and had significantly fewer outpatient visits for all causes ($p < 0.001$) and IV iron treatment ($p < 0.001$). Patients receiving FCM required fewer outpatient visits than those receiving FM and other treatments, including a subgroup of patients who initially received >1000 mg IV iron.

Conclusion: Higher doses of IV iron within 3 weeks of first dose may reduce further IV iron treatment needs and outpatient visits.

Keywords: anemia, ferric carboxymaltose injection, ferumoxytol, iron sucrose

Introduction

Iron deficiency anemia is the most prevalent form of anemia globally and can occur in patients with chronic diseases and inflammation, sometimes alongside anemia of chronic disease (ACD).^{1–3} Improved diets and living standards have resulted in the global burden of iron deficiency anemia decreasing over time, from 11.2% of all years of life with disability in 1990 to 8.8% in 2010.³ Anemia is now most prevalent in women of reproductive age (primarily due to menstruation) and in children, and a high prevalence of iron deficiency anemia also exists in people with gastrointestinal disorders.^{4,5} Symptoms of anemia include weakness and fatigue, and reduced aerobic capacity, which can impact work performance.^{3,6,7} If not properly treated, severe anemia can lead to hypoxia, potentially resulting in organ damage.⁸

Replacement iron is the mainstay therapy for iron deficiency anemia.^{5,8,9} Oral iron therapy is often prescribed for iron deficiency anemia, but its effectiveness is diminished and uptake from the gut is limited in patients with gastrointestinal diseases. Although blood transfusion is an effective treatment in patients with active bleeding, it only offers a temporary solution and does not address the underlying problem for iron deficiency anemia.⁵ Consequently, parenteral (intravenous [IV]) iron therapy is often necessary for patients with iron deficiency anemia associated with chronic diseases (often

gastrointestinal diseases that make oral iron supplements ineffective), those with a large or sustained loss of iron, and those who cannot tolerate oral iron.^{5,8,10}

Parenteral iron therapies that are currently available in the US vary in dose and administration schedule, and include ferric carboxymaltose (FCM), ferumoxytol (FM), iron sucrose, ferric derisomaltose, sodium ferric gluconate, and iron dextran.¹¹

Currently, data on the association between initial IV iron dose given, durability of treatment effectiveness, and the risk of iron deficiency relapse are limited. In a previous investigation of initial IV iron dose and retreatment in patients treated with iron isomaltoside (Scandinavian NIMO study),¹² an initial >1000 mg dose of iron isomaltoside significantly reduced the need for retreatment up to 12 months and had a higher treatment response rate compared to an initial dose of 1000 mg or lower.¹² However, the evaluation was limited to a single IV iron product in a study sample mostly composed of patients with inflammatory bowel diseases. It remains unclear if the findings are generalizable to a broader patient population receiving parenteral iron replacement therapy in US clinical practice.

The objective of this analysis was to characterize patterns of IV iron dosing in US clinical practice and examine the association between initial IV iron dose and real-world treatment effectiveness as assessed by the need for additional IV iron treatment and serum hemoglobin normalization.

Methods

Patient Data Source and Eligibility

Records data were obtained from the DRG real-world evidence (RWE) data repository US database; this database includes medical and pharmacy claims and electronic health record (EHR) data for >300 million patients from 2011 to the time of the study, covering approximately 98% of US health plans. Data from all sources is linked via a direct-matching algorithm and then de-identified at the patient level, enabling longitudinal tracking of patients across data sources and sites of care. Linked claims data and EHRs were available for approximately 75 million patients.¹³

Patients were included if they were adults (aged ≥ 18 years) who received IV iron between 1st March 2015 and 28th February 2017 and had anemia, defined as a baseline serum hemoglobin (Hgb) level below normal (<12 g/dL for females and <13.5 g/dL for males) in the 30 days before or on the date of the index (first) IV iron claim. Patients were excluded if they had received IV iron within 6 weeks of index date, were on dialysis, or if their total iron dose exceeded 4000 mg (inclusive of index dose) over 21 days. Iron doses in excess of 4000 mg were deemed an unlikely and unsafe amount of iron, indicating a potential error in data reporting.

Analysis

The design of the study is summarized in Figure 1. Iron deficit was estimated using the modified Ganzoni equation.¹⁴ Eligible patients were classified into four dose groups (not mutually exclusive): 1) patients with a total IV iron dose ≥ 1500 mg, 2) patients with a total IV iron dose <1500 mg, 3) patients receiving a total IV iron dose of >1000 mg and 4) patients with a total IV iron dose ≤ 1000 mg, within 21 days of 1st dose. The 21-day cut-off was chosen because the US prescribing information for FM and FCM recommend completing a full course within 7–14 days; a further 7 days were allowed in order to account for potential scheduling issues arising in the real-world setting. In addition, patients were classified into three mutually exclusive treatment groups based on the index IV iron product that they received: 1) patients who received FCM; 2) patients who received FM; and 3) patients who received other IV iron products except FCM and FM.

Treatment effectiveness was assessed by the proportion of patients achieving normalized serum Hgb level within 12 months of index dose, defined as ≥ 12 g/dL for females and ≥ 13.5 g/dL for males. Durability of iron replacement therapy was operationally defined as the absence of additional IV iron dose 30 days after initial dose. As such, the proportion of patients receiving additional IV iron after 30 days of the initial dose was compared between dose groups of ≥ 1500 mg vs <1500 mg initial IV iron dose using Cox proportional hazards regression, adjusted for comorbidities, baseline serum Hgb and baseline iron deficit level. In addition, mean number of all-cause outpatient visits and visits associated with IV iron

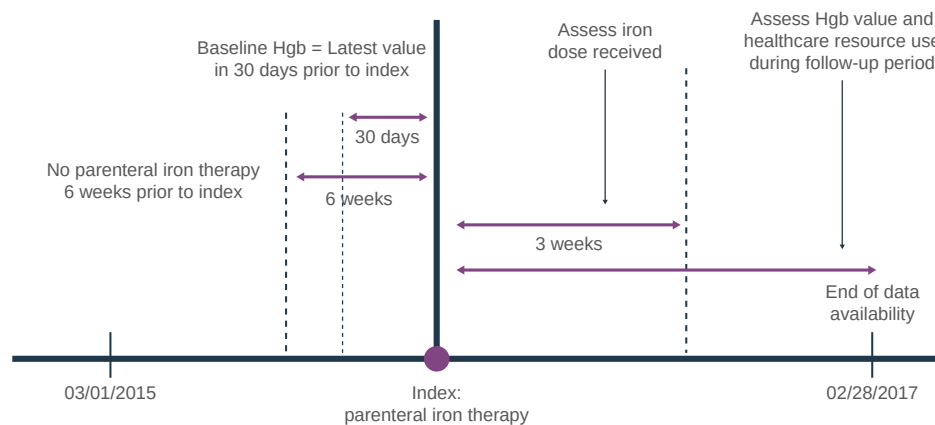


Figure 1 Study design.

Abbreviation: Hgb, hemoglobin.

infusion were assessed. Similar analyses were performed comparing patients who received >1000 mg vs ≤ 1000 mg initial IV iron dose.

Ethical Considerations

This study was conducted in accordance with ethical guidelines outlined in the Declaration of Helsinki. The research protocol followed in this retrospective study used de-identified secondary administration claims and electronic medical records (EMRs) that were collected for routine medical care and billing purposes. These data were compliant with the Health Insurance Portability and Accountability Act (HIPAA) Privacy rule, and thus the study did not require institutional review board (IRB) review. The claims and EHR data are linked together by a HIPAA-compliant encrypted patient key generated by a third party. It was not possible to contact patients to obtain informed consent as the data were de-identified.

Results

Patient Characteristics

In total, 2959 patients were included in the analysis (Figure 2; Table 1). At baseline, 58.4% were aged ≥ 65 years; 68.1% were female; mean (standard deviation [SD]) baseline Hgb was 9.7 g/dL (1.4); and median (SD) follow-up period length was 107 weeks (27.8). In total, 43% of patients (1285) had an iron deficit of >1000 mg.

IV Iron Treatment Received

Iron sucrose was the most frequently used treatment type, received by 1309 patients (44.2%), followed by FCM ($n = 763$; 25.8%) and FM ($n = 422$; 14.3%; Table 2). Only 567 patients (19%) received ≥ 1500 mg of total IV iron, and 942 patients (32%) received >1000 mg total IV iron within the first 21 days.

Mean (SD) index dose was 743 mg (148) for FCM, 466 mg (154) for FM, and 197 mg (222) for patients receiving other IV iron products. Two-thirds (66%) of patients treated with FCM received ≥ 1500 mg of total IV iron during the first 21 days (Figure 3), compared with 6% of patients treated with FM and 2% of patients receiving other iron products. Approximately 73% of patients treated with FM received >1000 mg of total IV iron during the first 21 days (Figure 3), compared with 66% of FCM patients and 7% of patients receiving other iron products.

Patient demographics, comorbidities, and mean baseline serum Hgb were similar for those receiving >1000 mg and ≤ 1000 mg of IV iron within the first 21 days. Patients receiving ≥ 1500 mg of IV iron had a lower proportion of chronic kidney disease and congestive heart failure but a higher proportion of inflammatory bowel disease and cancer compared with patients who received <1500 mg of IV iron within the first 21 days. Mean (SD) baseline iron deficit for the overall study sample was 1001 mg (312) and it was similar for those receiving >1000 mg, ≤ 1000 mg, ≥ 1500 mg and <1500 mg

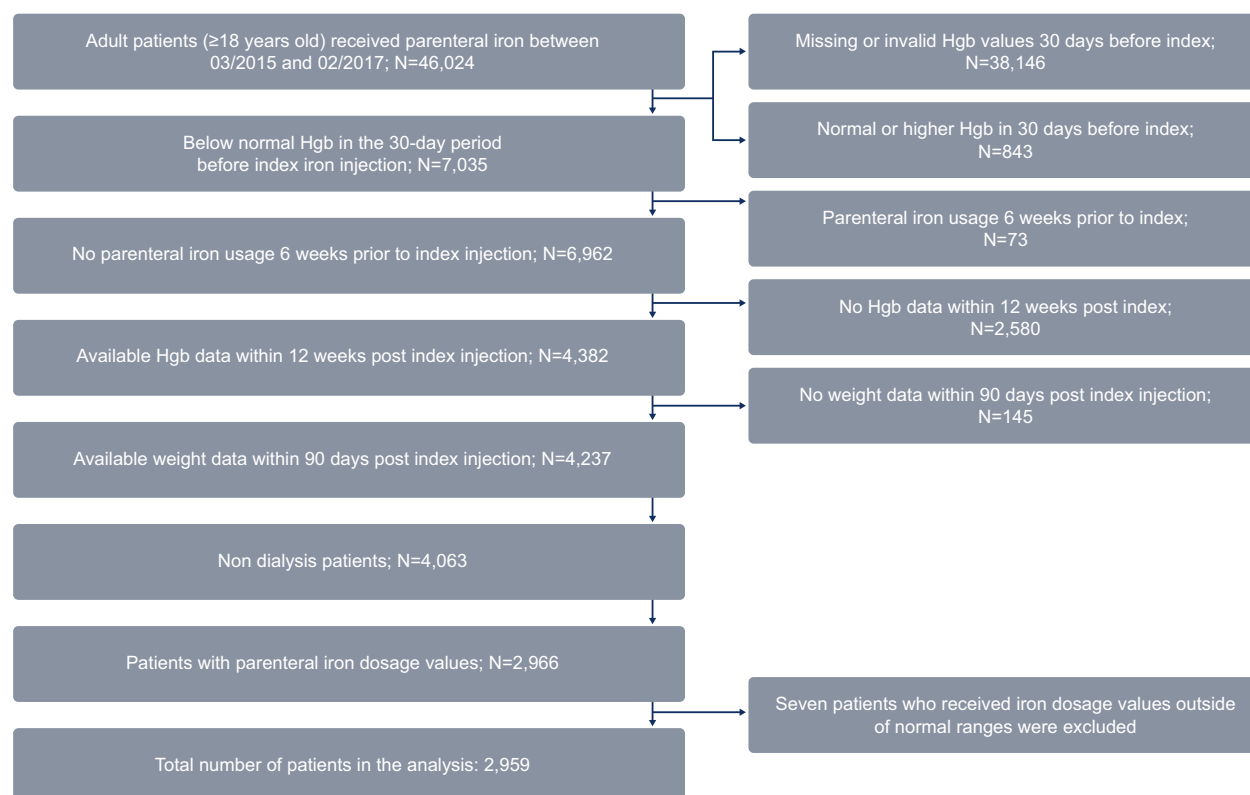


Figure 2 Patient sample attrition.

Abbreviation: Hgb, hemoglobin.

of IV iron within the first 21 days. Mean (SD) total IV iron dose was 777 mg (558) over the first 21 days after the index dose in the overall study sample. Mean (SD) doses were 1414 mg [405] for those receiving >1000 mg and 1642 mg [368] for those receiving ≥ 1500 mg of IV iron. For those receiving ≤ 1000 mg and <1500 mg of IV iron, the mean (SD) doses were 479 mg (320) and 572 mg (366), respectively, over the first 21 days.

Serum Hemoglobin Normalization and Further IV Iron Treatment Requirements

Within 12 months of the index dose, the proportion of patients achieving normalized serum Hgb was highest for those receiving ≥ 1500 mg (62%), followed by patients receiving >1000 mg (56%) as compared to those receiving <1500 mg (45%) and ≤ 1000 mg (44%) over the first 21 days ($p < 0.001$; Figure 4). The mean (standard error [SE]) number of all-cause outpatient visits and the mean (SE) number of outpatient visits for IV iron treatment were significantly lower in patients receiving ≥ 1500 mg than <1500 mg iron (all-cause visits: 10 [0.59] and 13 [0.33], respectively [$p < 0.001$]; IV iron treatment visits: 2 [0.08] and 4 [0.06], respectively [$p < 0.001$]; Figure 4). Similarly, the mean (SE) number of all-cause outpatient visits and the mean (SE) number of outpatient visits for IV iron treatment was significantly lower in patients receiving >1000 mg than ≤ 1000 mg iron required significantly fewer outpatient visits (all-cause visits: 11 [0.46] vs 14 [0.37], respectively, $p < 0.001$; IV iron treatment visits: 3 [0.08] vs 4 [0.07], respectively, $p < 0.001$; Figure 4). During the 12-month period after index IV dose, the proportion of patients requiring ≥ 3 IV iron infusions was lowest among patients who received an initial dose of ≥ 1500 mg during the first 21 days (39%), followed by patients who received an initial dose of >1000 mg (43%), patients who received <1500 mg (56%) and those who received <1000 mg (57%) over the first 21 days.

After adjusting for comorbidities, baseline serum Hgb level and baseline iron deficit level, patients who received an initial dose of ≥ 1500 mg had a 32% lower probability of receiving additional IV iron 30 days after index IV iron dose than those who received <1500 mg (adjusted hazard ratio [HR]: 0.68 [95% confidence interval (CI): 0.58, 0.81]; Figure 4) during the entire study follow-up period. For those who received an initial dose of >1000 mg during the

Table 1 Cohort Demographics and Characteristics

	All Patients	Received >1000 mg	Received ≤1000 mg	Received ≥1500 mg	Received <1500 mg
Total, n (%)	2959	942 (31.8)	2017 (68.2)	567 (19.2)	2392 (80.8)
Female gender, n (%)	2014 (68.1)	620 (65.8)	1394 (69.1)	375 (66.1)	1639 (68.5)
Age, n (%)					
18–44	441 (14.9)	118 (12.5)	323 (16.0)	80 (14.1)	361 (15.1)
45–54	351 (11.8)	107 (11.4)	244 (12.1)	79 (13.9)	272 (11.4)
55–64	438 (14.8)	125 (13.3)	313 (15.5)	79 (13.9)	359 (15.0)
≥65	1729 (58.4)	592 (62.8)	1137 (56.4)	329 (58.0)	1400 (58.5)
Comorbidities, n (%)					
Abnormal uterine bleeding	117 (4.0)	37 (3.9)	80 (4.0)	24 (4.2)	93 (3.9)
CKD (non-dialysis)	840 (28.4)	285 (30.3)	555 (27.5)	120 (21.2)	720 (30.1)
CHF	524 (17.7)	160 (17.0)	364 (18.0)	69 (12.2)	455 (19.0)
IBD	62 (2.1)	27 (2.9)	35 (1.7)	18 (3.2)	44 (1.8)
Post-partum anemia	1 (0.0)	-	1 (0.0)	-	1 (0.0)
Cancer	667 (22.5)	233 (24.7)	434 (21.5)	150 (26.5)	517 (21.6)
Prescription oral therapy, n (%)					
3 months pre-index	71 (2.4)	29 (3.1)	42 (2.1)	22 (3.9)	49 (2.1)
3 months post-index	37 (1.3)	4 (0.4)	33 (1.6)	1 (0.0)	36 (1.5)
3 months pre- and post-index	41 (1.4)	11 (1.2)	30 (1.5)	7 (1.2)	34 (1.4)
Mean weight, kg (SD)	83 (23.7)	84 (23.5)	82 (23.8)	85 (23.9)	82 (23.6)
Mean baseline Hgb, g/dL (SD)	9.66 (1.43)	9.72 (1.40)	9.64 (1.45)	9.79 (1.42)	9.63 (1.44)
Mean iron deficit per modified Ganzoni, mg (SD)	1001 (312)	1007 (320)	998 (308)	998 (315)	1001 (311)
Mean IV iron dose received in first 3 weeks, mg (SD)	777 (558)	1414 (405)	479 (320)	1642 (368)	572 (366)

Abbreviations: CHF, chronic heart failure; CKD, chronic kidney disease; Hgb, hemoglobin; IBD, inflammatory bowel disease; IV, intravenous; SD, standard deviation.

Table 2 Type of IV Iron Treatments Received by Cohort

IV Iron Treatment Received	Patients	
	N	%
Iron sucrose	1309	44.2
FCM	763	25.8
FM	422	14.3
Sodium ferric gluconate	108	3.6
Iron dextran	357	12.1

Abbreviations: FCM, ferric carboxymaltose; FM, ferumoxytol; IV, intravenous.

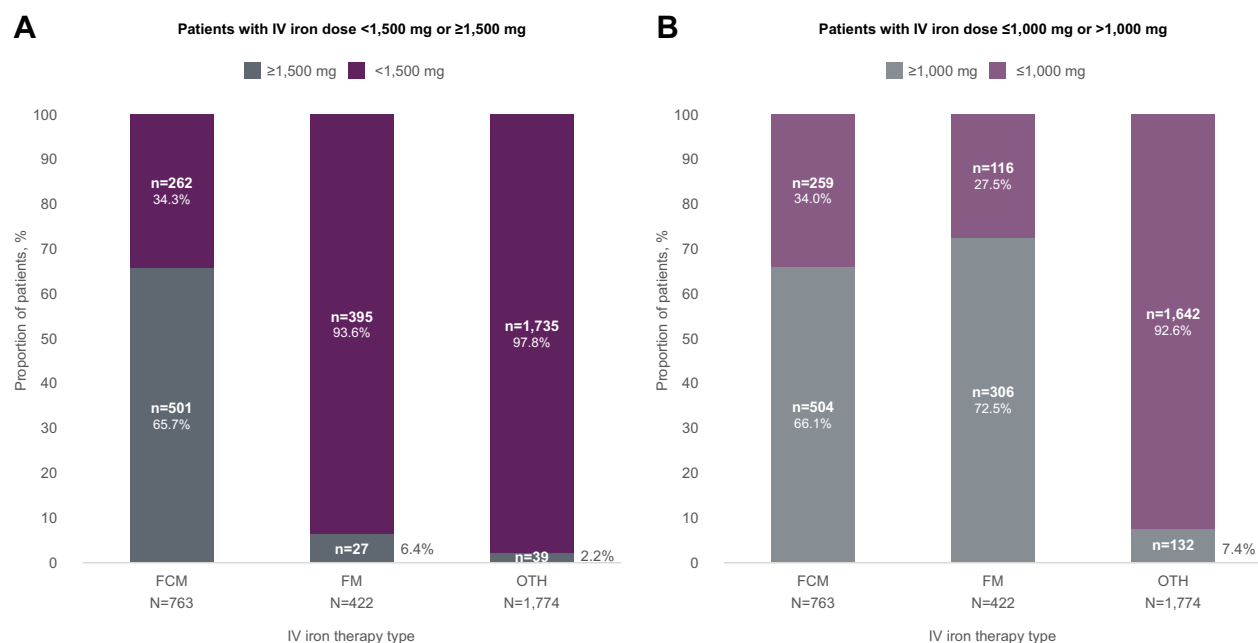


Figure 3 Proportion of patients receiving IV iron products within 21 days of index by dose (A) <1500 mg and ≥1500 mg and (B) ≤1000 mg and >1000 mg. **Abbreviations:** FCM, ferric carboxymaltose; FM, ferumoxytol; IV, intravenous; OTH, other.

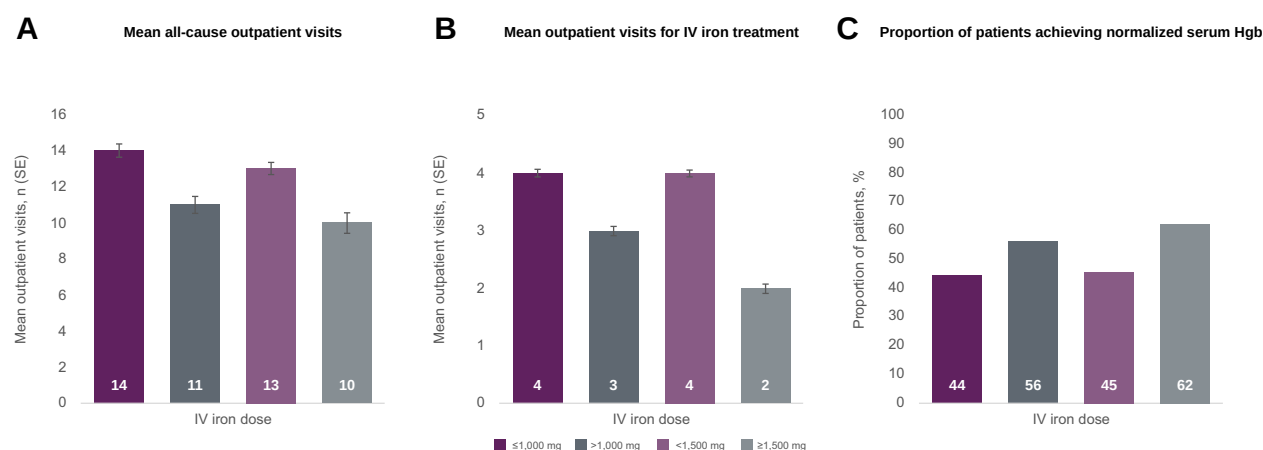


Figure 4 Outcomes in patients receiving an initial dose of IV iron. (A) Mean all-cause outpatient visits, (B) mean outpatient visits for IV iron treatment and (C) proportion of patients achieving normalized serum Hgb. **Abbreviations:** IV, intravenous; SE, standard error.

first 21 days after index IV iron dose, the probability of receiving additional IV iron 30 days after index IV iron dose was 41% lower than for those who received ≤1000 mg (adjusted HR: 0.59 [95% CI; 0.52, 0.67]; Figure 5).

Among the three IV iron product groups, FCM had the highest proportion of patients achieving normal serum Hgb level (61%) and the lowest proportion of patients requiring ≥3 IV iron infusions within 12 months of index IV iron dose (27%) (Figure 6). The mean number of all-cause outpatient visits and visits related to IV infusion within 12 months of treatment was highest among patients treated with “other” IV iron therapies, and the lowest proportion was of those treated with FCM (Figure 6). Treatment group differences were similar in the subgroup of patients with a >1000 mg deficit.

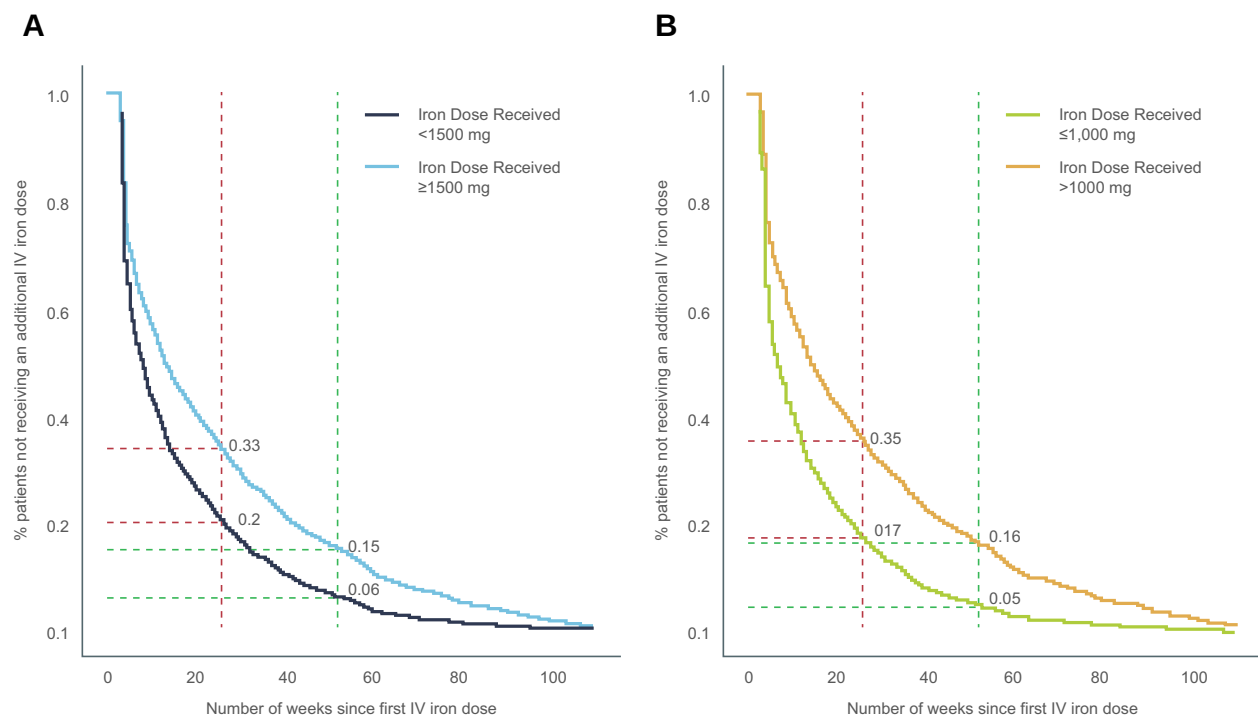


Figure 5 Probability of receiving an additional IV iron dose during study follow-up period in patients receiving (A) <1500 mg and ≥1500 mg and (B) ≤1000 mg and >1000 mg.

Abbreviation: IV, intravenous.

Discussion

This analysis showed that high doses of IV iron within three weeks of first dose may offer an effective treatment option that is convenient for patients with iron deficiency anemia in need of parenteral therapy. By reducing the need to return for further treatment, high iron doses may minimize the risk of treatment ineffectiveness due to patient non-compliance and reduce healthcare resource utilization in patients with iron deficiency anemia receiving IV iron therapy.

Results of these data suggest that underdosing is common in real-world practice and it should be a consideration when initiating treatment. Despite a mean iron deficit of 1001 mg in the study sample, the mean IV iron dose received during the 3 weeks from index was only 777 mg. Mean iron deficit levels did not differ across dose groups, corroborating previous findings that baseline iron deficits are often not considered when choosing between IV iron products and determining IV iron dose for repletion in real-world practice.¹⁵ Moreover, our data suggested that many patients did not receive IV iron consistent with a product's dosing recommendations. For example, during the first 21 days of treatment, our study showed that one in three patients treated with FCM received total cumulative IV iron dose lower than the recommended dose of 1500 mg of total IV iron. Similarly, approximately 1 in 4 of patients treated with FM received IV iron dose lower than the recommended cumulative total dose of 1020 mg. Although the reason for underdosing is unclear, possible barriers to adequate dosing might be poor follow-up or patients not returning to clinic after their first dose of IV iron.

At the time of this study, FCM and FM were the only IV iron products available on the US market providing a total cumulative dose >1000 mg in an initial course of IV iron therapy. In this study, treatment with IV iron of ≥1500 mg and >1000 mg was associated with significant reduction in the likelihood of additional IV iron treatment after 30 days. Treatment with FCM was also associated with lowest number of outpatient visits and the highest likelihood of normalized Hgb recovery among the three IV product groups, across both the overall cohort and the subgroup of patients with an iron deficit of >1000 mg. This indicates that the clinical benefit of high-dose IV iron is not only limited to patients with a high estimated iron deficit; rather, full replenishment of iron with a high dose IV product in the first 3 weeks of

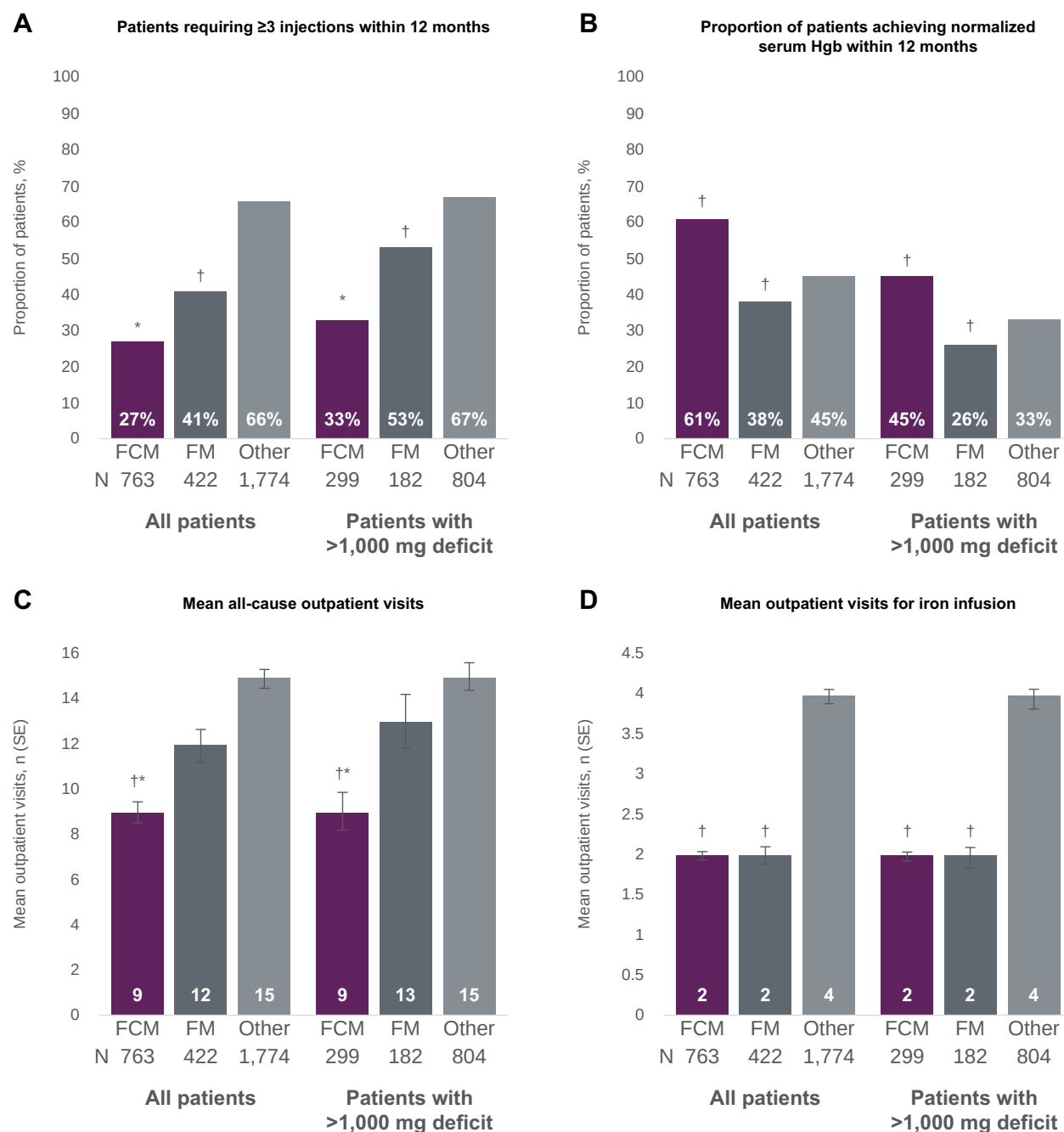


Figure 6 Post-infusion resource use by treatment type. **(A)** Patients requiring ≥ 3 injections within 12 months, **(B)** proportion of patients achieving normalized serum Hgb within 12 months, **(C)** mean all-cause outpatient visits, **(D)** mean outpatient visits for iron infusion.

Notes: *Statistically significant difference vs FM ($p < 0.001$); †statistically significant difference vs other ($p < 0.001$).

Abbreviations: FCM, ferric carboxymaltose; FM, ferumoxytol; Hgb, hemoglobin; IV, intravenous.

treatment appears to be important in reducing the clinical and economic burden of patients with iron deficiency anemia, irrespective of deficit. The mean number of infusion-related office visits in the low-dose IV iron group was highest and doubled that of patients treated with FCM and FM, but the proportion of patients achieving normalized serum Hgb was lowest for the low-dose IV iron group. These results suggest that giving low dose IV iron more frequently may be ineffective to provide sufficient iron repletion.

The study has several limitations. First, the study was a retrospective analysis of claims and EMR data from an open network. Claims data are often adjudicated on an ongoing basis. Healthcare services provided by out-of-network providers may not be captured by the database, which could lead to imprecision in the healthcare resource utilization results. Additionally, use of concomitant over-the-counter oral iron replacement medications was not captured by the database. We cannot completely rule out possible confounding from patient self-administered oral iron supplementation. Because the study required serum Hgb data, patients who did not have complete Hgb data recorded in EMRs were excluded, which could limit the generalizability of the study findings. A higher proportion of cancer patients and lower proportions of patients having non-dialysis dependent chronic kidney disease and congestive heart failure were observed among patients receiving ≥ 1500 mg dose group than the < 1500 mg group. Future studies are needed to determine the effect of these comorbidities on study results. Because ferritin data were limited in the database, we were unable to assess iron stores to further understand the durability of iron repletion. Ferric derisomaltose was recently approved in the US and its data were not available at the time of the study. Future studies including data from ferric derisomaltose will provide a more comprehensive assessment on the effect of high dose IV iron on clinical and economic burden of iron deficiency anemia among US patients. A longer-term assessment could provide better insights into long-term patient outcomes.

Conclusion

In a real-world US setting, initial treatment with higher total dose of IV iron was associated with improved serum hemoglobin levels, reduced need for further iron treatment and lower healthcare resource utilization.

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Disclosure

CL, IB, and SK were employees of Clarivate, a healthcare analytics consultancy, at the time of the study. WJK is an employee of Daiichi Sankyo Inc. RVB was a consultant to Daiichi Sankyo, Amgen, Bristol Myers Squibb, AMAG, SecuraBio, and was on the speaker's bureau of Rigel and Celgene during the time of this study. The authors report no other conflicts of interest in this work.

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Primary Diffuse Large B Cell Lymphoma of Bone: A Single-Center Experience

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Primary Diffuse Large B Cell Lymphoma of Bone: A Single-Center Experience

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Background: Primary diffuse large B-cell lymphoma of the bone (PB-DLBCL) is a rare type of extra-nodal lymphoma. This study aimed to examine the clinical characteristics, outcomes, treatment modalities and risk of central nervous system relapse (CNSR) among adult Jordanian patients with PB-DLBCL.

Methods: This retrospective study included patients aged >16 years who were diagnosed with PB-DLBCL and treated at our hospital between 2002 and 2021. Clinical characteristics, treatment modalities, outcomes and CNSR events were extracted from the hospital's data system and analysed. Patients were categorised into unifocal (UF) and multifocal (MF) PB-DLBCL groups according to the number of bone sites involved. The involvement of only one site was defined as UF, whereas the involvement of two or more sites was defined as MF.

Results: In total, 12 patients were diagnosed with PB-DLBCL. Their median age was 47.5 years (range, 17–80 years). The male:female ratio was 1:1. There were eight patients in the UF PB-DLBCL group and four in the MF PB-DLBCL group. All patients received treatment with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone. In the UF PB-DLBCL group, the male:female ratio was 5:3, the median age was 41 years, and the follow-up duration was 9–135 (mean, 83.3) months. In the MF PB-DLBCL group, the male:female ratio was 1:3, the median age was 51.5 years, and the survival time was 3–11 (mean, 7) months. Three patients with vertebral UF PB-DLBCL underwent early vertebroplasty without complications. The most common site involved was the vertebral column. Most patients with UF PB-DLBCL achieved complete remission (CR), whereas no patients with MF PB-DLBCL achieved CR.

Conclusion: PB-DLBCL is rare in adult Jordanian patients. UF PB-DLBCL is more common than MF PB-DLBCL. Patients with UF PB-DLBCL had a good prognosis. Patients with MF PB-DLBCL had a high international prognostic index score, risk of CNSR and short survival time.

Keywords: primary diffuse large B-cell lymphoma of the bone, WHO classification of primary bone lymphoma, unifocal, multifocal

Introduction

Primary bone lymphoma is a rare type of neoplastic disorder. It is estimated to constitute 1% of all lymphomas, with diffuse large B-cell lymphoma (DLBCL) being the most common histological type.^{1–6}

Studies describing the clinical manifestations and treatment modalities of PB-DLBCL remain controversial as most of the research results are from Western countries. Therefore, we conducted this retrospective study to better understand the clinical characteristics, treatment modalities and outcomes of PB-DLBCL in adult Jordanian patients.

Materials and Methods

This 19-year retrospective study was approved by the Institutional Review Board Committee (IRB) of King Abdullah University Hospital (KAUH). The need for informed consent was waived by the IRB due to the retrospective nature of

the study, and the data was anonymised. This study included patients diagnosed with PB-DLBCL and treated at KAUH between 2002 and 2021.

PB-DLBCL was diagnosed via guided bone biopsy, histological examination, and immunohistochemical (IHC) analysis for CD20, PAX-5, CD10, Bcl-2, Bcl-6 and MUM-1. Accordingly, each lymphoma was categorised into germinal centre B-cell (GCB) and non-germinal centre B-cell (NGCB) types. The inclusion criteria were as follows: having undergone radiological examination and positron emission tomography (PET) upon diagnosis (Figure 1, Figure 2A and B), having a follow-up time of ≥ 3 months and not presenting with significant lymphadenopathy or any other extra-nodal site involvements other than the bone, with the exception of bone marrow involvement, as all patients included in the study underwent bone marrow biopsy as part of cancer staging at the time of diagnosis.

Categorisation: According to PET scan results, patients were categorised into unifocal (UF) and multifocal (MF) PB-DLBCL groups according to the number of bone sites involved. The involvement of only one site was defined as UF, whereas the involvement of two or more sites was defined as MF. We considered more than one vertebral involvement as MF.

Data on patient characteristics, such as age, sex, clinical manifestations, including B symptoms upon diagnosis, symptom duration (from symptom onset until diagnosis), treatment modalities and survival duration after treatment were extracted from the computer registry file system of KAUH. Survival time was calculated from the time of diagnosis to the time of the last hospital visit, and information about the time of death was extracted from the Jordanian Civil Status and Passport Department. Additionally, data about risk factors, such as serum lactate dehydrogenase (LDH) levels, were obtained. Human immunodeficiency virus (HIV) testing is not routinely performed as most patients do not want to



Figure 1 PET scan showing unifocal bone involvement of the scapula with mass.

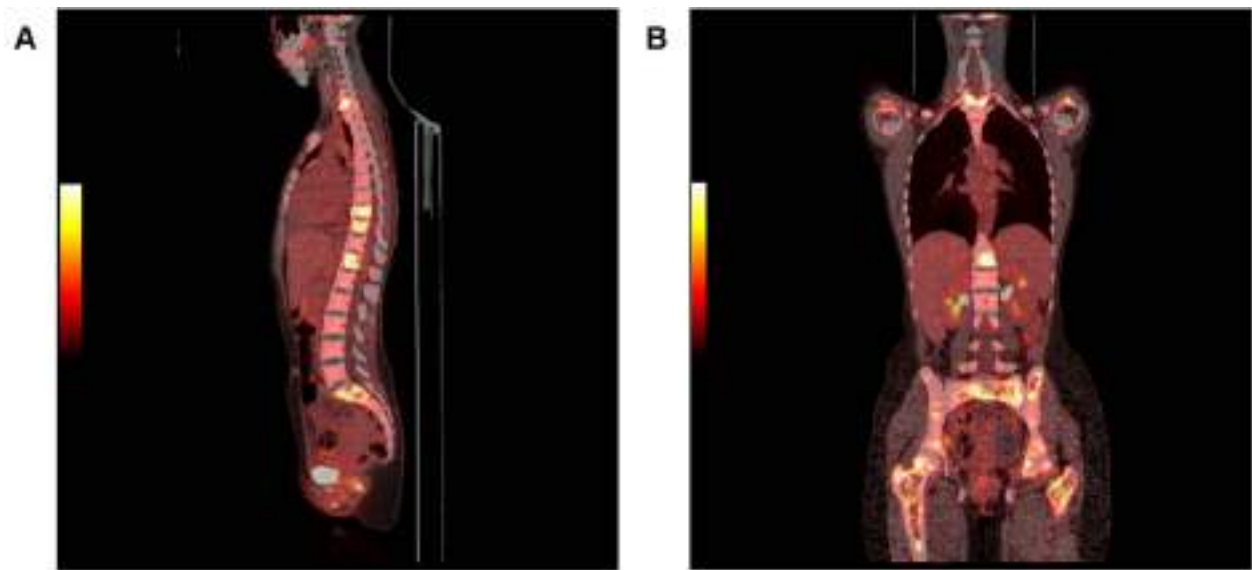


Figure 2 (A) PET scan showing with multifocal bone involvement of the vertebral column. **(B)** PET scan showing multifocal bone involvement pelvis, femur, vertebra, and scapulae.

undergo it and the prevalence of HIV among Jordanians is low ($<0.1\%$).⁷ CNSR was diagnosed via lumbar puncture to examine the cerebrospinal fluid (CSF). The presence of lymphoma cells was determined via cytology, flow cytometry of the CSF and contrast-enhanced radiological examination.⁸

For response assessment, we used the response criteria based on the International Working Group consensus response evaluation criteria in lymphoma (RECIL 2017).⁹

International Prognostic Index (IPI) score

The patients' IPI score was calculated based on the following, with 1 point being assigned to each: age of ≥ 60 years, increased LDH levels, stage III–IV disease, Eastern Cooperative Oncology Group performance status score and >1 extra-nodal site of disease.¹⁰

Results

Patient Characteristics

The clinical characteristics of patients are shown in Tables 1 and 2. In total, 261 patients aged >16 years were diagnosed with at KAUH. Among them, 12 (4.6%) were diagnosed with PB-DLBCL, with a median age of 47.5 years (range, 17–80 years) and a male:female ratio of 1:1.

In the UF PB-DLBCL group, there were 8 patients, with a male:female ratio of 5:3, median age of 41 years (range, 17–80 years), mean age of 42 years and follow-up duration of 9–135 (mean, 83.3) months. In the MF PB-DLBCL group, there were 4 patients, with a male:female ratio of 1:3, median age of 51.5 years (range, 22–71 years) and survival time of 3–11 (mean, 7) months. Among patients with UF PB-DLBCL, there were 5 with GCB and 3 with NGCB. Among patients with MF PB-DLBCL, 2 had GCB and 2 had NGCB.

Sites of Bone Involvement

The vertebrae ($n = 7$) were the most common site of bone involvement, followed by the pelvis and femur ($n = 2$), scapulae ($n = 1$) and clavicle ($n = 1$). All patients with MF PB-DLBCL had vertebral involvement, and only one patient with MF PB-DLBCL presented with spinal as well as long-bone involvement.

Table 1 Demographic and Clinical Characteristics of Patients with UF PB -DLBL (n=8)

Cases								
Patient	1	2	3	4	5	6	7	8
Age	36	46	80	17	56	28	49	25
Sex	F	F	M	F	M	M	M	M
Site	Sacrum	Scapula	Clavicle	Femur	L3	Femur	T3	T10
Symptoms	Pain	Mass	Mass	Pain	Pain	Pain	Pain	Pain
Fracture	No	No	No	No	Yes	Yes	Yes	Yes
GC/NGC type	GC	GC	NGC	GC	NGC	GC	NGC	NGC
Duration of symptoms (m)	6	2	4	3	6	5	8	3
Serum LDH	H	H	N	H	H	H	H	H
IPI index	I	I	3	I	I	I	I	I
Survival in months	143	97	81	132	108	33	*9	95

Abbreviations: UFPB-DLBCL, unifocal, primary bone diffuse large B-cell lymphoma; F, female; M, male; L3, third lumbar vertebra; T3, third thoracic vertebrae; P, pelvis; H, high; N, normal; * 9 patients died owing to severe left ventricular impairment; GC, germinal center; NGC, non-germinal center.

Table 2 Demographic and Clinical Characteristics of Patients with MF PB -DLBL (n=4)

Patients	1	2	3	4
Age	54	22	49	71
Sex	M	F	F	F
Site	T12-L5 F, H, P	C7- T3, H,P	T5-L3	T1-L3
Symptoms	Pain	Pain	Pain	Pain, SC
Duration of symptoms (m)	14	18	23	25
Serum LDH	H	H	N	H
IPI index	3	4	4	4
Survival in months	11	7	7	3
GC/NGC	GC	GC	NGC	NGC
CNSR	Yes	Yes	Yes	No

Abbreviations: MF PB-DLBCL, multifocal primary bone diffuse large B-cell lymphoma; F, female; M, male; T12-L5, third thoracic vertebra-fifth lumbar vertebra; C7-T3, seventh cervical vertebra-third thoracic vertebra; T5-L3, fifth thoracic vertebra-third lumbar vertebra; T1-L3, first thoracic vertebra-third lumbar vertebra; P, pelvis; H, high; N, normal; m, month; GC, germinal center; NGC, non-germinal center.

Clinical Presentation of PB-DLBCL

Pain was present at the site of bone involvement in all patients. Three patients with MF bone involvement had B symptoms, but no patients with UF involvement had B symptoms. All patients with vertebral involvement had fractures. Three patients had masses in the scapulae, clavicles and femur. The median symptom duration in the UF PB-DLBCL group was 4.5 (range, 2–8) months. In the MF PB-DLBCL group, the median symptom duration was 20.5 (range, 18–25) months.

Treatment Modalities

All patients received rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP), and only one patient with scapula involvement was treated with R-CHOP combined with radiotherapy. Three patients with UF vertebral involvement underwent early vertebroplasty prior to chemotherapy during bone curettage biopsy.

IPI Score

In the UF PB-DLBCL group, 7 patients had an IPI score of 1 and 1 patient had an IPI score of 3. In the MF PB-DLBCL group, 3 patients had an IPI score of 4 and 1 patient had an IPI score of 3.

Central Nervous System Relapse

Three of four patients with MF PB-DLBCL had CNSR, but none with UF PB-DLBCL had CNSR.

Bone Marrow Involvement

Bone marrow involvement was present in three patients with MF PB-DLBCL but none of the patients with UF PB-DLBCL.

Chemotherapy Complications

One patient with spinal MF involvement had vertebral collapse after the first cycle of R-CHOP, thereby resulting in spinal cord compression and, eventually, death.

Response Criteria

Seven patients with UF PB-DLBCL achieved complete remission (CR), but none of the patients with MF PB-DLBCL achieved CR.

Discussion

To the best of our knowledge, this the first study to describe Jordanian patients with PB-DLBCL. In this study, PB-DLBCL constituted for 4.6% of all cases of PB-DLBCL, which is less than what has been reported in other studies^{11,12} and different from a report from China that showed a lower frequency of 0.01%.¹³ The median age of our patients with PB-DLBCL was younger than that reported in other studies.¹⁴ No sex bias was observed for PB-DLBCL.¹⁵ However, comparing PB-DLBCL subtypes, we noticed male predominance among those with UF PB-DLBCL and female predominance among those with MF PB-DLBCL. This finding differs from the results reported in other studies showing male predominance in both types.^{14–16} Female predominance in MF PB-DLBCL in our study may be due to the fact that the diagnosis was delayed in most of our female patients because the bone pain was attributed to rheumatological causes, resulting in a long symptom duration before diagnosis.

Regarding IHC subtypes, we found that the GCB type was predominant among those with UF PB-DLBCL, whereas both GCB and NGCB subtypes occurred in equal proportions among those with MF PB-DLBCL. As our study included a small number of patients, the effect of GCB and NGCB subtypes on prognosis was not clear.^{14,17,18}

The main symptoms were pain, masses and fractures. Fractures were present in patients with vertebral involvement and have been shown to be the most common symptom of PB-DLBCL.^{1,3,5,8–14,14,15,15–17}

Our study revealed the absence of B symptoms in patients with UF PB-DLBCL as opposed to their presence in patients with MF PB-DLBCL. Additionally, patients with MF PB-DLBCL had higher IPI scores, bone marrow involvement and CNSR. These observations in our patients suggest that MF PB-DLBCL represents an advanced stage of the disease.

In our study, the vertebrae were the most common site of involvement in both types of PB-DLBCL, which corroborates the results of other previous studies.^{5,18} Notably, another study showed the predominance of appendicular bone involvement.¹⁴

All patients received R-CHOP chemotherapy. Out of eight patients with UF PB-DLBCL, six achieved CR, but none of the patients with MF PB-DLBCL achieved CR, which is in agreement with the results of another study.¹⁴

Three patients with UF vertebral involvement underwent early percutaneous vertebroplasty before starting R-CHOP chemotherapy and none of them relapsed. This suggests that early vertebroplasty may be beneficial in patients with vertebral UF PB-DLBCL, as shown in a previous case report.¹⁹ One patient with MF vertebral involvement developed

vertebral collapse with spinal cord compression after the first dose of R-CHOP. Hence, vertebroplasty should also be considered in cases of MF vertebral involvement.

In conclusion, our retrospective study with a small number of patients, which reflects the rarity of PB-DLBCL, showed that UF PB-DLBCL has a better prognosis than MF PB-DLBCL and that MF PB-DLBCL represents an advanced stage of the disease.

Ethics Statement and Informed Consent

All procedures performed in studies involving human participants were following 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. The Ethics Committee of King Abdullah University Hospital (785/2018) approved this retrospective study. The need to obtain informed consent was waived, as the study was a retrospective study, and there was no additional risk to patients. All data were anonymized to maintain patient privacy.

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Disclosure

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Influence of α_2 -Macroglobulin, Anti-Parasite IgM and ABO Blood Group on Rosetting in *Plasmodium falciparum* Clinical Isolates and Their Associations with Disease Severity in a Ghanaian Population

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Purpose: The severity of *Plasmodium falciparum* infections is associated with the ability of the infected red blood cells to cytoadhere to host vascular endothelial surfaces and to uninfected RBCs. Host blood group antigens and two serum proteins α_2 -macroglobulin (α_2 M) and IgM have been implicated in rosette formation in laboratory-adapted *P. falciparum*. However, there is only limited information about these phenotypes in clinical isolates.

Methods: This was a hospital-based study involving children under 12 years-of-age reporting to the Hohoe Municipal Hospital with different clinical presentations of malaria. Parasite isolates were grown and rosette capabilities and characteristics were investigated by fluorescence microscopy. α_2 M and IgM were detected by ELISA.

Results: Rosette formation was observed in 46.8% (75/160) of the parasite isolates from all the blood groups tested. Rosettes were more prevalent (55%) among isolates from patients with severe malaria compared to isolates from patients with uncomplicated malaria (45%). Rosette prevalence was highest (30%) among patients with blood group O (30%) and B (29%), while the mean rosette frequency was higher in isolates from patients with blood group A (28.7). Rosette formation correlated negatively with age ($r = -0.09$, $P = 0.008$). Participants with severe malaria had a lower IgM concentration (3.683 ± 3.553) than those with uncomplicated malaria (5.256 ± 4.294) and the difference was significant ($P = 0.0228$). The mean concentrations of anti-parasite IgM measured among the clinical isolates which formed rosettes was lower (4.2 ± 3.930 mg/mL), than that in the non rosetting clinical isolates (4.604 ± 4.159 mg/mL) but the difference was not significant ($P = 0.2733$). There was no significant difference in plasma α_2 M concentration between rosetting and non rosetting isolates ($P = 0.442$).

Conclusion: *P. falciparum* parasite rosette formation was affected by blood group type and plasma concentration of IgM. A lower IgM concentration was associated with severe malaria whilst a higher α_2 M concentration was associated with uncomplicated malaria.

Keywords: *Plasmodium falciparum*, severe malaria, uncomplicated malaria, rosettes, blood group, alpha-2 macroglobulin, immunoglobulin M

Introduction

Despite the recent decline in *P. falciparum* infections globally, malaria continues to affect the African continent with most of the disease burden and mortalities occurring in this region.¹ Case incidence of malaria among children under 5 years-of-age was estimated to be 225/1000 individuals at risk in 2019, which was a slight decline from the year 2000 estimate. Malaria deaths in children less than 5 years declined between year 2000 and 2019 by 17% worldwide.¹

Plasmodium falciparum continues to be the dominant species in the sub-region, where the climate favors the *Anopheles* vector.² *P. falciparum* is virulent and is responsible for the majority of malaria mortality and severe morbidity.^{1,3}

The pathogenesis of *P. falciparum* malaria is directly linked to the ability of the parasite to express variant surface antigens (VSA) on the surface of IRBCs, which allows the parasites to establish long-lasting chronic infection.⁴ Since the publication of the full genome of *P. falciparum* three major families of variant genes have been studied extensively.^{5,6} The proteins expressed by these variant genes include Erythrocyte Membrane Protein 1 (PfEMP1); RIFIN (Repetitive Interspersed family of polypeptides) proteins; STEVOR (Sub telomeric variable open reading frame) proteins.⁷ In *P. falciparum* infection, the VSA expressed is mainly for evading the host immune responses by allowing IRBCs to sequester in tissues, thereby avoiding their removal by the spleen.^{8,9} These parasite ligands (RIFINs, STEVOR, PfEMP1) have all been studied and known to be released onto IRBC at specific times in the parasite life cycle. PfEMP1 is transcribed at the ring stage and exported to the surface of the IRBC at the late trophozoite stage whilst RIFINs and STEVORs are transcribed and expressed later in the pigmented trophozoite and schizont stages.^{10–12}

The ability of the IRBC to attach to uninfected RBCs is another binding process, known as rosetting, which has been found to be associated with severe malaria in African children.^{13–15} Some host factors including complement receptor 1 (CR1), heparan sulphate (HS), blood group antigens and recently host serum proteins such immunoglobulin M (IgM) IgM and α_2 M have been identified to be associated with rosette formation.^{15–17} For instance, polymorphism in CR1, thalassemia and red blood cells such as the AS trait has been shown to confer protection against severe malaria.^{18–20} Another important biological role of PfEMP1 is their affinity for binding to blood group A and B antigens, which has also been linked to severe malaria.²¹ Various studies have shown the involvement of IgM as one of the serum proteins involved in the attachment of rosette formation.^{22,23} Whilst IgM remains the largest and first immunoglobulin to appear in response to malaria antigens, the structure of IgM and its pentameric nature make it an effective immune effector molecule.^{16,24} Previous studies have implicated serum IgM in rosette formation in children and their contribution to the development of severe malaria.^{15,25}

Another serum protein identified to be involved in rosette formation is α_2 M, which is a non-immunoglobulin molecule and one of the most abundant proteins found in peripheral blood circulation in humans.^{16,25} α_2 M is a protease inhibitor, which is produced by the liver and locally synthesised by macrophages, adrenocortical cells and fibroblast and part of the innate immune system.²⁶ In humans, α_2 M has been demonstrated to play an important role in maintaining homeostasis of cytokines and growth factors.²⁷ Increased α_2 M plasma levels have consistently been seen during periods of life characterized by growth, development, and differentiation such as embryogenesis, pregnancy, and childhood.^{26,28}

Laboratory-adapted parasites strains¹⁶ and patient parasite isolates²⁵ have previously been used to show that serum α_2 M works in synergy with IgM to increase avidity of binding multiple PfEMP1 molecules on the IRBC surface to mediate rosetting. This study seeks to investigate further the associations of anti-parasite IgM and α_2 M plasma concentrations, as well as the effect of ABO blood group types on rosette formation among field isolates and their association with severity of malaria infection.

Materials and Methods

Ethical Statement

Ethical clearance for this study was obtained from by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, Ghana (Study number 026/13-14) and the Ethical Review Committee of the Ghana Health Service (MOH; file GHS-ERC 08/05/14).

The study complied with the Helsinki declaration for conducting research involving human subjects. Written informed consent was obtained from each parent/guardian of all the study participants.

Study Design and Population

The study was cross-sectional and involved 206 children less than 12 years of age who were admitted to or attended the outpatient department (OPD) of the Hohoe Municipal Hospital with different malaria pathologies (uncomplicated malaria and severe malaria). In this study, severe malaria was defined as a child presenting with signs and symptoms of malaria and in the presence of *P. falciparum* asexual parasitaemia with one or more of either impaired consciousness (Blantyre coma score of <3), acidosis, hypoglycaemia, hyperparasitaemia, renal impairment (acute kidney injury), severe malarial anaemia (haemoglobin concentration of <5 g/dl), Jaundice and others as defined by the Physician in-Charge. Uncomplicated malaria was also defined as children presented with both microscopy and RDT positive for malaria but with no features of severe malaria.

Rosetting is defined as the binding of uninfected red blood cells to a centrally *Plasmodium falciparum* infected red blood cell. Different categories of rosetting include small rosette defined as the binding of an infected RBC by 2 to 3 uninfected RBCs. A medium rosette is a *Plasmodium falciparum* infected RBC bound to 4 or 5 uninfected RBCs. A large rosette is an infected RBC bound by more than 5 uninfected RBCs. In this study, rosette frequency is defined as the percentage of total mature-parasite-infected cells forming rosettes against two hundred cells infected with matured parasites whilst rosette prevalence referred to proportion of the total parasite isolates collected throughout the study period that formed rosettes.

Sample Collection

In brief, the study was explained to the parents/guardians after which informed consent was sought from them. Approximately 3 mL of whole blood was collected after rapid diagnostic test (RDT) gave a positive result. In addition, the presence of parasites when viewed under a light microscopy confirmed malaria infection and a PCR test was used to confirm the *Plasmodium falciparum* malaria cases and differentiation of species of mixed infection cases. Haemoglobin levels were measured using automated haematology analyser (Sysmex KX-21N) and blood groups determined using standard protocols as described elsewhere.²⁹ The blood samples were centrifuged and separated into infected red blood cells and plasma. The plasma was stored in a -80°C freezer until ready to be used for the measurement of IgM and α_2 M levels by ELISA. Approximately, 200uL of the patients' pellet RBC was used for short term in vitro cultures in the patients' own blood and the rest stored in liquid nitrogen for long term storage.

ABO and Rhesus Blood Grouping

Approximately three drops (20 μ L) of whole blood were put on a clean white tile. The blood was individually mixed with an equal volume of anti-A, anti-B or anti-D (Fortress Diagnostic Limited, UK.) after which, the tile was rocked gently for 5–10 minutes. The blood drops were observed for agglutination. The observation of agglutination in the blood drops indicated the presence of rhesus (Rh) antigen corresponding to the anti-sera used. Lack of agglutination for both A and B indicated the blood group O.

Parasitemia Estimation by Light Microscopy

Thick and thin blood smears were prepared on microscopic glass slide for all RDT confirmed malaria positive samples. The thick blood smear was prepared by placing a drop of blood on the slide and spreading it out evenly in a circular motion. On the same slide, a thin blood smear was also prepared by placing a drop of blood on the slide and using another slide held at 45 degrees to the drop on the first slide. The thin film side was immersed in methanol (VWR, USA) to fix the cells and dried whilst the thick film was only air dried. The dried slides were stained with 10% Giemsa stain (Merck, Germany) for 15 minutes. Slides were washed under running water and air dried. A drop of immersion oil (Sigma Aldrich, USA) was put on the slide and observed under a light microscope at 100X objective lens. The parasitaemia was calculated as the number of parasites divided by the number of white blood cells all multiplied by 8000 and the species differentiation was done to confirm that sample was positive for *P. falciparum*.

Short Term in vitro Cultivation of Fresh *Plasmodium falciparum* Clinical Isolates

The *Plasmodium* parasite isolates were maintained in short term culture (one cycle) in the erythrocytes of the participants with RPMI-1640 medium supplemented with 1% L-glutamine, (Sigma Aldrich, USA, Cat no. R8758) (50 µg/mL gentamycin (Thermo Fisher, Cat no. 15750060), 50 mg/L hypoxanthine (Sigma Aldrich, UK, H9377), and 10% heat-inactivated type AB⁺ human serum (Sigma Aldrich, USA, Cat no. H6914). Cultures were incubated at 37 °C with a gas mixture of 5% O₂, 2% CO₂, balanced with N₂ (Air Liquide, UK, Cat No. 4687). The parasites were maintained until the late trophozoite and schizont stage when the rosette capability was assessed using a fluorescent microscope.

Rosette Formation Analysis by Fluorescence Microscopy

Rosetting was assessed in the first cycle of the in vitro growth when parasites reached late trophozoite stages. In brief, aliquot of 100 µL of the parasite culture was picked from the 5mL culture flask into a 1.5mL tube followed by the addition of 2µL of 25µg/mL ethidium bromide and incubated for 15 minutes at 37°C. About 10µL of the ethidium bromide-stained parasites were pipetted onto a fluorescence microscope slide and covered with a coverslip and read under fluorescence and at X40 magnification using Olympus BX 43 (Olympus, Japan). Rosettes were counted per 200 infected red blood cells and images recorded. Isolates rosettes were then categorized into small, medium and large based on the number of uninfected RBCs bound to a centrally IRBC. In isolates with different sized rosettes, the predominant rosette type is used to categorise it.

Measurement of Alpha-2-Macroglobulin Concentration Using ELISA

The plasma concentration of α₂M was measured using a commercial ELISA kit (RAB 0600: Sigma Aldrich) following the manufacturer's instructions and protocols. In brief, samples (diluted at 1:2000), controls, and standards were added to pre-coated plates at 200 µL/well. Plates were incubated for two and half hours at room temperature with gentle shaking at 200rpm. Plates were washed with 300µL of wash buffer and detection antibody was added at 100 µL/well and incubated for one hour at RT. Streptavidin solution was added at 100µL/well and incubated for 45 minutes. The plates were developed with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate reagent at 100 µL/well for 30 minutes in the dark. Reaction was stopped with 50 µL of Stop solution and plate read immediately at 450 nm.

Measurement of Plasma Anti-Parasite IgM Concentrations Using ELISA

The plasma concentration of anti-parasite IgM was measured using indirect ELISA technique. High binding 96 - well ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with soluble crude malaria antigen (0.5 X 10⁶ schizonts/ well) at 50µL/well and incubated at 4°C overnight. Plates were retrieved and washed 3X with 200µL/well of washing buffer (PBST; 1X PBS supplemented with 0.05% Tween 20 at pH 7.2) and blocked with 200µL per well blocking buffer (5% non-fat dry milk (Marvel, UK) in PBS) and incubated for 1 hour at room temperature on a shaker (150rpm). Plasma and controls were added at 50µL/well diluted 100X in assay buffer (1% milk/PBS). Positive control (pool of hyperimmune Ghanaian adults) and negative controls (pool of unexposed Europeans) were added alongside the samples in their respective wells for each plate. The plates were incubated for 1 hour at room temperature. Anti-parasite IgM was detected using 100µL/well of goat anti-human IgM conjugated Horseradish Peroxidase (HRP-IgM) (Invitrogen, USA) at a dilution of 1:1000 for 1 hour. Plates were developed with 50L/well TMB for 10 minutes and stopped with 50µL/well of 0.2M H₂SO₄. The optical density was read at 450nm Biotek plate reader (VT, USA) and converted to concentrations using ADAMSEL (version b040; Ed Remarque).

Statistical Analysis

Spearman rank correlation was performed to analyse the associations between the clinical variables and the concentration of anti-parasite IgM or α₂M. Clinical isolates were stratified as rosetting and non rosetting and differences between the clinical variables were compared by students *t*-test and Mann Whitney test where appropriate. A linear regression analysis was used to determine the relationship between host variables and rosette formation. P<0.05 were considered statistically significant. All the data collected was analysed using Graph pad prism 5.0 (Graph pad Software, Inc).

Results

Clinical Characteristics of Study Population

A total of 230 children were enrolled into the study over a two-year period. In the first year, 116 participants were recruited whilst the second year had 114. At the end of recruitment, the data base was cleaned to exclude all participants who were positive by RDT but had no parasites present in their sample after light microscopy. Most of them had taken medication before hospital visit and their in vitro cultures were not successful. Based on our criteria for participant enrollment, malaria cases were carefully classified into either severe or uncomplicated. There were more males (110) than females (96) in the study and the mean age of the children was 5.0 years (7 months – 11 years). The mean haemoglobin level was 9.3g/dL (3.6–13.9g/dL) for the general population. When this was stratified, anaemia was seen in 90 (43.6%) whilst severe anaemia was seen in 4 (1.9%) of the study participant. In 52 (25%) of the participants, parasite density (parasitemia) was above 100,000 parasites /ul. Most of the participants were of blood group O type (94/206). The clinical characteristics of participants with severe cases was compared to those with uncomplicated cases. The mean age for the uncomplicated malaria was 5 years whilst for the severe cases it was 3.8 years. Haemoglobin level was lower in the severe cases (8.4g/dL) than the uncomplicated (10.3g/dl). There was no significant difference in the mean parasitemia between the uncomplicated and the severe cases ($P = 0.31$). Patients with severe disease had a lower body weight than those with uncomplicated malaria (Table 1).

Rosette Capabilities Among *P. falciparum* Clinical Isolates

Rosetting assays were run on parasite isolates when they were at the trophozoite stage at a parasitemia of $>2\%$. Infected RBC are shown as bright spots not bound by uninfected RBCs (Figure 1A). Rosettes are shown as IRBCs (bright spot) bound by two or more uninfected RBCs as observed under the fluorescence microscope (Figure 1B). Rosettes were categorized as small (Figure 1C), medium (Figure 1D) or large, defined by the number of uninfected RBCs bound to the IRBC. Out of the 206 parasite isolates put into short term culture, 160 were successful and were used for the rosetting assay. Rosette formation was observed in 75 (46.8%) of the clinical isolates comprising 42 from the severe malaria category and 33 from the uncomplicated malaria category but the difference in rosette formation capabilities was not statistically significant ($P=0.3$). Rosetting rate of 56% (42/75) among parasites from severe disease cases was higher than those found among uncomplicated malaria cases (44%, 33/75). Majority (30) of the samples had large rosettes followed by those with small rosettes (24) with 21 of them forming medium rosettes (Figure 2).

Table 1 Clinical Characteristics of Study Participants

Parameter	Severe Malaria N = 79 Mean ($\pm$ SD)	Uncomplicated Malaria N = 127 Mean ($\pm$ SD)	P values
Sex			
Male n (%)	47 (22.8%)	65 (31.5%)	
Female n (%)	32 (15.5%)	62 (30.1%)	
Age (years)	3.79 ($\pm$ 1.97)	5.0 ($\pm$ 2.93)	0.006
Haemoglobin (g/dl)	8.4 ($\pm$ 2.29)	10.3 ($\pm$ 1.95)	0.0001
Body Weight (kg)	14.7 ($\pm$ 4.92)	17.1 ($\pm$ 8.07)	0.014
Body Temperature ($^{\circ}$ C)	38.7 ($\pm$ 1.44)	38.6 ($\pm$ 1.23)	0.530
α_2 M conc (mg/mL)	2.27 ($\pm$ 0.16)	1.916 ($\pm$ 0.1814)	0.149
Parasitemia (Parasites /ul)	77,274 ($\pm$ 14,124)	58,196 ($\pm$ 12,292)	0.3110
Glucose (mmol/dl) $\pm$ SD	6.207 $\pm$ 0.1730	7.135 $\pm$ 0.2609	0.0041
Blood Group Type n (%)			
A	18 (8.73%)	29 (14.1%)	
B	32 (15.5%)	25 (12.1%)	
AB	4 (1.9%)	4 (1.9%)	
O	44 (21.4%)	50 (24.3%)	

Notes: Values are represented as mean and standard deviation or proportions between groups. Values highlighted represent comparison with significant P-values < 0.05 . Significant P-values are highlighted in bold.

Abbreviations: N, number; SD, standard deviation.

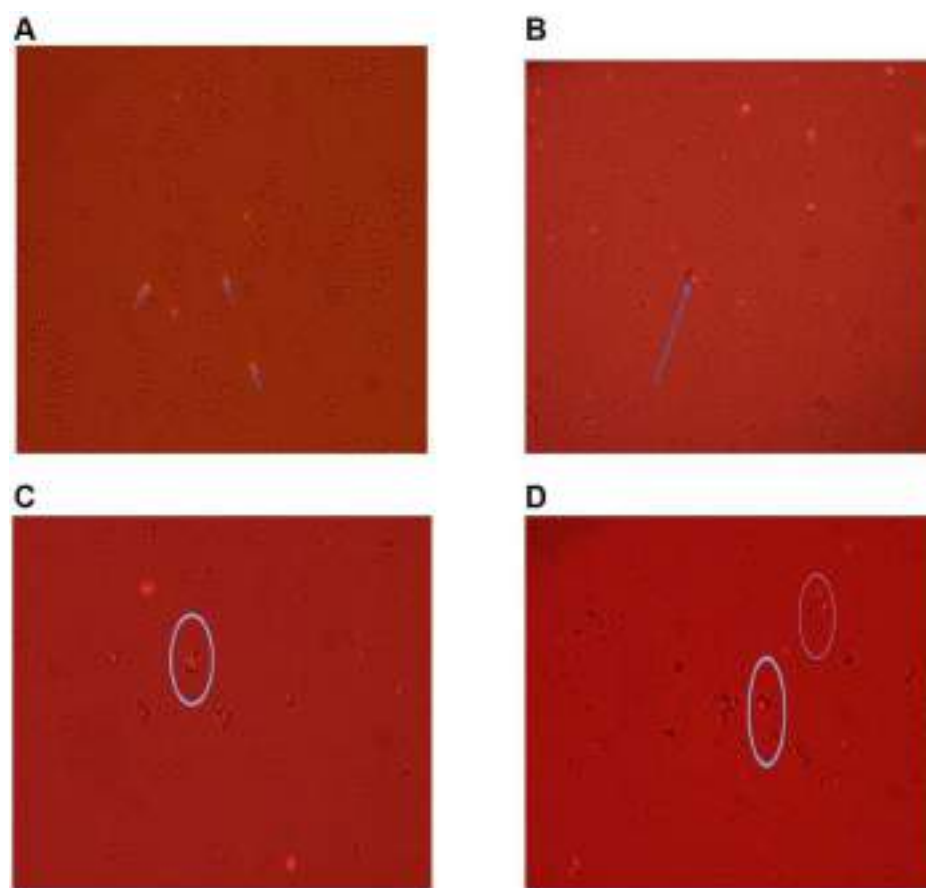


Figure 1 Micrographs of rosetting parasites. (A) infected RBC with parasite showing as bright spots, (B) infected RBC bound by uninfected RBC, (C) small rosette with 2 or 3 uninfected RBC and (D) large rosette with >3 uninfected RBCs.

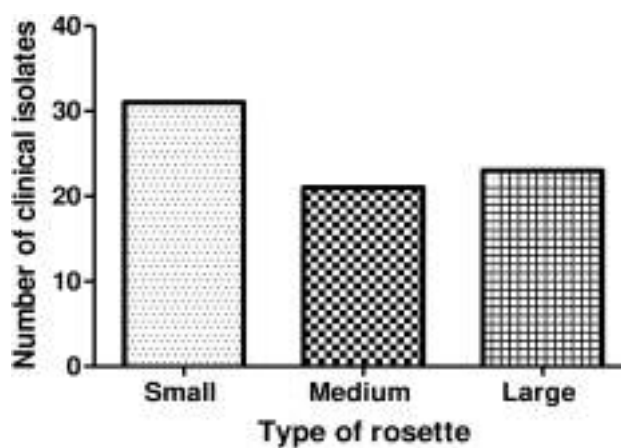


Figure 2 Categorizing Parasite Isolates by their rosetting capabilities. Large rosettes = infected RBC bound by > 5 uninfected RBCs, Medium = bound by 3–5 uninfected RBCs, Small rosettes = bound by 2 or 3 uninfected RBC.

Interestingly, when the rosettes were categorized according to the source of the parasite isolate, whether from severe malaria or uncomplicated malaria, it was observed that small rosettes were from parasites collected from severe malaria patients and the difference was statistically significant ($P=0.015$) (Table 2).

Table 2 Rosette Categorization in Severe and Uncomplicated Malaria Isolates

Rosette Categorization	SM	UM	P value
Large rosettes	14	16	0.3
Medium rosette	10	11	0.2
Small rosette	18	6	0.015
Total	42	33	

Note: Significant P-values are highlighted in bold.

Abbreviations: SM, severe malaria isolate UM, uncomplicated malaria isolate.

Rosette Formation Was Observed in All Blood Group Types in Different Proportions

Rosette formation was observed in all blood groups with rosettes frequently seen in isolates from blood group O followed by blood group B type. Rosetting frequency was highest in parasite isolates from blood group A. There was no statistical difference in mean rosetting frequency among the blood groups ($P=0.5$). Rosette characteristics were different among the blood groups.

Large rosettes (bound by >5 uninfected RBC) were seen in blood group types A and B whilst group O showed smaller rosettes (Figure 3).

Rosetting Capabilities of *P. falciparum* Clinical Isolates from Different Disease Categories in Different Blood Group Types

Rosette formation was seen in all four blood group types in different proportions, but the number of rosettes did not differ significantly between disease categories (Figure 4). The chance of finding a rosette (rosette prevalence) among the clinical isolate was higher in blood group O type (30.1%) and blood group B type (29.8%). On average, blood group A had the highest mean frequency (rosettes per 200 IRBC) of 28.7 rosettes per isolate as compared to 16.7 rosettes per isolate observed among blood group O type isolates. The highest number of rosettes per isolate was recorded in blood group B followed by A with the least in blood group AB. Generally, there was rosette formation among parasites from all disease categories grown in all blood group types with the only exception being among blood group AB where rosetting was not observed in the severe malaria group (Figure 4).

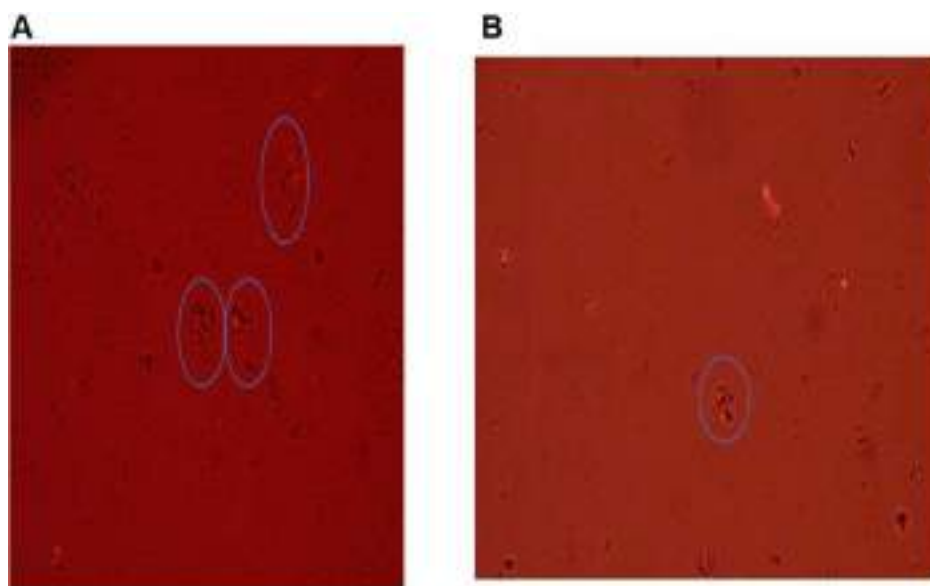


Figure 3 Micrograph showing parasite isolate rosettes from (A) blood group A type rosette and (B) blood group O type. (A) Large rosettes seen in blood group type B (B) Small rosettes seen in blood group type O.

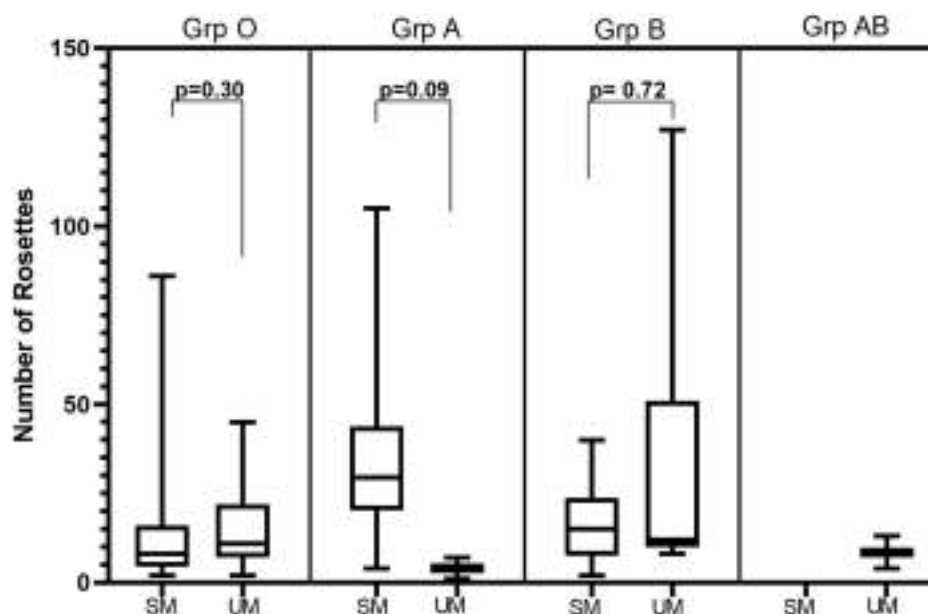


Figure 4 Association between number of rosettes and disease categories in different ABO blood group types. The middle line represents the median and the lower and upper bars are the minimum and maximum number of rosettes.

Abbreviations: SM, severe malaria; UN, uncomplicated malaria; Grp, group.

Host Factors That Affect Rosette Formation

When the measured clinical variables were analysed between rosetting and non rosetting clinical isolates, it was observed that parasitemia was higher among rosetting isolates compare to non rosetting isolates ($P=0.002$) (Table 3). The age of the participant was not associated with rosetting although rosetting was commonly found among parasite isolates from younger children than parasites from older children ($p<0.9$) (Table 4). The relationship between rosette formation and measured parameters were assessed using a linear regression analysis.

Parasitemia and Rosette Formation

When the measured clinical variables were analysed between rosetting and non rosetting clinical isolates, it was observed that parasitemia was higher among rosetting isolates as compare to non rosetting isolates ($P=0.002$) (Table 3).

Association of Plasma Concentrations of α_2 M on Rosette Formation and Malaria Severity

The α_2 M concentration weakly correlated positively with rosette frequencies ($r=0.080$ but the difference was not significant ($p=0.52$) (Figure 5A). Although α_2 M concentration was found to be higher in uncomplicated malaria (mean = 2.27 mg/mL) than in severe malaria (mean=1.92 mg/mL), ($P = 0.2$), the difference was not statistically significant. A significantly higher α_2 M concentration was observed in patients with a higher parasitemia ($P= 0.02$) (Table 4).

Table 3 Effect of Patient Variables on Rosetting and Non Rosetting Isolates

Parameter	Mean Values Non Rosetting	Mean Values Rosetting	P-values
Malaria parasites (parasites/uL)	65,403	74,014	0.002
Haemoglobin (g/L)	9.621	10.19	0.4
Age	4.695	4.698	0.2

Note: Significant P-values are highlighted in bold.

Table 4 Association Between Patients' Parameters and Plasma Levels of α_2 M Concentration

Parameter	r	P
Age (years)	4.752e-005	0.9
Glucose (mmol/L)	0.0006348	0.8
Haemoglobin levels (g/dl)	0.0003071	0.9
Disease category	0.02139	0.2
Parasitemia (parasite/uL)	0.123	0.02
Weight	0.003972	0.6

Notes: Spearman rank correlation analysis of patient parameters and the concentration of α_2 M. Significant P values have been highlighted. Plasma alpha 2 macroglobulin concentration in study participants of varying clinical presentations and rosette formation. Significant P values are highlighted in bold.

Also, in correlation analysis of non resetting parasite isolates, only parasitemia was found to correlate with α_2 M concentration ($P=0.04$) whilst age and hemoglobin levels did not show any significant association (Table 5).

To determine the effect of α_2 M concentration on rosette formation, the mean plasma concentration of α_2 M was compared between rosette formation by isolates from severe and uncomplicated malaria patients. It was observed that the

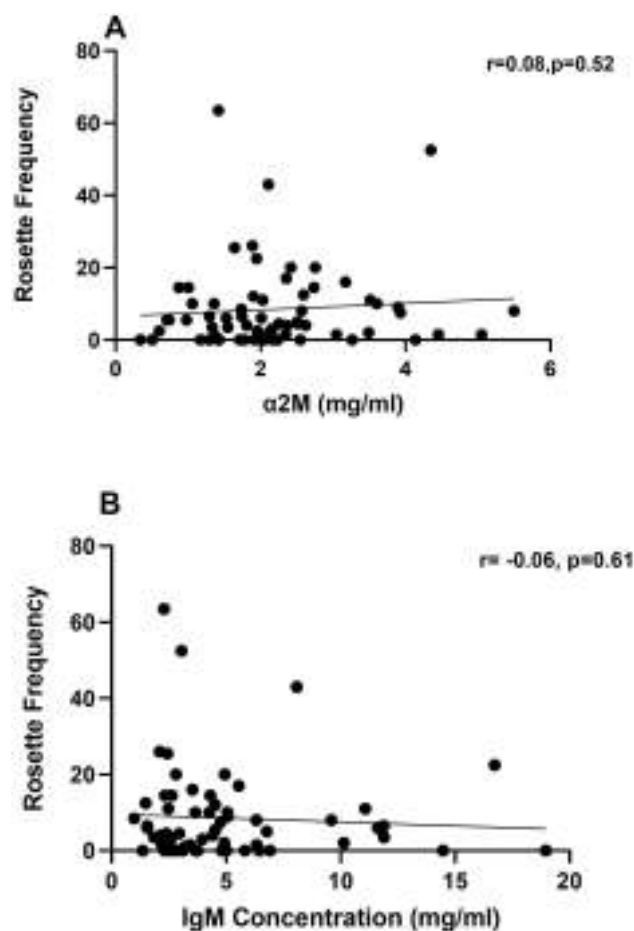


Figure 5 Scatter plot showing the relationship between rosette frequency and plasma α_2 M and IgM concentrations. (A) scatter plot of rosette frequencies and plasma α_2 M concentrations and (B) scatter plot of rosette frequencies and plasma anti-parasite IgM concentrations.

Table 5 Association of α_2 M Concentration on Non Rosetting Isolates

	r	P value
Age (years)	0.091	0.6
Haemoglobin levels (g/dL)	0.223	0.2
Parasitemia (IRBCs/ μ L)	0.334	0.04

Note: Spearman Rank correlation analysis of non rosetting isolates only. Significant P values are highlighted in bold.

Table 6 Association of Anti-Parasite IgM Concentration on Clinical Variables Among Rosetting Parasite Isolates

	Spearman r value	P value
Haemoglobin	0.3012	0.02
Number of rosettes	0.0039	0.5
Parasitemia	0.1260	0.2
Age	0.1793	0.1

Notes: Spearman Rank correlation of anti-parasite IgM and measured parameters among the rosetting isolates only. Significant values are highlighted in bold.

mean concentration of rosetting isolates [2.08 mg/mL (0.3mg/mL - 5.4mg/mL)] was similar to that of non rosetting isolates [2.02mg/mL (0.3mg/mL - 4.9mg/mL)] ($P=0.3534$) ([Figure S1](#)).

Association of Plasma Concentration of Anti-Parasite IgM on Rosette Formation and Disease Severity

The anti-parasite IgM concentrations did not correlate with rosette frequencies among all the isolates studied ([Figure 5B](#)) although a weak negative correlation was observed it was not statistically significant ($r=-0.06$, $p=0.61$). Also, the mean plasma concentration of anti-parasite IgM among the study population was 4.5 mg/mL (± 4.0). Males had higher mean anti-parasite IgM levels (4.6 ± 4.4 mg/mL) compared to the females (4.2 ± 3.7 mg/mL), $P < 0.05$. Participants with severe malaria had a lower mean anti-parasite IgM concentration (3.7 ± 3.6 mg/mL) as compared to those with uncomplicated malaria (5.3 ± 4.3) and the difference was statistically significant ($P=0.02$) ([Figure S2](#)).

The mean plasma concentration of anti-parasite IgM measured among patients with rosetting forming isolates was lower at 4.2 (± 3.930) as compared to the mean anti-parasite IgM concentration of (4.604 (± 4.159)) from patients whose parasite isolates did not form rosettes.

In correlation analysis, among the rosetting clinical isolates, anti-parasite IgM concentration was affected by only haemoglobin levels ($P=0.02$) whilst all the other variables were not significantly affected by the anti-parasite IgM concentration ([Table 6](#)).

Discussion

Studies have shown that among patients, rosetting rate is dependent on the disease category of either severe or uncomplicated and that among severe malaria, typically therefore, rosette formation is more pronounced in severe malaria than in uncomplicated malaria cases. In this study, rosette formation was observed in 46.8% (75/160) of the parasite isolates tested. This level of rosetting is lower as compared to higher rosetting rates found in other studies,¹³ but not unexpected as this could be as a result of the relatively fewer number of isolates obtained from the participants with severe malaria in the study. Even so, rosetting rate was higher (56%) among the severe malaria cases compared to the uncomplicated (44%). The difference was however not statistically significant. This agrees with other studies which

showed that among severe malaria cases, there was higher rate of rosetting compared to uncomplicated malaria, though rosette formation was found in all categories of disease in different proportions.^{13,25,30,31}

A number of reasons could account for the lower rosetting rate observed among the parasite isolates. Barragan et al showed that among children from Kenya and Gabon, repeated malaria attacks in high malaria transmission areas resulted in exposure to antigens which prompted an antibody mediated response that recognises many rosetting antigens.³² These anti-rosetting antibodies produced in response to repeated exposure to parasite ligand has been linked to a reduced rosetting rates and gradual acquisition of immunity in children.³³ In support of this, others found high levels of antibodies to a parasite ligand (NTS-DBL1 α) known to be associated with rosetting in children in malaria endemic areas^{34,35} which resulted in a reduced level of rosetting in the clinical isolates from that endemic community.

Parasite ligand PfEMP1 being expressed by *var* genes have the ability to produce clonally different phenotypes of the genes (antigenic variation) with different binding affinities that protect the parasite from immune clearance.³⁶ Other factors such as host immunity, genetics and polymorphisms in certain host factors may be involved in the development of severe malaria aside rosetting.³⁷

Rosette formation was enhanced by blood group A antigens and the age of the child. This is consistent with a study done elsewhere which supports the high frequency of rosetting found in isolates from blood group A type.²¹ Though rosette formation was prevalent in groups O and B, it was blood group A isolates that produced the largest rosettes supporting similar findings in other studies.²¹ Remarkably, small rosettes were associated with severe disease compared to large ones but this is contrary to other studies where larger rosettes were found to be associated with severe malaria.^{14,38} The dominant blood group in our study area was blood group O and this blood group has been associated with formation of small sized rosette and in essence protect against severe disease.³⁹

Parasitemia also affected rosette formation and there is conflicting data on the effect of parasitemia on rosetting among African children. There is conflicting data on the effect of parasitemia on rosetting among African children. Whilst some studies found positive correlation between parasitemia and rosetting,^{14,40,41} other studies show no association of rosette formation with high parasitemia.^{42,43} Our findings agree with studies by Marsh et al, as well as the Heddini and later Rowe which showed a positive correlation between parasitemia (parasite densities) and rosetting in African isolates.^{13,41,42} A previous study conducted in India which investigated DBL- α (parasite ligand that support rosetting) diversity in severe disease in Odisha population showed significantly higher parasitemia among rosetting isolates.⁴⁴ These findings implicate rosette formation to contribute to elevated parasite densities that favor invasion of the host cells from bursting schizont.¹⁴ Therefore, the elevated parasitemia seen among the rosetting isolate is probably advantageous to ensuring the survival and replication of the parasite.^{12,45}

Our study showed a lower anti-parasite IgM concentration among rosetting isolates and this was positively correlated with rosette frequency consistent with findings by others that showed a positive association between rosette frequency and IgM concentrations in Kenyan children.^{15,46} Conversely, higher anti-parasite IgM concentrations were found among patients whose parasite isolate did not form rosettes and this supports recent findings that show that high IgM concentrations among children in endemic areas is protective against severe malaria.^{16,47,48}

A higher mean α_2 M serum concentration was found among uncomplicated malaria patients than severe malaria patients, who had a lower mean α_2 M concentration. Similar observations were made in a study in Thailand where higher α_2 M concentrations were found in women with malaria parasites compared to women who did not have parasites.⁴⁹ Increased α_2 M concentration has also been shown in children suffering from “chagasis” (*Trypanosoma cruzi* infection) in Bolivia.⁵⁰

A high α_2 M concentration among the non rosetting isolates contradicts findings elsewhere which showed otherwise.¹⁶ However, α_2 M concentration was positively associated with parasitemia among non rosetting isolates only, a phenomenon not observed among the rosetting isolates studied. During malaria infections, merozoites use proteolytic enzymes in re-invading red blood cells whilst schizont degradation of hemoglobin involves the use of these same proteolytic enzymes. Therefore, α_2 M is produced by the body in response to high proteolytic enzymes to binds irreversibly to these proteolytic enzymes and protecting the tissues from tissue damage.⁵¹ The findings also shows that α_2 M may elicit high parasitemia in non rosetting isolates and therefore α_2 M may be used as a biological marker for high parasitemia in malaria infected individuals.

Parasite isolates from patients with high α_2 M concentrations had more rosettes than those from patients with low α_2 M concentration. This confirms the role of α_2 M in enhancing rosette formation by increasing the avidity of binding of PfEMP1 molecules on the surface of the IRBC consistent with others studies.^{16,25}

This may confirm the role of this serum protein in supporting high parasite growth in lieu of severe disease. Increases in α_2 M concentration has been documented in diseases that result in high plasma and tissue proteinase levels such as cystic fibrosis rheumatoid arthritis and pancreatitis.⁵⁰ During *Plasmodium* infections, are used in the rapturing and re-invading of red blood cells and this process generates high amounts of proteinases that effectively trigger high production of α_2 M to remove the excess proteinases.⁵¹

Conclusion

This study has shown that rosette formation in *P. falciparum* clinical isolates is affected by the blood group type of the patient with isolates from blood group A type producing the highest number of rosettes per isolates.

A low IgM concentration was associated with severe disease whilst a high α_2 M concentration was associated with uncomplicated malaria. In relation to disease outcome, blood group A type and low anti-parasite IgM concentration increased the risk of severe malaria among participants.

Abbreviations

α_2 M, alpha 2 macroglobulin; CR1, complement receptor 1; ELISA, enzyme linked immunosorbent assay; ERC, ethical review committee; GHS, Ghana health service; HRP, horse radish peroxidase; HS, heparan sulphate; IgM, immunoglobulin M; IRBC, infected red blood cells; MOH, ministry of health; NHS, normal human serum; OD, optical density; OPD, out patient department; PBS, phosphate buffered saline; PBST, phosphate buffered saline 0.1% Tween 20 detergent; PfEMP1, plasmodium falciparum erythrocyte membrane protein 1; RBC, red blood cells; RDT, rapid diagnostic test; RIFIN, *P. falciparum*-encoded repetitive interspersed families of polypeptides; RPMI, Roswell Parks Memorial Institute; SM, severe malaria; STEVOR, subtelomeric variable open reading frame; TMB, 3,3',5,5'-tetramethylbenzidine; UM, uncomplicated malaria; VSA, variant surface antigens.

Data Sharing Statement

The datasets analysed in this study are available from the corresponding author on reasonable request.

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Author Contributions

All authors made significant contribution to the work, whether in the conception, study design, implementation, acquisition of data, analysis or interpretation. All the authors were involved in the drafting, revising and critical reviewing of the article. They all agreed on the journal to which the article has been submitted and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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Thrombin Use in Surgery: An Evidence-Based Review of Its Clinical Use [Retraction]

Ham SW, Lew WK, Weaver F. *J Blood Med*. 2010;1:135–142.

The Editor and Publisher of *Journal of Blood Medicine* wish to retract the published article. It has come to the journals attention that the article contains a significant amount of overlap to an article published previously by two of the same authors:

- Clinical use of topical thrombin as a surgical hemostat. Lew WK, Weaver FA, *Biologics: Targets and Therapy*, 2008;2(4):593-599 (<https://doi.org/10.2147/BTT.S2435>).

The authors did not respond to our queries and have been notified of the decision to retract.

We have been informed in our decision-making by our policy on publishing ethics and integrity and the COPE guidelines on retractions.

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

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Waldenstrom's Macroglobulinemia and Ascites: A Case Report

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Waldenstrom's Macroglobulinemia and Ascites: A Case Report

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Background: Waldenstrom's disease is characterized by the presence of pathological changes in the B lymphocytes that are in the last stages of maturation. One characteristic of WM is the production of an abnormal high amount of IgM and hyper viscosity syndrome. The MW gets worse, symptoms such as fatigue, weight loss, night sweats, fever, recurrent infections and swollen lymph nodes develop in patients who have a known history of MGUS. In this clinical case, our patient without history of MGUS, presents for the first time for medical observation only for ascites and the presence of an interportocaval lymph node package. An atypical presentation of the disease that makes us reflect on the difficulty of making a diagnosis in the elderly patient and on pathogenetic hypotheses of ascites not yet explored.

Case Presentation: Seventy-three-year-old patient, hospitalized for the onset of ascites with sloping edema, diffuse left pulmonary opacification. At the ultrasound check, cava and portal vessels patent and of regular caliber, however with inversion of flow in correspondence with the right branch and of the door to the hilum, with a subdiaphragmatic retrocaval focus with a maximum diameter of about 3 cm, which cannot be better viewed. CT scan of the abdomen with confirmation of the presence of an interportocaval lymph node package. After evidence of the electrophoretic protein picture of a double component, probably monoclonal with positive urinary immunofixation for free K chains. IgM dosage equal to 2190 mg. Serum immunofixation practice that confirms the diagnosis of type B lymphoproliferative syndrome as per Waldenstrom's disease, confirmed by bone marrow aspiration with morphological and flow cytometric study. Immediately begin chemotherapy with Bendamustine 120 mg. After 4 weeks of therapy with the reduction of IgM values, the patient no longer presented ascites.

Conclusion: This case has an unusual presentation of this disease and we could shed a new light on the possible pathogenesis of portal hypertension in Waldenstrom's disease.

Keywords: ascites, portal hypertension, Waldenstrom's disease, elderly

Introduction

Background

Waldenström's macroglobulinemia is a rare form of cancer, the estimated incidence of which, according to the Italian Association against Leukemia-Lymphomas and Myeloma (AIL), is 2.5 cases per 1,000,000 people. Waldenstrom's disease is characterized by the presence of pathological changes in the B lymphocytes that are in the last stages of maturation.¹ The presence of these alterations determines the uncontrolled growth of abnormal cells, generating numerous clones, mainly in the lymph nodes and, subsequently, in the medulla. Abnormal cells can also accumulate in other organs and tissues, such as the liver and spleen, resulting in an increase in volume (hepatosplenomegaly). Cancer cells produce only one type of immunoglobulin, called the monoclonal component or the M protein. Most people with LPS (90–95%) produce monoclonal immunoglobulin type M (IgM) and rarely produce other antibody isotypes such as IgA or IgG. Additionally please include that WM is considered a non-hodgkin's lymphoma that it is related to hyperviscosity syndrome and describe resulting complications in more detail.

Since MW makes up the majority of cases of LPL, the two terms are sometimes used interchangeably. Usually, MW is preceded by a condition known as a monoclonal gammopathy of undetermined significance (MGUS IgM), which may or may not progress to MW. Any progression of MGUS IgM into MW takes a long time, usually years. Patients with MGUS IgM are asymptomatic; the identification of the pathology can occur following the execution of blood tests during routine investigations.^{2,3} A detectable (usually low) amount of abnormal blood IgM may be found in these individuals. The initial phase of MW is characterized by the absence of symptoms (asymptomatic MW) and requires close monitoring. If the MW gets worse, symptoms such as fatigue, weight loss, night sweats, fever, recurrent infections and/or swollen lymph nodes may develop. The increase in plasma viscosity (commonly refers to plasma when we speak of “hyperviscosity”) leads to the appearance of neurological symptoms due to the slow flow of blood through the vessels of the microcirculation: paraesthesia, headache may appear; the localization of vaso-occlusive phenomena in the brain can cause drowsiness, ataxia, disturbances in alertness, up to coma.

Case Presentation

73-year-old patient with arterial hypertension, diabetes on oral hypoglycemic therapy, no history of liver disease. He is hospitalized for the onset of ascites with sloping edema, diffuse left pulmonary opacification. On a vigilant and well-oriented physical examination in time and space, with globally reduced vesicular murmur and hypophonesis in the left lung area, globose abdomen with abundant presence of ascites, non-significant pericardial circumferential detachment on the ecobedside and moderate left pleural effusion with cardiac kinetics in the norm, fibrosclerotic valve systems. On the ECG BAV I degree with atypia of repolarization. In the blood tests, moderate increase in cholestasis indices and only very slight increase in GOT, mild hypoalbuminemia, slight decline in PT, normal bilirubin, normal platelets. Major negative hepatitis markers, no alcohol intake reported. Negative tests on ascitic fluid with serum-ascites deponent gradient calculation for transudate equal to 1.3. At the ultrasound check, following the first paracentesis, liver volume increased to a finely inhomogeneous echostructure and with irregular margins, non-dilated biliary tract, suprahepatic veins, cava and portal vessels patent and of regular caliber, however with inversion of flow in correspondence with the right branch and of the door to the hilum, with a subdiaphragmatic retrocaval focus with a maximum diameter of about 3 cm, which cannot be better viewed. CT scan of the abdomen with confirmation of the presence of an interportocaval lymph node package. The PET exam shows accumulation of the metabolic tracer of a diffuse character due to the note consolidation of the upper lobe and lingula of the left lung highlighted on CT (SUV max 5.9). Concomitant further accumulation of the tracer is found in some lymph nodes in the intercavo-aortic area, interportocaval and hepatic hilum (Figure 1). After evidence of the electrophoretic protein picture of a double component, probably monoclonal with positive urinary immunofixation for free K chains. IgM dosage equal to 2190 mg. Serum immunofixation practice that confirms the diagnosis of type B lymphoproliferative syndrome as per Waldenström's disease, confirmed by bone marrow aspiration with morphological and flow cytometric study. Genetic research in Waldenström's macroglobulinemia has taken a decisive step forward in 2011, thanks to the discovery of a mutation in the MYD88 gene with a frequency of 90% or higher in moles WM patients. This same study showed that the MYD88 gene mutation, named MYD88 L265P, was not frequently found in other types of lymphoma or multiple myeloma, confirmed by subsequent follow-up studies conducted by researchers on WM around the world. The current guidelines recommend allele-specific PCR testing to detect the MYD88 L265P mutation inside the bone marrow cells in suspected cases of WM, indicating it as an essential exam for the diagnosis of the disease. Our patient had this mutation. Immediately begin chemotherapy with Bendamustine 120 mg. The patient did not receive combination therapy with rituximab because he showed intolerance to rituximab.

Discussion

This case is of importance due to severity and due to lack of information regarding this rare disease. In this clinical case, the disease started with no previous history of MGUS and with the only symptom represented by ascites. Only three cases of Waldenström with malignant ascites in 2004 by Stoffel,⁴ Marinella⁵ in 1994, Simos⁶ in 1978 have already been described in the literature.

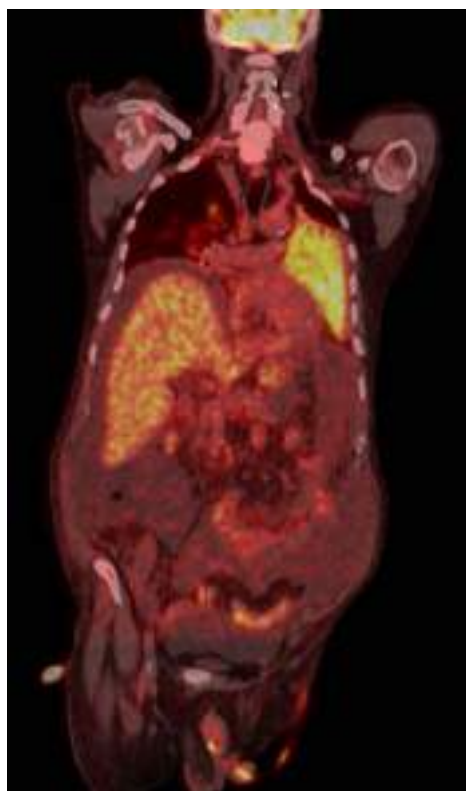


Figure 1 The PET-CT examination shows accumulation of the metabolic tracer of a diffuse character due to pulmonary thickening, with ground glass. Concomitant further accumulation of the tracer is found in some lymph nodes in the intercavo-aortic area, inter-portocaval and hepatic hilum. Limited to the resolution capacity of the method (4 mm) no further pathological areas are highlighted accumulation of the radiopharmaceutical in the remaining areas of the body examined.

In the first case report,⁴ massive peritoneal ascites developed over a short period of time in a patient without any pre-existing history of liver disease and when the liver biopsy did not show frank cirrhosis, other precipitants of ascites were considered. In patients with cirrhosis, a portal pressure gradient of 10–12 mmHg is defined as clinically significant portal hypertension and is required as a minimum for the development of ascites. The hypothesis that hyperviscosity caused the ascites is supported by the disappearance of ascites during treatment of the lymphoproliferative disorder and normalization of the serum IgM levels. Hyperviscosity that had aggravated pre-existing portal hypertension; in Marinella's case,⁵ the ascites was attributed to peritoneal lymphomatosis. In Simos's case Waldenström's macroglobulinemia presents with pleural effusion and ascites.

Our patient presents ascites as the only clinical manifestation. There is no peritoneal lymphomatosis, no pleurisy or signs of hyperviscosity. Cava and portal vessels patent and of regular caliber, however with inversion of flow in correspondence with the right branch and of the door to the hilum, with a subdiaphragmatic retrocaval focus with a maximum diameter of about 3 cm, which cannot be better viewed. CT scan of the abdomen with confirmation of the presence of an interportocaval lymph node package. We tried to identify the pathogenetic mechanisms responsible for ascites, advance a hypothesis never described before in the literature: the flow inversion of the right portal branch was an expression of a slowing of the flow secondary to an intrahepatic obstacle and in particular of the small portal vessels due to probable infiltration of the wall by lymphoplasmacellular elements.

Conclusion

This case has an unusual presentation of this disease and we could shed a new light on the possible pathogenesis of this:

1. Portal hypertension may be due to increased blood flow from the splenic vein secondary to splenomegaly.

2. Lymph node compression may have been a contributing cause to the onset of portal hypertension (in particular on the splenomegaly aspect and sloping edema).
3. The flow inversion of the right portal branch was an expression of a slowing of the flow secondary to an intrahepatic obstacle and in particular of the small portal vessels due to probable infiltration of the wall by lymphoplasmacellular elements.

Abbreviations

MGUS, Monoclonal Gammopathy of undetermined significance; MW, Macroglobulinemia Waldenstrom; ECG, electrocardiography; BAV, atrioventricular block; PT, prothrombin time.

Consent of the Patient

The authors have the consent of the patient for the publication of the case. (CARE guidelines). The authors used the patient consent form (provided by the Taylor & Francis group, of which Dove Medical Press is a part) completed, signed and securely saved. Consent to publication has been obtained: the ethics committee of the ASL Napoli 1 approved the publication of the case. The authors are able to share it with the editors of the journal if requested.

Author Contributions

CB wrote the original manuscript and edited the manuscript, AC performed ultrasound examination, MG, PM, AG, AF, MGC, ML and PT managed the patient, MS provided pathological images with descriptions, PDM performed clinical supervision. 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 report no conflicts of interest in this work.

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Assessment of Blood Transfusion Utilization and Patient Outcomes at Yekatit-12 Hospital, Addis Ababa, Ethiopia

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Assessment of Blood Transfusion Utilization and Patient Outcomes at Yekatit-12 Hospital, Addis Ababa, Ethiopia

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Background: The goal of blood transfusion is to provide a safe, sufficient, and timely supply of blood components to the recipients by ensuring that donation is safe and no harm to the recipient. So the current study was aimed to assess blood transfusion utilization and its outcome in patients at Yekatit-12 Hospital, Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted from May 01 to July 30, 2021, on 616 individuals who have requested blood transfusion at Yekatit-12 Hospital, Addis Ababa, Ethiopia. Information on blood and blood component utilization was collected by using a checklist. Finally, data were analyzed using SPSS version 21.0, and those variables in which P-value is less than 0.05 were considered statistically clinically significant.

Results: A total of 1540 cross-match units were requested for 616 (53.2% male) patients with a mean age of 43 years. Out of 1540 cross-matched blood units, 1498 units of blood were transfused for 615 individuals with a mean of 2.43 units. The most widely used blood component was whole blood 694 (46.3%) and 1252 (83.6%) of the blood unit were “RH” positive. From a total of 68 (11.1%) none improved reported cases, 48 (10.5%) were those transfused with whole blood transfusion. The proportion of improvement after transfusion according to the service ranged from 83.3.0% to 100% and no post-transfusion reactions were reported. A significant improvement was seen in the hematological profile (hemoglobin, red blood cells, hematocrit, platelets, and white blood cells) findings of the transfused individuals after blood unit transfusion ($P < 0.05$).

Conclusion: The overall utilization rate and improvement status after transfusion were high even if the utilization rate has some limitations. The overall ratios of cross-match to transfusion ratio, transfusion probability, and transfusion index were 1.03, 99.8%, and 0.97, respectively.

Keywords: blood transfusion, utilization, outcome, Yekatit-12 Hospital, Ethiopia

Background

Blood transfusion is a procedure in which whole blood or parts of blood are put into a patient's bloodstream through a vein to compensate for any type of blood loss or to treat anemia.¹ As recent WHO and different national blood bank reports indicated, the rational use of blood has increased and it needs evidence-based patient blood transfusion management and transfusion medicine practice.² Many clinical data indicate transfusion of blood components is the most common medical procedure in health institutes because the demand for safe blood transfusion is increasing with the increment of a blood disorder.¹ On the other hand; if blood transfusion is misused, it may expose recipients to undesirable risks, which causes additional costs for the health system and waste a scarce resource.³

As Many findings indicated, blood transfusion in the clinical area has significant importance and its therapeutic benefit was achieved by taking and implementing evidence-based activities through continuous review and evaluation of demographic and clinical characteristics, clinical response of transfusion recipients, and appropriate clinical utilization of blood and blood products.²

Patient Blood Management (PBM) takes an individualized, multidisciplinary approach to the management of a patient's blood after transfusion of blood components, through the development of a management plan to optimize a patient's blood, minimize blood loss, and optimize tolerance of anemia.⁴ Although blood transfusion order is a common practice in most health care institutes, no clear data is indicated on the utilization and outcomes of blood transfusion. So the assessment of utilization and outcomes of blood transfusion are vital things to provide a rational and evidence-based, case-specific practical framework to avoid unnecessary expenses imposed on patients and institutions, and thereby increases the availability of safe blood for those who are in real need. Thus this study aimed to assess transfusion utilization and patient outcomes at Yekatit-12 Hospital, Addis Ababa, Ethiopia; August 2021.

Materials and Methods

Study Area and Setting

A cross-sectional study was conducted from May 01 to July 30, 2021, at Yekatit-12 Hospital which is found in Addis Ababa; the capital city of Ethiopia under the Addis Ababa Health Bureau; to assess the utilization of blood and blood components.

Source Population

The study population was all patients who have transfused blood and blood components at Yekatit-12 Hospital during the study period.

Study Population

The study population transfused individuals and those who have full records, of blood, transfused, units of blood, the reason for transfusion, a component of transfused, CBC results before and after transfusion, and outcomes of transfusion based on WHO guidelines.

Inclusion and Exclusion Criteria

Inclusion Criteria

The study population transfused individuals and those who have full record of blood transfused, unit of blood, the reason for transfusion, the component of transfused, CBC result before and after transfusion, and outcomes of transfusion based on WHO guidelines Yekatit-12 Hospital from September 01, 2019, to May 20, 2021.

Exclusion Criteria

Recipients with incomplete records based on WHO guideline.

Sample Size Calculation and Sampling Method

Sample Size Calculation

All transfused individuals during the study period and fulfilling our inclusion criteria were included. 3 months of prospective data were collected from the records of transfused patients in all wards of Yekatit 12 Hospital from May 01, 2021, to the end of July 2021. The total number of patients who have transfused during the study period and fulfilled the inclusion criteria was 616.

Sampling Method

A simple random cross-sectional study was conducted from May 01, 2021, to July 30 2021 on transfused individuals at Yekatit-12 Hospital, Addis Ababa, Ethiopia.

Data Collection Procedure

The data was collected by using a checklist which was developed following a thorough review of the literature from different sources and including information related to the socio-demographic condition and medical status. The checklist contains sections for assessing demographics, blood transfusion-related information, and associated factors. The questions and statements are grouped and arranged according to the particular objectives that are aimed to address. Records regarding the reason for transfusion, unit of blood requested, type of blood group requested, type of blood component

requested, a unit of blood cross-matched, units of blood utilized after cross-match, and outcomes of recipients after transfusion were collected by reviewing the recipient's cards, and laboratory blood bank logbooks by using structured pretested check-list. Details including gender, age, a hematological profile of the patient, the number of units that were expired (outdated) before utilization, type and amount of blood components transfused, the health professional who ordered the transfusion, the department from which the request was issued and patient improvement status was noted. Transfused patients with the absence of clinical symptoms, with improvement in hematological profile after transfusion and/or reduced length of stay in the hospital were defined as showing better improvement.

Quality Assurance

The data were collected using a checklist after orientation has given to all data collectors regarding the procedures to be followed during the data collection by hematology experts. In addition, the collected data were reviewed and checked for completeness and consistency daily by the PI and two supervisors.

Data Analysis and Interpretation

Data was cleared, edited, checked for completeness manually, and entered to Software Package for the Social Science (SPSS) for version 20.0 for Windows[®] (SPSS Inc., Chicago, IL, USA for analysis. After organizing and cleaning the data, frequencies and percentages were calculated for all variables that are related to the objectives of the study. The extent of blood prescribed, utilized, and transfusion outcome was checked. The level of statistical significance was set at a 95% confidence interval. A P-value of less than 0.05 was considered statistically clinically significant. Finally, the result is presented by using tables and narrative forms.

Operational Definitions

- Recipients card: Patient cards that have blood transfusion records.
- Prescribed blood: Unit of blood requested by the physician for transfusion.
- Donated Blood: Units of blood utilized by the patient/ recipients.
- Returned Blood: Blood returned to the lab after cross-matching.
- Missed Blood: Blood was not donated by different factors.
- Moreover, the following transfusion utilization and outcome-related operational definitions were used as a guideline for this study.^{5,6}
 - Cross-match to transfusion ratio (C/T ratio) = number of units blood cross-matched to the number of units of blood transfused and a ratio of C/T less than or equal to 2.5 is considered as indicative of significant/efficient blood usage.
 - Transfusion probability (TP) = calculated by the number of patients transfused divided by the number of patients cross-matched and then multiplied by 100. A TP value of greater than or equal to 30% was considered an indication of efficient blood usage.
 - Transfusion index (TI) = defined by a ratio of the number of blood units transfused to the number of patients cross-matched and a TI value of greater than or equal to 0.5 or was considered as an indication of significant blood utilization.

Ethical Considerations

The study was conducted after ethical approval was obtained from the Research and Ethics Institutional Review Board of ECUSTA Health Science Institute. An official permission letter was submitted to the Addis Ababa Health Bureau and Yekatit-12 Hospital. The informed written consent was obtained for participants with ages of greater than 18 years old and informed written assent was also obtained from each participant with the age of fewer than 18 years from their parents or guardians based on Helsinki declaration or protocol before the actual data collection.

Result

A total of 1540 blood units were requested for 616 study subjects. From a total of 616 transfused patients, 328 (53.2%) were males and the rest 288 (46.8%) were females. The median age of the study subjects was 43 years, with the highest proportion (43.5%) being in the age group 18–35 years. All total 1540 blood units requested for cross-matching were cross-matched in the laboratory and 1498 blood units were transfused with a mean of unit 2.43. From a total of 1498 blood units transfused, 709 (47.3%) of the blood transfused was blood group O and 1252 (83.6%) of the blood was Rh-positive. From a total of 616 subjects, 110 (17.9%) of the patients were given four units of blood, 196 (31.8%) of the patients were given three units of blood, 160 (26.0%) of the patients were given two units of blood and the rest 150 (24.4%) of the patients were given one unit of blood. Additionally from 1540; 1127 (73.15%) of the blood transfusions were requested or authorized by specialist doctors and the rest was requested by general practitioners and specialized nurses (Table 1).

Table 1 Demographic Characteristics of Transfused Patients with Different Blood Components at Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, from May 01 to July 30, 2021

Variable			Male		Female		Total Recipients	
			Absolute	%	Absolute	%	Absolute	%
Age group in years	<18		41	6.7	22	3.6	63	8.6
	18–35		154	25	114	18.5	268	43.5
	36–45		68	11.0	40	6.5	108	17.5
	46–65		51	8.3	61	9.9	112	18.2
	>65		14	2.3	51	8.3	65	10.6
	Total		328	53.2	288	46.8	616	100
ABO blood group	A	Rh +	111	7.4	62	4.1	173	11.5
		Rh-	31	2.1	23	1.5	54	3.6
	B	Rh +	151	10.1	60	4.0	211	14.1
		Rh-	32	2.1	19	1.3	51	3.4
	AB	Rh +	154	10.3	107	7.1	261	17.4
		Rh-	23	1.5	12	0.8	35	2.3
	O	Rh +	366	24.4	241	16.1	607	40.5
		Rh-	82	5.5	24	1.6	106	7.1
No of units transfused	One		105	17.0	92	14.9	197	32.0
	Two		55	8.9	47	7.6	102	16.6
	Three		120	19.5	51	8.3	171	27.8
	Four		85	13.8	61	9.9	146	23.7
Cross-match is authorized by	GP		378					24.6%
	BSC Nurses		35					2.3
	Specialist		1127					73.2

Abbreviations: A, blood group “A”; B, blood group “B”; AB, blood group “AB”; O, blood group “O”; GP, general practitioner; Rh +, rhesus-positive; Rh, rhesus-negative.

According to this study found the most widely used blood unit component was the whole blood unit which accounted for 990 (66.1%), followed by packed red cell volume which was 208 (13.9%), and then plasma only which was 155 (10.3%), platelets only was 82 (5.5%) and more than one component was 63 (4.2) (Table 2).

As the study findings indicated, two and more than two blood components are transfused for a single patient (like whole blood and plasma, whole blood and platelet, whole blood and PRC, and platelet and PRC, whole blood, Plasma & platelet, and whole blood, plasma and PRC) even if whole blood is the most utilized blood component more than one unit for a single individual (98.75%). In the current study, the blood component transfusion utilization, and outcome pattern at the department level was also assessed and the findings indicated that the majority of the individuals transfused were from medical wards which accounted for 239 (38.8%) and the lowest 17 (2.8%) in the maternity ward. A total of 467 (31.2%) units of blood are utilized by the medical ward; of which 231 (49.5%) were whole blood components. Furthermore, as the finding indicated, the utilization of more than one blood unit for a single person was high in this ward relative to others which accounts for 358 (27.5%) out of 1301 blood units utilized more than one unit, followed by the emergency ward which is 329 (25.3%) (Table 3).

Selected Hematological Parameters Both in Case of Pre-Transfusion and Post-Transfusion

In the current study, the pre-transfusion and post-transfusion hematological parameter results (hemoglobin, hematocrit, PLT, RBC index, RBC, and WBC) of transfused individuals were measured. The finding of the laboratory results for those individuals indicates better improvement in all blood parameters (Table 4). Additionally, the findings of the current research indicated a significant change in post-transfusion blood cell parameters (hemoglobin, hematocrit, PLT, RBC index, RBC, and WBC) were seen compared with the laboratory results of the pre-transfused hematological findings of the individuals (P value < 0.05).

Table 2 Types of Blood Components Transfused with Blood from Patients Admitted in Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, from May 01 to July 30, 2021

Types of Blood Component	Number of Transfused Individuals		Numbers of Blood Units Utilized	
	Absolute	Percentage	Absolute	Percentage
Whole blood	457	74.3	990	66.1
Only Plasma only	42	6.8	155	10.3
Platelet only	33	5.4	82	5.5
Packed Red Blood Cells only	71	11.5	208	13.9
More than one components	13	2.1	63	4.2

Table 3 Distribution of Blood and Blood Component Utilization by Different Departments at Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, May 01 to July 30, 2021

Blood Component	Department and Number of Blood Units Utilized by Each Ward					Total
	Medical	Surgery	Emergency	Gynecology	Others	
Whole blood	231 (49.5%)	222 (65.5%)	224 (79.4%)	102 (63.4%)	211 (84.7%)	990 (66.1%)
only Plasma only	57 (12.2%)	31 (9.1%)	16 (5.7%)	34 (21.1%)	17 (6.8%)	155 (10.3%)
Platelet only	63 (13.5%)	8 (2.4%)	1 (0.4%)	7 (4.3%)	3 (1.2%)	82 (5.5%)
PRBC Cells only	101 (21.6%)	57 (16.8%)	23 (8.2%)	12 (7.5%)	15 (6.0%)	208 (13.9%)
> one blood unit	15 (3.2%)	21 (6.2%)	18 (6.4%)	6 (3.7%)	3 (1.2%)	63 (4.2%)
Total	467 (31.2%)	339 (22.6%)	282 (18.8)	161 (10.8%)	249 (16.6%)	1498 (100%)

Abbreviations: PRBC, packed red blood cell; Others, includes intensive care ward, pediatrics wards.

Table 4 Selected Hematological Parameters Before and After Whole Blood and Blood Component Transfusion at Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, May 01 to July 30, 2021

Variable	Level	Pre-Transfusion No	Post -Transfusion No	P-value
Hemoglobin (g/dl)	<7 g/dl	288 (46.8%)	65 (10.6%)	0.001
	7–10 g/dl	220 (35.7%)	87 (14.1%)	0.002
	>10g/dl	108 (17.5%)	464 (75.3%)	<0.001
	Total	616 (100%)	616 (100%)	NA
Hematocrit (%)	<34%	385 (62.5%)	51 (8.3%)	<0.001
	37–54%	157 (25.5%)	363 (58.9%)	0.002
	> 55%	74 (12.0%)	202 (32.8%)	0.03
	Total	616 (100%)	616 (100%)	NA
RBC count ($10^{12}/L$)	< $3.6 \times 10^{12}/L$	496 (80.5%)	152 (24.7%)	<0.001
	$3.6\text{--}6.0 \times 10^{12}/L$	80 (13.0%)	259 (42.0%)	<0.001
	> $6 \times 10^{12}/L$	40 (6.5%)	206 (33.4%)	<0.001
	Total	616 (100%)	616 (100%)	NA
WBC count ($10^6/L$)	< $3 \times 10^9/L$	197 (32.0%)	80 (13.0%)	0.012
	$3\text{--}10.2 \times 10^9/L$	322 (52.3%)	482 (78.2%)	0.022
	> $10.2 \times 10^9/L$	97 (15.7%)	54 (8.8%)	0.001
	Total	616 (100%)	616 (100%)	NA
Platelet count ($10^9/L$)	< $150 \times 10^9/L$	223 (36.2%)	37 (6.0%)	0.001
	$200\text{--}450 \times 10^9/L$	341 (55.4%)	501 (81.3%)	0.001
	> $450 \times 10^9/L$	52 (8.4%)	78 (12.7%)	0.03
	Total	616 (100%)	616 (100%)	NA

Patient's Response After Transfusion

Out of a total of 616 transfusion episodes during the study period; 525 (85.2%) of the patients showed better improvement after transfusion, 68 (11.1%) of the patients did not show improvement after transfusion and the rest 23 (3.7%) had no report regarding their response after transfusion. From a total of 457 individuals transfused with whole blood components; 396 (86.6%) cases were improved after transfusion. Out of the total, 68 (11.1%), non-improved cases; 48 (70.6%) were individuals who have transfused with whole blood components; and the rest 20 (29.4%) individuals were patients transfused with other blood component transfusions (Table 5).

According to the current study finding, the improvement status in all departments was better, which were 210 (87.9%) out of 239 patients transfused in a medical ward, 152 (92.1%) out of 165 transfused patients from the surgical ward, and 79 (74.5%) out of 106 transfused patients in the emergency ward (Table 6).

A total of 1540 cross-matched blood units; 1498 blood units were transfused; for 615 patients and the overall ratios of C/T, %T, and TI index were 1.03, 99.8%, and 0.97, respectively (Table 7).

As the finding of this study indicated; a significant association was seen between the hematological (hemoglobin, hematocrit, red blood cell, platelet, and white blood cells) parameters of pre-transfused results with that of post transfused result (P-value <0.05)(Table 4).

Table 5 Patient Improvement Status After Blood Transfusion at Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, May 01 to July 30, 2021 (N =616)

Patient Status				
Type of Blood Product	Improved	Not Improved	No Report	Total
Whole blood	396 (86.7%)	48 (10.5%)	13 (2.8%)	457 (74.2%)
Only Plasma only	34 (73.9%)	6 (13.0%)	6 (13.1%)	46 (7.5%)
Platelet only	28 (75.7%)	8 (21.6%)	1 (2.7%)	37 (6.0%)
PRBC Cells only	67 (88.2%)	6 (7.9%)	3 (3.9%)	76 (12.3%)
Total	525 (82.5%)	68 (11.1%)	23 (3.7%)	616 (100%)

Abbreviation: PRBC, packed red blood cell.

Table 6 Patient Improvement Status Regarding Different Departments at Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, May 01 to July 30, 2021 (N=616)

Departments	Patient Status			Total
	Improved	Not Improved	No Response Data	
Medical Ward	210 (87.9%)	25 (10.5%)	4 (1.7%)	239 (38.8%)
Surgery	152 (92.1%)	7 (4.2%)	6 (3.6%)	165 (26.8%)
Emergency	79 (74.5%)	20 (18.9%)	7 (6.6%)	106 (17.2%)
Gynecology	14 (82.4%)	1 (5.9%)	2 (11.8%)	17 (2.8%)
Others	70 (78.7%)	15 (16.9%)	4 (4.5%)	89 (14.4%)
Total response	525 (82.5%)	68 (11.1%)	23 (3.7%)	616 (100%)

Note: Others= includes intensive care ward, pediatrics wards.

Table 7 Overall Blood Utilization Among Patients Transfused at Yekatit-12 Specialized Hospital; Addis Ababa, Ethiopia, May 01 to July 30, 2021 (N=616)

Blood Transfusion Indicators	Value	Utilization Status
Cross match to transfusion ratio (C/T ratio)	1540/1498 = 1.03	Significant blood usage
Transfusion probability (TP)	615/616 X 100 =99.8	Significant blood usage
Transfusion index (TI)	1498/1540 = 0.97	Efficient blood usage

Discussions

In the current study, out of a total of 616 transfused patients, 328 (53.2%) were males and the mean age of the patients was 43 with a higher percentage in the age range 18–35 which accounts for 268 (43.5%) and this finding was similar with a study finding conducted in Gondar,⁷ Nigeria⁸ and the age group was somewhat higher with a study conducted in Jimma, Ethiopia.⁹ A total of 1540 blood units were requested for 616 patients from different wards in Yekatit-12 Hospital from May 01 to July 30, 2021, of which 1498 (93.7%) blood units were transfused for each patient with a mean of 2.43 blood units which is relatively in line with the finding of different studies conducted in Gondar Hospital, Northwest Ethiopia,⁷ higher relative to a study conducted in Jimma, Ethiopia,¹⁰ Southern Nigeria,⁸ Saudi Arabia¹¹ and lower than with a cross-sectional study conducted in Ethiopia.¹² From a total of 1498 blood units utilized; 990 (66.1%) were whole blood components, 709 (47.3%) of the blood transfused was blood group O type and 1252 (83.6%) of the blood was Rh-positive which is similar to the study conducted in Jimma, Ethiopia,^{9,10} Southern Nigeria.⁸

Among 1498 blood units transfused for 615 patients, 713 (47.6%) were blood group “O”, 227 (15.2%) were blood type “A”, 262 (17.5%) were blood type “B” and the rest 296 (19.8%) were blood type AB. In addition from a total of

1498 transfused blood units, most of the blood units; 1252 (83.6%) were RH positive and the rest 246 (16.4%) blood units were RH negative (Table 1). This finding was in line with the findings of the study conducted at Tikur Anbessa Specialised Hospital, Ethiopia,¹² an institution-based cross-sectional study conducted in 2018 at Jimma, Ethiopia,¹⁰ a cross-sectional study conducted at Gondar Hospital, Ethiopia,⁷ year hospital-based study conducted in Black Lion Specialized Hospital, Ethiopia¹³ and a study conducted in.¹⁴

In our study the overall ratios of cross-match to transfusion ratio (C/T), transfusion probability (%T), and transfusion index (TI) were 1.03, 99.8%, and 0.97, respectively (Table 4); which indicates better utilization of blood units at different wards of Yekatit-12 Referral Hospital (C/T < 2.5%, T > 30% and TI > 0.5). Similar to the current study findings; a study conducted in Jimma, Ethiopia,¹⁰ in Gondar, Ethiopia,⁷ in teaching hospitals of India,¹⁵ and a study conducted in Northern India¹⁶ were reported better utilization of blood units. However, the current study indicated a better utilization rate relative to a study conducted in Nigeria,¹⁴ a cross-sectional study in Rajouri,¹⁵ and a study conducted in the blood bank of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Nigeria.¹⁴ In addition, the finding of the transfusion probability of the current study was indicated significant blood utilization relative to the finding of the study conducted in large tertiary hospitals in Ethiopia.¹²

As the findings of this study indicated; out of 615 patients, 419 (68.1%) individuals have utilized two or more than two blood units. Of which 102 (16.6%) individuals were transfused with two blood units, 171 (27.8%) individuals were transfused with three blood units, and the rest 146 (23.7%) individuals were transfused with four blood units (Table 1) and a nearly similar finding was reported by the finding of the study conducted in black lion hospital, Ethiopia [25].

Out of 615 transfused individuals; most of the patients were from the medical ward which accounts for 239 (38.9%), followed by the surgical ward which is 165 (26.8%) (Table 6), and this finding was relatively in line with the finding of a study conducted by.¹⁰ Whole blood was the abundantly transfused blood component (55.9% in a medical ward, 65.5% in the surgical ward, 79.2% in an emergency ward, 63.4% in gynecology wards, and 84.7% in other words) (Table 3) which is in line with studies finding conducted in Ethiopia,¹² in Gondar, Ethiopia,⁷ and BLSH, Ethiopia.¹³

In the current study; a significant association was seen between the hematological (hemoglobin, hematocrit, red blood cell, platelet, and white blood cells) parameters of pre-transfused results with that of post transfused result (P-value < 0.05) (Table 4) which is in line with the study conducted in India,¹⁷ in Italy¹⁸ and a study conducted in BLSH, Ethiopia.¹³ The overall transfusion rate of this study was 97.3% which is higher than a study conducted in India which reports 90.0%,¹⁹ retrospective study conducted in India which reports 62.6%,²⁰ a study conducted in Jimma, Ethiopia which reports 82.1%,¹⁰ a study conducted in Gondar, Ethiopia which reports 47%,⁷ and nearly similar with a study finding conducted in Nigeria which reports 94.42%.¹⁴ However, in the current study, the utilization of blood components rather than whole blood was minimal, which is less than 12.5%. The main reason for this may be the backward tradition of preparation of blood components based on the requirement of the patient. This was due to the use of whole blood as a sole component for transfusion in resource-limited areas making whole blood is seldom used as a component for blood transfusion rather than its therapeutic products.

Conclusion

Out of 616 individuals requested for cross-match with a mean age of 43 years, 53.2% were males; 1498 blood units were transfused for 615 individuals with an overall average transfused blood unit of 2.43. In the current study, the most utilized blood type was blood group O (47.6%), and RH type was RH positive (83.6%). The most utilized blood component was the whole blood component which accounts for 55.9% in a medical ward, 65.5% in the surgical ward, 79.2% in an emergency ward, 63.4% in gynecology wards, and 84.7% in other wards.

In the current study; from 615 transfused individuals, 68.1% individuals have utilized two or more blood units (16.6% individuals have utilized two blood units, 27.8% individuals have utilized two blood units three blood units, and 23.7% individuals have utilized two blood units four blood units). Also in our finding; a significant improvement was seen in hematological parameters (hemoglobin, hematocrit, red blood cell, platelet, and white blood cells) after transfusion (P-value < 0.05). The overall C/T, %T, and TI were 1.03, 99.8%, and 0.97, respectively; which indicates better utilization of blood units (C/T < 2.5%, T > 30% and TI > 0.5). The overall prevalence rate of improvement status after transfusion was greater than 73.9 and the overall prevalence rate of non-improvement status after transfusion was 11.11%. The study also showed that there were no post-transfusion reactions during the study period.

Recommendations

According to our findings, we would like to recommend transfusing separated blood components will be more advantageous than transfusing whole blood to reduce the shortage and waste of blood. Training on blood component usage for those engaged in the service is mandatory to use this precious product economically. Finally, regular auditing of blood and blood component utilization could improve the transfusion service and alleviate an increased demand for blood and blood components.

Abbreviations

BLSH, Black Lion Specialized Hospital; CDC, Center of Disease Control Center; CTR or C/T, cross-match to transfusion ratio; ECUSTA, Ethiopian Catholic University Institute; HBV, hepatitis B virus; HCV, hepatitis C virus; Hgb, haemoglobin; HIV, human immunodeficiency virus; NBB, National Blood Bank; PBM, patient blood management; PLT, platelet; PRBC, packed red blood cell; RBC, red blood cell; SPSS, Software Package for the Social Science; WBC, white blood cell; WHO, World Health Organization.

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Disclosure

The authors report no conflicts of interest in this work.

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Evaluation of the Wateen App in the Blood-Donation Process in Saudi Arabia

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Background: Blood transfusions are essential for many medical procedures, but current supplies of blood are insufficient to meet the needs of all patients. Apps offer a means to improve donor recruitment and enhance blood-donation systems, if they are usable and useful. The Saudi Ministry of Health launched a blood-donation app, “Wateen”. However, there is currently no evidence of the app’s usability and usefulness among users.

Purpose: The aim of this research was to evaluate the usability, user satisfaction and perceived usefulness of this blood-donation app in Saudi Arabia.

Methods: A mixed-method study was conducted comprising a quantitative questionnaire with donors, and qualitative semi-structured interviews with healthcare professionals. Descriptive analysis was used for the quantitative data and a thematic approach for the qualitative data.

Results: A total of 401 donors completed the questionnaire, 53.7% of whom were males. Most participants were highly satisfied with the Wateen app and found this app easy to use and navigate. Older people found the app less easy to use compared with younger respondents. Key benefits identified by questionnaire respondents included the potential to encourage donation, and improve communication. A total of 12 healthcare professionals were interviewed. Most healthcare professionals expressed that the app was generally acceptable and easy to use. They felt that the app has the potential to be effective in enhancing donor awareness, facilitating communication between donors and healthcare professionals, and improving the efficiency of the donation process. Some accessibility issues were identified that need to be considered. It was also suggested that more be done to expand the app functionality and increase awareness of the app.

Conclusion: This blood-donation app is highly usable and acceptable among donors and healthcare professionals in Saudi Arabia, offering several benefits. Some accessibility issues were identified, along with possibilities for improving accessibility and expanding the app’s functionality.

Keywords: apps, blood donation, usability, user satisfaction, Wateen

Introduction

Blood transfusions are an essential part of many medical procedures and save lives. Without access to regular blood donations, healthcare systems cannot meet the need for transfusions, leading to lack of treatment for certain conditions, and losses of life. According to the World Health Organization, 3–5% of the population should donate blood annually to ensure adequate supplies.¹ Several studies have identified a number of factors that influence blood-donation rates, including being medically unfit,² lack of awareness or knowledge,³ fear of infections, lack of time,⁴ and lack of invitation.⁵

The Saudi blood transfusion system utilizes a system of blood banks at hospitals, in which individual hospitals house blood banks to meet the needs of their patients, with blood being donated from voluntary donors, or from replacement donors (comprising friends, family members or colleagues).² However, donation rates across Saudi Arabia are not adequate to meet the needs of patients, often leading patients and their families to seek donations via alternative routes, including social media requests,^{6–8} Official statistics record the number of blood donors in Saudi Arabia as 341,688 in

2011; to reach the target WHO rate of 3–5%, this figure should have been 814,109–1,356,849.⁹ This discrepancy between supply and demand is set to increase as the Saudi population grows.^{10–12}

In recent years, the potential for mobile and communications technology to drive blood donation has begun to be recognized.⁶ Several countries have launched apps to encourage blood donations, such as the UK's NHS Give Blood app. The Saudi Ministry of Health (MOH) recently launched its own smartphone application "Wateen", with the intention of improving awareness of blood donation among the Saudi population in an effort to increase availability of blood.¹³ Wateen is integrated with the SMS (text messaging) system, enabling it to send SMS messages to prospective donors of a specific blood type, and to signpost local donation facilities.¹²

It has been found that the usability of blood-donation apps, and levels of satisfaction with these apps, are important factors that influence donors' intent to use them, and to return for future donation.^{2,14,15} Previous studies have identified several key factors that influence mHealth adoption and efficiency, including user satisfaction with apps, and perceptions of their usefulness and ease of use.^{12,16} However, to date the investigation of these factors in the context of blood-donation apps has not been adequately examined globally, and even less research has been conducted specific to Saudi Arabia or the surrounding Gulf Region. One recent study assessed Saudi donors' opinions regarding the Wateen app via a mostly closed-ended questionnaire, without investigating donors' satisfaction with the usability of the app, or their perceptions of its usefulness.¹⁷ This study also did not consider healthcare professionals' views, despite their central role in the blood-donation process. The present study therefore employs a mixed-methods (quantitative and qualitative) design involving both donors and healthcare professionals to provide an in-depth picture of the usability of and user satisfaction with the Wateen app, and perceptions of its usefulness to the blood donation process in Saudi Arabia.

Materials and Methods

This study adopted a convergent mixed-methods design, utilizing both qualitative and quantitative data to evaluate the use of the Wateen app in the blood-donation process in Saudi Arabia.¹⁸ The study examined usability, usefulness and satisfaction from the perspectives of both donors and healthcare professionals.

Quantitative data collection utilized a self-administered questionnaire to explore donors' attitudes towards the Wateen app, whereas qualitative data was gathered via semi-structured interviews with doctors, to gain their expert opinions regarding the Wateen app in their own words. These data strands were gathered concurrently, and analyzed separately, before being integrated and synthesized so they could be interpreted together, leading to a higher knowledge yield and a fuller picture of the research topic.

The study was conducted at blood-donation centers, located in Riyadh Saudi Arabia during Oct-Dec 2021. The ethical approval for this study was obtained from Ministry of Health (MHO).

Study Instruments

To collect donors' opinions, a validated, structured, self-administered questionnaire was adapted from a previous study,¹⁷ with some questions modified to collect useful supplementary information. The questionnaire was translated into the Arabic language, before being validated using the WHO Process of Translation and Adaptation of Instruments (WHO). A pilot study was conducted, which found the questionnaire to have a Cronbach α of 0.9 and a scale level content validity index of 0.90.

To collect healthcare professionals' in-depth expert opinions regarding the Wateen app, a semi-structured interview was used. The interview topic guide was based on a literature review,^{2,5} and was designed to elicit healthcare professionals' opinions regarding the app's tasks/functions, their satisfaction towards the app, the usability of the app, the potential benefits, any perceived limitations or concerns, and their suggestions for improvement. The interview also collected demographic participant information including age.

Participants

The target population for this study was healthy adults who were eligible to donate blood, and who had previously used the Wateen app, as well as physicians, nurses, and phlebotomists involved with donation centers.

The sample size for the donor group was estimated at 382 participants, allowing for a 95% confidence interval, an alpha error of 0.05, an accuracy of $\pm 5\%$, and power of 80%. These sample sizes were calculated using the Raosoft

Sample Size Calculator.¹⁹ Healthcare professionals were recruited until data saturation was reached, ie, the point at which new interviewees no longer disclosed new information or data.

The inclusion criteria were as follows: for donors, eligible participants were adults aged over 18 years who had previously donated blood via the Wateen app at least once, who were able to provide informed consent and participate actively in the research; for the healthcare professional group, eligible participants were adults aged 24 years or over who had at least 6 months' experience in donation centers, and who were able to provide consent and participate actively.

Participant Recruitment

Questionnaire

A random sampling technique was adopted to recruit donor participants,¹⁸ in which the researcher approached each fourth donor who entered the waiting area at the donation centers. Willing donors were asked to complete a consent form, before being provided with a copy of the study questionnaire. Assistance with completing the questionnaire was provided if requested.

Semi-Structured Interviews

Healthcare professionals were approached purposively to participate.¹⁸ After assessing whether they met the study criteria, interested and eligible participants were asked to complete a consent form, which included publication of anonymised responses. An interview was then conducted with each participant by the researcher. Each interview lasted between 25 and 45 minutes. Interviews were conducted until data saturation was reached, ie when no new information was being revealed.²⁰ Interviews were recorded, then transcribed, with transcriptions checked for accuracy against the original recordings.

Data Analysis

Quantitative data analysis was conducted using SPSS software package 19 to calculate descriptive statistics, eg means and standard deviations.²⁰ Interview transcripts were subjected to thematic analysis using NVivo 12 software. This analysis comprised the stages of: (1) data familiarization, (2) creation of initial codes, (3) collection of codes into broader themes, (4) specification of themes, (5) review of themes, and (6) writing the report.^{18,20} The qualitative and quantitative results of the study were integrated and analyzed together, recognizing any divergences and convergences between these results. The matrix can be seen in [Appendix 1](#).

Results

Quantitative Data (Questionnaire)

Socio-Demographic Characteristics

Of 401 donors included in the study, 53.7% were males. Most participants (32.6%) were in age the 25–34 age group. More than two thirds (67.2%) of the donors were Saudis, while others (32.2%) were foreign nationals, mostly from Yemen, India, Egypt and Syria ([Table 1](#)). A full breakdown of demographic factors is provided in [Table 1](#).

Ease of Use, Satisfaction and Benefits of Using the App

This section presents the findings of the questionnaire segments on Ease of Use, Satisfaction, and Potential Benefits. Overall results for each questionnaire segment was calculated by aggregating the average scores for each question in that segment. This showed the participants found the app easy to use (with a mean score of 4.06, and an SD of 0.15), that participants were satisfied with the app and its interface (mean 4.14, SD 0.27), and that they felt that use of the app would be beneficial (mean 4.30, SD 0.18). Mean scores and standard deviations for each questionnaire question are given in [Appendix 2](#).

Results from the “Ease of Use” segment of the questionnaire (as shown in [Appendix 2](#)) found that ease of use, navigation between screens, information available on the application, and the easiness of undoing mistakes were all rated as very good by >70% of respondents. In terms of demographic differences, the results indicated a large discrepancy in ease-of-use scores between older (≥ 55 years) and younger (<55 years) participants, suggesting older participants found the Wateen app less easy to use.

Table 1 Donors Demographic Characteristics

Gender	
Male	(53.7%)
Female	(46.3%)
Age	
18–24	(28.1%)
25–34	(32.6%)
35–44	(17.5%)
45–54	(10.4%)
55–64	(7.6%)
>65	(3.8%)
Nationality	
Saudi	(67.2%)
Non-Saudi	(32.8%)
Education Level	
Secondary education degree	19.7%
Diploma degree	20%
Bachelor's degree	35.4%
Master's degree	21.7%
PhD degree	3.2%
Employment	
Yes	81%
No	19%

Results from the “Satisfaction” segment found that participants’ satisfaction levels with respect to various aspects of the Wateen app were also high. Convenience in accessing blood centers by finding the suitable time, location and date, time required to find and book, quality of the interface, relevance of tasks and functions on the application were aspects with which the participants were highly satisfied, and were rated very good by almost 60% of the participants.

The participants’ perceptions of the various benefits of the Wateen app are shown in [Appendix 2](#). In summary, these were as follows: providing easier access to blood donation centers, playing a key role in improving the blood supply, facilitating convenient and confidential communication with healthcare providers, delivering effective services remotely, saving time and encouraging users to donate more. Some participants reported that the rewards function helped to increase their motivation to donate. However, some reported that there were technical issues that affected their engagement with the app, such as failing to present rewards, leading them to contact IT support to resolve this issue.

Qualitative Data (Semi Structured Interviews)

Participant Characteristics

Twelve healthcare professionals participated in the semi-structured interviews. Eight of the participants were males (66.7%), and four were females (33.3%). Most participants (eight of twelve) were between 24 and 64 years old. A range of healthcare professionals were represented, including nurses (three), physicians (three), lab workers (three), and phlebotomists (three). A full breakdown of demographic information is provided in [Table 2](#).

Table 2 Healthcare Professionals Demographic Characteristics

Gender	
Male	(66.7%)
Female	(33.3%)
Age	
24–34	(33.3%)
35–44	(33.3%)
45–54	(25%)
55–64	(8.4%)
Nationality	
Saudi	(67.2%)
Non-Saudi	(32.8%)
Job Role	
Physician	3
Lab workers	3
Nurse	3
Phlebotomists	3

Qualitative analysis of the interview data identified three primary themes: usability and satisfaction, information and communication, and impact of the app on the blood-donation process.

Theme 1: Usability and Satisfaction

Overall Usability and Satisfaction. Most participants generally satisfied with the app and they found this app is easy to use, easy to learn, and easy to navigate. They reported that tasks such as booking a donation appointment could be completed in just a few steps, and that no instructions or training were required. Some participants noted that people of all ages attended donation centers having booked an appointment using the app, suggesting it is usable by people of all age groups, including older users.

I believed the app is very easy to use because it just requires simple interaction to achieve a task; just select and click to book a donation appointment; no more steps. (Phlebotomists 3)

Donors did not need training or instructions; they can use it immediately because the app needs only a few clicks to do a task. (Nurse 1)

During my work period in this center I noticed many donors who are aged from 18 to 70 years old who book their donation appointment using this app, so I think this app is easy to use for everyone. (Lab worker 2)

App Accessibility. Some participants believed that some donors, particularly older people or those with low vision, may have difficulty reading text within the app as it is small, and suggested it would be good to enable users to magnify text, or to change the font size in the app overall.

I think this application is excellent and users will not face any challenges but the size of the text might be a big barrier because it is too difficult to read for some people. (Doctor 2)

Customizing the size of fonts would help the app to be more accessible for all people, including older people, or people with vision problems. (Phlebotomists 1)

Including other languages would help to make the app more accessible to all people living in Saudi Arabia.

The app is available only in Arabic and English language. Other users who [...] do not know Arabic/English [...] cannot use it and engage in donation. (Nurse 3)

Theme 2: Information and Communication

App as an Informative Tool. Participants reported that the educational information in the app plays a key role in increasing donors' awareness about the blood-donation process, motivating and attracting more people to donate. However, including more attractive educational materials, eg. clips, would help to reduce users' anxieties surrounding transmission of infectious diseases, needles, dizziness etc. The inclusion of educational materials was also recommended to advise donors how to manage the post-donation period, and improve users' engagement and attitudes towards blood-donation.

I believe this app helps a lot in increasing donors' knowledge and awareness about blood donation but I think it would be good if [it included] more information about what they [donors] should do after their donation (Nurse2)

Educational information is not improved awareness alone but it enhances their overall opinions towards blood donation (Doctor1)

App as a Communication Tool. The app enhances communication with donors compared with traditional methods of recruitment, making it easier to find, communicate with, and recruit donors. For, example, the app has the ability to send notifications when particular blood types are in short supply, which users can then share with other donors, making donors feel more motivated to donate and help others.

Before Wateen there were no official channels to call for donors who can offer specific blood types for patients that are in demand the app now could help to send requests in this case for people who have the same blood [type] and they can also share it with friends/relatives (Doctor1)

Healthcare professionals had confidence in the app's protection of donors' confidential information as this app is only used for donation purposes. Some healthcare professionals suggested that it would be more efficient if the app presented the results of blood screening after donation. However, other healthcare professionals disagreed with this as this may increase users' stress, particularly when concerns were identified, which require explanation from a healthcare professional.

Many people visit the center asking about their blood results ... I suggest adding the blood results in the app to promote people to donate more, save their time and effort, and increase their communication through the app.

(Lab worker3) If the result is positive the patients will be stressed and of course it should be discussed with their doctors. I do not think it is the most appropriate way to send these via the app. (Doctor2)

Theme 3: Impact of the App on the Blood-Donation Process

Time and Effort. The analysis revealed that the quality of services was a key factor influencing users' engagement with the blood-donation process. Healthcare professionals noted that using this app saves time and effort for both donors and

healthcare professionals. They further reported that the app reduced waiting times for donors, and allowed centers to operate more efficiently, by enabling donors to select a specific time, date and location in advance.

Before donors spent a long time in some centers to donate and there were long queues ... however, nowadays using this app donors have the ability to book the time and date and come to that appointment where they can be served easily by the center staff. (Nurse 3)

Features That Impact Donor Engagement. This app has the ability to record the number of times users donated, in addition to issuing reminders. These features increase people's motivation to engage with the app and to undertake more blood donation, by increasing their feelings of satisfaction.

The app encourages donors to donate particularly for humanitarian reasons, eg., in order to save people's lives, as they usually do not know them ..., so being a volunteer helps them to feel satisfied and happy. (Phlebotomists 2)

Some doctors reported that although the app posts the names of the donors who achieve a high number of donations as a reward, additional reward features could improve engagement with blood donation, increasing donation rates in Saudi Arabia.

I suggest that if donors donate a specific number of times, let's say 11–12 times a year, they [could be rewarded by] waiving specific fines or even given shopping coupons, which could be integrated easily in the app by linking with government entities. (Doctor 1)

Information on Donation Centers. Some healthcare professionals reported that whilst the app helps donors to find the nearest donation location with accurate information including GPS, distance (the time needed to reach), and opening hours, the app could improve the blood-donation service further if it also offered information about which hospitals have the most urgent need.

Sometimes donors come and ask whether the center has an urgent need for blood, and say they would like to donate to those in most need. (Lab worker 1)

Awareness of the App. Despite the benefits of the Wateen app, there is a need for official advertisement about the app, as some donors who came to donate were unaware of it.

Some donors until now did not know about this app ... More advertisements would help people to know about it. (Nurse 2)

Discussion

This research evaluated the usability, usefulness and user satisfaction of the Wateen app in the blood-donation process in Saudi Arabia. The results presented in this paper show high levels of satisfaction regarding the app and its usefulness, and good usability outcomes among participants. Whilst these findings suggest positive experiences with the Wateen app, they should be interpreted in the specific Saudi context. Previous research suggests that positive attitudes towards blood donation are already prevalent across Saudi Arabia, and that these attitudes may influence prospective donors' engagement with recruitment methods.¹¹ At the same time, such contextual factors highlight the importance of the current research, which examined the Wateen app in this specific cultural context. This research into Saudi donors' and healthcare professionals' experiences with the Wateen app identified a number of possibilities for improvement, relating to how the app could further enhance blood donation, and meet users' differing needs, expectations and preferences in Saudi Arabia.

Several recent studies have identified perceived ease of use as a key factor that influences potential users' engagement with e-health applications.^{12,16,21} The findings of the present research into the ease of use of the Wateen app revealed that both donors and healthcare professionals found the app generally easy to use, learn, and to navigate. Ease of navigation and interaction with the app, ease of recovery following mistakes, and ease of accessing information were rated as high by the majority of donors. Similarly, healthcare professionals expressed that the app is easy to use and navigate and noted that the app is used by donors from different age groups, highlighting its usability by older people.

The questionnaire, however, found that younger people reported good usability more often, and were more motivated, than older people. These findings are in line with prior research, which has found that older users may be less engaged and/or experience greater issues with technological interventions.^{22–24} Some concerns were raised by healthcare professionals regarding app accessibility, for example, the lack of a zoom feature and inability to change font sizes, which may hinder older people or people with impaired vision. Whilst these accessibility issues are likely to affect older people disproportionately, this population also has a higher frequency of underlying health conditions, meaning they are less likely to donate blood anyway.^{23,25} Such issues surrounding age and digital competence are also likely to lessen as younger, technologically literate people grow into old age. In the meantime, training could enable older users to engage more with this app, and other e-health technologies.^{22,26} Ouhbi et al showed that very few donation apps are available in multiple languages, limiting the number of potential donors they can reach.^{14,15} The present research also found that widening the language provision of the Wateen app beyond Arabic and English could increase its accessibility and user base. These findings suggest that understanding the needs of older adults, people with impaired vision, and Non-Arabic Speakers is crucial to helping maximize apps' usability and reach within this context.

Findings from previous research indicate that training could be one way to improve users' competence and confidence with an e-Health app, and therefore improve their acceptance.^{27,28} However, the majority of healthcare professionals in the present research revealed that donors use the Wateen app without receiving any training, and donors also reported that they typically did not require help to use it. The lack of training required to use e-health apps is associated with high levels of usability.^{22,27}

There was disagreement between interviewees as to the benefit of sharing blood screening results with donors via the app. Some interviewees thought this would be a beneficial feature, which could encourage prospective donors to engage with the app and to donate. However, other healthcare professionals expressed concerns with such practices, related to confidentiality and increased stress for users, particularly in the case of concerning test results. This latter finding is in keeping with those of Batis and Albarrak, who assert that sharing results via apps is not good practice, and that confidentiality is a more important consideration than convenience in these situations.² Both donors and healthcare professionals expressed confidence in the app's protection of donors' confidential information where it is only used for blood-donation purposes. Some interviewees expressed concerns about ensuring confidentiality if the functionality of the app were expanded in future to include sharing of blood screening results with donors.

Users' perceptions of the effectiveness of m-health apps play a key role in determining their intention to use such applications.^{28,29} Guglielmetti et al determined that the quality of blood-donation services specifically influences prospective donors' engagement.³⁰ The Wateen app performed well in this regard, with donors and healthcare professionals identifying a number of benefits it could bring to users, and to the blood-donation process. Findings from both study groups indicate that the app offers a more effective route for users to donate blood versus previous donation methods, and has the potential to reduce latency between the need for blood and blood donation, a finding in line with previous research.¹¹ Participants agreed that the app enhances knowledge and awareness by offering information, that it improves communication between donors and donation centers, and that it improves efficiency by enabling easier recruitment of donors and by directing donors to the most convenient donation centers. These reported benefits are in line with those identified in previous research.^{2,30,31}

Previous research highlighted that the lack of availability of qualified healthcare professionals and high waiting times are two of the biggest factors that prevent prospective donors from donating.¹⁰ In keeping with previous research suggesting that m-health applications are associated with increased efficiency, the present research found that the Wateen app can help to reduce these pressures.^{32,33} Specifically, participants reported that it helps to strengthen the blood-donation service by reducing the time and effort contributed by qualified healthcare professionals, and by reducing waiting times at donor centers.

Strengths and Limitations

The main strength of this study is in its use of a rigorous, mixed-methods approach to deliver insight into the attitudes of donors and healthcare professionals towards the Wateen blood-donation app, and their impressions of its usability and perceived benefits. This design allowed for a nuanced picture of the app, as evidenced by the sometimes-divergent

findings between the study groups. Some potential limitations of this study pertain to the representativeness of the study sample. The small number of female participants relative to male is one consideration, but reflects the method of recruiting participants and the relatively low number of females among the population of existing donors in Saudi Arabia.¹¹ The low number of older versus younger participants is another factor, which may be of particular concern due to the relative difficulty with technology typically experienced by older users. The findings' generalizability beyond the Saudi context may be limited, given its particular cultural factors, and the particulars of the blood-donation process in Saudi Arabia. Finally, the qualitative data was analyzed by a single researcher. However, the analysis was discussed with an expert in qualitative data to ensure its credibility.

Recommendation for Further Studies

This study examined key stakeholders' perceptions of the benefits of this app in facilitating blood donations. Future research could empirically assess the impact of this or other blood donation apps on key aspects of blood donation, such as healthcare professionals' workloads, waiting times and knowledge for donors. The results also suggest there is a need for ongoing assessment of the needs and preferences of target users for this and other m-health apps.

Conclusion

This study found that a blood donation app was highly usable and acceptable by donors and healthcare professionals in Saudi Arabia, offering benefits to prospective donors, healthcare professionals, and the blood donation process. Some accessibility issues were identified, along with possibilities for improving accessibility and expanding the app's functionality.

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Disclosure

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Concizumab as a Subcutaneous Prophylactic Treatment Option for Patients with Hemophilia A or B: A Review of the Evidence and Patient's Perspectives

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Abstract: Concizumab is a monoclonal, humanized IgG4 antibody specific for the Kunitz-2 domain of Tissue Factor Pathway Inhibitor (TFPI). Preclinical studies in vitro or on animal models and in vivo have demonstrated the ability of concizumab to restore thrombin generation, promoting the establishment of a procoagulant action; all these results were subsequently confirmed in the studies of EXPLORER program. Concizumab may represent a new opportunity for the treatment of persons with hemophilia, so there is much anticipation for the results of the ongoing trials still. This review retraces all the studies on concizumab published to date, with a brief discussion about the patient's perspectives.

Keywords: hemophilia, concizumab, anti-TFPI antibodies, new hemophilia treatments

Introduction

Hemophilia is a rare hereditary, X-linked, hemorrhagic disorder characterized by deficiency of coagulation factor VIII (hemophilia A) or IX (hemophilia B). A typical expression of this disease is spontaneous or traumatic bleeding which can cause the patient severe disability and, in the worst scenario, death. Prophylaxis with factor concentrates is started from an early age in children with severe or moderate hemophilia to minimize this hemorrhagic risk.

Despite the great efforts of clinical research, until recently there were no treatments other than replacement ones, but today a bispecific monoclonal antibody, emicizumab, is available to clinicians for the subcutaneous treatment of hemophilia A, while other subcutaneous drugs for the treatment of both, hemophilia A and B, are now in experimental phase, such as fitusiran, an antithrombin inhibitor, and the anti-TFPI (Tissue Factor Pathway Inhibitor) antibodies, as marstacimab or concizumab.

The long history of concizumab starts from afar, when it was still called *mab2021*, in fact the first report published by Petersen¹ that highlighted the hemostatic properties of this anti-TFPI antibody dates to 2012.

The idea that this antibody could be useful in the hemophilia treatment arises from the in vitro and animal model evaluation of its efficacy in blocking TFPI, thus allowing the tissue factor/activated factor VII (TF/FVIIa) complex to exert its action within the coagulation cascade, which ends with the clot formation. Butterfield et al² described the mechanism of action of different anti-TFPI antibodies in development, including concizumab, and the role these may play in the treatment of hemophilia. Here we retrace the concizumab history.

The purpose of this work is to review all reports on concizumab from in vitro studies to those dealing with its use in people with hemophilia A and B, and published from January 2012 to December 2021.

Methods

A MEDLINE search using the key terms “concizumab”, AND “mab2021” showed a total of 40 published reports. Two of them were immediately excluded, one because dealing with recombinant factor VIIa (rFVIIa) - the SMART study -, the other because “erratum”. Among remaining 38 articles, thirteen deal exclusively with concizumab, four with TFPI inhibitors. New treatments for hemophilia are generically discussed in 18 reports, while the remaining 3 articles consider data regarding treatment costs, pharmacovigilance, and the impact of different treatments on joints.

Since the subject of the review is concizumab, only the 13 reports that deal with it specifically will be considered in this manuscript. In Table 1 the complete reviewed literature. Finally, a short section will discuss patients' perspectives regarding this new anti-TFPI antibody, still under study, compared to those of the available drugs.

Results

In 1998 Roberts et al³ described the key role played by the complex TF/FVIIa in the coagulation cascade initiation. The authors highlighted in their in vitro model, and which mimics the in vivo condition, how this pathway initiates the coagulation by activating both factor IX (FIX) and factor X (FX). The result of these activations will be the conversion of prothrombin into thrombin, resulting in the fibrin clot formation. TFPI turns off this reaction, thus allowing proper hemostasis to be maintained. Structure, isoforms and mechanism of action of this serine protease inhibitor, have been comprehensively reported by Broze & Girard⁴ and by Wood et al⁵. The TFPI gene was cloned and completely characterized in 1988,⁶ it measures approximately 90 Kb and is located on the long arm of chromosome 2 (q32), contains 10 exons and nine introns. Scientists have isolated several isoforms:^{4,5} the TFPI α purified from plasma and conditioned media of HepG2 cells. It consists of an acidic aminoterminal (N-) region followed by three tandem Kunitz domains encoded by exons 4-6-9 and the intermediate peptides which are allocated among Kunitz domains encoded by exons 5 and 7, and by a carboxyterminal (C-) region; the TFPI β initially discovered in mice, but also present in the ECV304 human cells of a bladder cancer presenting some endothelial characteristics; and the TFPI δ whose mRNA was found to be more expressed in the liver. In healthy people mean plasma TFPI concentration is about 70 ng/mL. TFPI circulates in plasma bound to lipoproteins, so its concentration depends on the low-density lipoproteins (LDL) amount, which can in turn be affected by the diet. Most plasma TFPI has a molecular weight between 34 and 41 kDa and is composed of 276 amino acids. In vitro models showed that TFPI α can be proteolytically degraded by a different proteinase, such as thrombin, plasmin, neutrophil elastase, or FXa if its molar quantity exceeds that of the TFPI α itself. Plasma concentrations of TFPI α were also reduced in patients with factor V (FV) deficiency, protein S (PS) deficiency and presumably also in those with FVIII deficiency. TFPI turns off the coagulation cascade with an irreversible process that requires the presence of calcium, and which is enhanced by the action of protein S. The structure of TFPI α , the most present isoform in plasma, is illustrated in Figure 1.

Table 1 All Concizumab Papers Considered in This Review

Reference (n)	Authors	Year	Model	Phase (trial)
[10]	Hilden I et al.	2012	Animal (rabbits)	
[11]	Agersø H et al.	2014	Animal (monkeys)	
[12]	Hansen L et al.	2014	Animal (rats, rabbits)	
[13]	Lauritzen B & Hilden I	2019	Animal (rabbits)	
[14]	Lauritzen B et al.	2019	Animal (monkeys, rabbits)	
[15]	Chowdary P et al.	2015	Human	I (Explorer I)
[16]	Chowdary P et al.	2018	Human	I (Explorer I)
[17]	Waters EK et al.	2017	Human	2 (Explorer 2)
[18]	Eichler H et al.	2018	Human	3 (Explorer 3)
[19]	Shapiro AD et al.	2019	Human	2 (Explorer 4/5)
[20]	Shapiro AD et al.	2021	Human	2 (Explorer 4/5)
[21]	Yuan et al.	2019	Human/Animal (rats/rabbits)	
[22]	Kjalke M et al.	2021	In vitro	

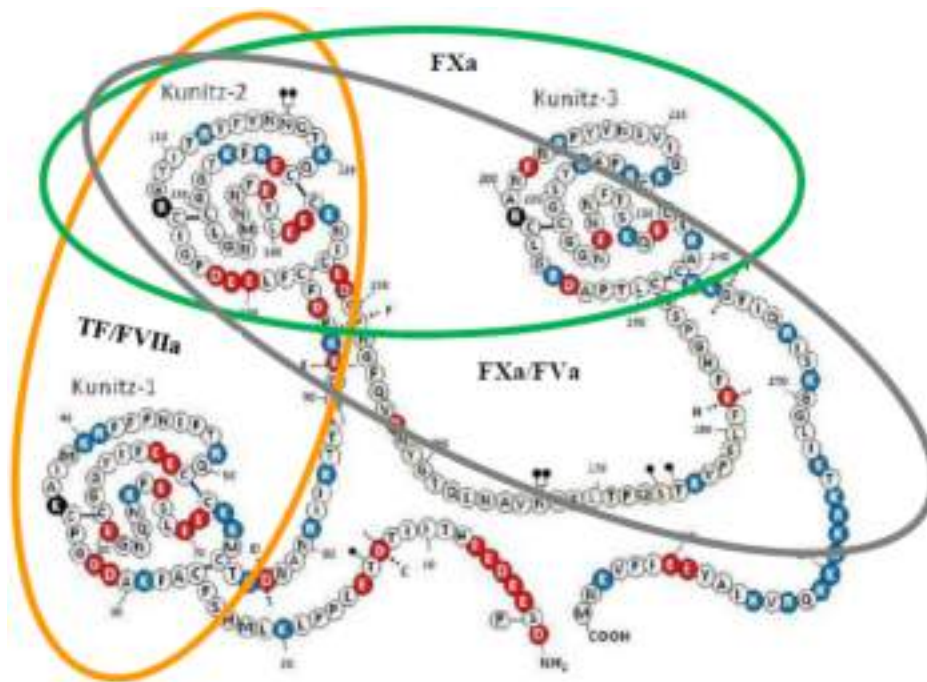


Figure 1 Structure of TFPI.

Notes: The domains required for inhibition of FXa, and of TF/FVII and FXa/FVa complexes are enclosed in the green, Orange, and grey ovals, respectively. Republished with permission of Frontiers in Bioscience, from Tissue factor pathway inhibitor: structure-function, Broze GJ, Girard TJ, Volume 17, Edition 1, 2012; permission conveyed through Copyright Clearance Center, Inc.⁴

The TFPI activity takes place at the level of the Kunitz domains. The first domain inhibits the TF/FVIIa complex, the second one inhibits FXa, while the third is only involved in binding to lipoproteins and heparins, without any known inhibitory function. The C-terminal region seems to favor contemporary interaction of TFPI and FXa to the phospholipid surface, and it is also involved in the FXa inhibition. Some in vitro models have indeed shown that the TFPI molecules lacking from the C-terminal region have a reduced anti-Xa and anticoagulant effect. All these inhibitory actions of the TFPI, which consequently lead to a drastic reduction of thrombin generation, may help explain the excessive bleeding in patients with hemophilia. Therefore, inhibiting the action of TFPI can be a strategy to be used in new drugs, such as concizumab, for the treatment of this hemorrhagic disease. The mechanism of action of TFPI is reported in Figure 2.

Concizumab is a monoclonal, humanized IgG4 antibody specific for the Kunitz-2 domain. Preclinical studies in vitro or on animal models and in vivo have demonstrated the ability of concizumab to restore thrombin generation, promoting the establishment of a procoagulant action.^{1,7-9}

Animal Models

In 2012 Hilden et al¹⁰ published the results of four independent studies performed in rabbit models after induction of hemophilia with a FVIII antibody (Ab). The first two were a dose-response studies in which the rabbits, induced cuticle bleeding, were randomized to receive *mab2021* intravenously or isotype control Ab. In the third study hemophilic rabbits, induced cuticle bleeding, were instead randomized to receive rFVIIa, *mab2021*, or isotype control Ab. The last study tested the efficacy of *mab2021* administered subcutaneously. In all studies use of *mab2021* reduced the blood loss, shortening the bleeding time. Very encouraging for the future of this new monoclonal antibody was its efficacy highlighted also in case of subcutaneous administration.

Two years later Agersø et al¹¹ published an interesting pharmacokinetic (PK) report in which two different studies performed in Cynomolgus Monkeys were described. In the first study twelve animals were divided in five different groups. The first three groups (2 monkeys/group) were included in a multiple dose PK study with escalating dose levels

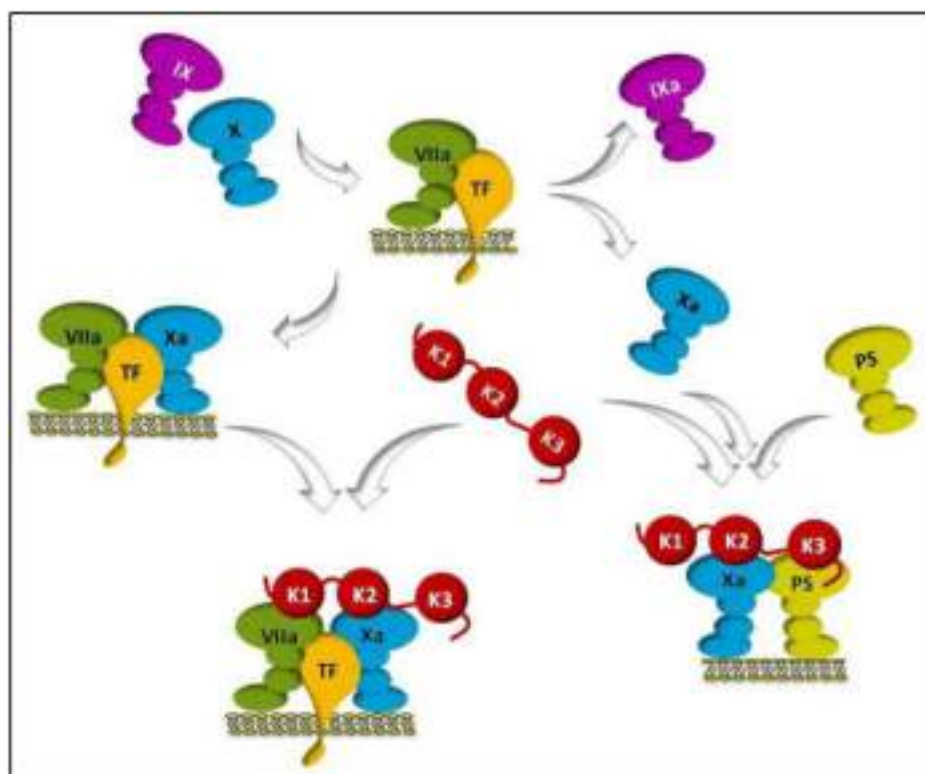


Figure 2 Mechanism of action of TFPI.

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administered every two weeks. For each group, blood samples were subsequently collected pre-dose and at different times. The remaining two groups were included in a single dose PK-study in which 20 mg/kg of concizumab were administered to monkeys intravenously or subcutaneously. Blood samples were subsequently collected pre-dose and at fourteen different times after drug administration. The second study was a thirteen-week toxicity study performed in fifty animals divided into four different groups based on the dose of administered drug and route of administration. Blood samples were after collected at different times for each group. These studies showed that the PK of concizumab was characterized by a high bioavailability (93%) after subcutaneous administration, with a clearance of 0.14 mL/h/kg, and reaching steady state after one week, slower in case of daily drug administration due to clearance-mediated target saturation. The potential efficacy of concizumab administered subcutaneously highlighted in the hemophilic rabbits' study is therefore confirmed by these pharmacokinetic results.

Similar data were obtained in the same year by Hansen et al¹² In their study the role of concizumab in neutralizing the TFPI inhibition in human endothelial cell-like immortalized cell line EA. hy926WT, derived from umbilical vein cells, was proven, while the PK profile was studied in both rats and rabbits.

Lauritzen & Hilden in their letter to editor, recently published,¹³ described three independent studies performed on hemophilic rabbits. These studies confirmed previous data: escalating doses of concizumab administered within five minutes from cuticle bleeding induction are equally effective to reduce the ongoing bleeds, but concizumab resulted less effective than rFVIIa if administered 15–30 minutes after bleeding induction. These data suggest that the use of rFVIIa is more suitable for the treatment of acute bleeding, while the use of concizumab is more appropriate for prophylaxis.

Another report always published by Lauritzen et al¹⁴ explored the efficacy and the safety of concizumab and rFVIIa when simultaneously administered. Three different studies were described. The first was an in vitro study in which the

human blood was taken from healthy donors and the platelet rich plasma (PRP) was subsequently prepared by centrifuging the whole blood. Hemophilia was induced *ex vivo* by adding a polyclonal sheep FVIII-antibody. Thrombin Generation Test (TGT) was then performed for PRP after adding concizumab, rFVIIa or a combination of concizumab and rFVIIa in equal parts. Both, concizumab and rFVIIa, increased thrombin peak in hemophilia A-like blood, while the combination of these two drugs caused a synergistic effect. Results subsequently confirmed with the thromboelastography.

In the second study the efficacy of concizumab, rFVIIa and a combination of concizumab/rFVIIa was tested in different groups of hemophilic rabbits after cuticle bleeding induction. The obtained data confirmed that both, concizumab and rFVIIa, were equally effective in reduce bleeds after cuticle bleeding induction, but the combination of these two drugs did not bring any additional effects and did not increase hemostatic efficacy.

The last study evaluated the safety of concizumab in a group of Cynomolgus Monkeys. Concizumab or vehicle control were daily administered to animals by subcutaneous injection for 28 days. A concizumab loading dose of 9.0 mg/kg was administered to monkeys for four days, followed by a dose of 1.0 mg/kg from day 5 to day 28. On day 28 the animals in the concizumab group also received three subsequent escalating doses of rFVIIa (0.25–0.5 – 1.0 mg/kg) two hours apart. Steady state of concizumab in plasma was reached after 27 days in all animals, except in one that had developed inhibitors against concizumab. No clinical adverse events nor thrombi during pathological examinations have been found. In conclusion concizumab in animal models resulted effective in reduce bleeding and safe.

The EXPLORER Trials

The Explorer 1^{15,16} was a first trial in humans. In this study escalating single doses of concizumab (intravenously or subcutaneously) were administered to healthy volunteers and persons with hemophilia (PWH) A or B. The study was a Phase 1, multicenter, randomized, double blind, placebo controlled, single dose and dose escalation trial. The participants enrolled in this study were randomized 3:1 to receive a single dose of concizumab (3 subjects) or placebo (1 subject). Dosing was initiated in healthy volunteers and, subsequently, continued in PWH. The primary end point of the study was safety, assessed at well-defined intervals up to 43 days after concizumab administration.

Pharmacokinetics (PK) and pharmacodynamics (PD) were also assessed throughout the entire study. 52 participants were overall included in the Explorer 1 trial, 28 were healthy volunteers, while 24 were PWH (21 with hemophilia A and 3 with hemophilia B). Throughout the trial 76 adverse events (AEs) were reported, 75% of them were mild, but none was a serious event. 19 AEs occurred in the placebo group. No correlation among different doses of concizumab and the onset of adverse event was found. The PK of concizumab was found to be non-linear and similar between PWH and healthy subjects. The PD analyses showed that the plasma unbound TFPI profiles were inversely related to PK ones, in fact the unbound TFPI concentration in plasma decreased if the concizumab concentration increased and remained decreased for over 14 days post dose at the highest concizumab levels. 24 bleeds were reported in 14 PWH (5 placebo; 9 concizumab), while only one bleed occurred in a healthy volunteer given concizumab. Except one, no bleeds were recorded in the subjects presenting in plasma high concizumab levels and low unbound TFPI concentrations. Concluding data from this study therefore demonstrate that also in humans concizumab can be considered a safe drug both intravenously or subcutaneously, as already shown in animal models.

Like the previous study, the Explorer 2 trial had as end points the evaluation of safety, PK and PD of concizumab administered subcutaneously to healthy male subjects and PWH. Waters et al¹⁷ investigated the role of concizumab in the thrombin generation, both in PWH and in healthy volunteers. A total of 22 participants were included in two different studies, 18 were hemophiliacs, while the remaining 4 were healthy males whose blood samples were obtained from the Explorer 2 trial. The thrombin generation, assessed by the thrombin generation assay (TGA), increased with concizumab after *ex vivo* spiking of hemophilia plasma and multiple subcutaneously doses in healthy subjects.

Another study on the safety of concizumab in PWH was the Explorer 3 trial.¹⁸ This multicenter, phase 1b trial investigated five escalating dose cohorts, each cohort had eight hemophilia patients and was divided into two different blocks. The study was placebo controlled, then two arms of treatment were defined: the concizumab arm and the placebo one. The assessed end points were safety, PK and PD, and the subcutaneous immunogenicity of concizumab at different doses. Overall, 24 participants were enrolled in this trial, equally divided in three different concizumab dose groups

(0.25–0.5–0.8 mg/kg) subcutaneously administered every four days. In each cohort six patients received concizumab and two placebo. A total of 56 adverse events occurred in 19 patients were reported, 54 of them mild and the remaining two moderate. The number of AEs was not related to the concizumab dose increase. PK and PD obtained in this trial confirmed as described in the previous studies. 91 bleeds were described, 28 of them in five patients in placebo group. Almost all bleeds were mild, except for two severe episodes, one spontaneous hemarthrosis and one traumatic muscle bleeding, which occurred in the concizumab 0.5 mg/kg group and the placebo group, respectively.

Explorer 4 and Explorer 5 trials^{19,20} were two different Phase 2 studies created to assess the efficacy and safety of subcutaneous concizumab administration in persons with hemophilia A or B (PWH-A or -B) with inhibitors (Explorer 4) and in severe PWH-A without inhibitors (Explorer 5), and to establish the optimal dose of concizumab for the subsequent Phase 3 studies. 36 PWH-A, 9 PWH-A with inhibitors and 8 PWH-B with inhibitors were enrolled in these studies and treated with concizumab 0.15 mg/kg, with potential dose escalation to 0.20 and 0.25 mg/kg in case of three spontaneous bleeds within twelve weeks of study drug treatment. The assessed PK/PD parameters confirmed the results of phase 1 trials. Estimated annual bleeding rate (ABR) was lower in patients with inhibitors than in those without (3.0 vs 5.9), and only two of seventeen inhibitor patients escalate the dose. All patients chose to participate to the extension phase of the trials. In these trials concizumab resulted safe and well tolerated, no severe event adverse or thromboembolic episodes were reported. Three patients in each study (Explorer 4 and Explorer 5) developed antibodies against the drug (ADA), without clinical manifestations. All three patients in the non-inhibitor trial developed a very low ADA titer (1.0–16.0 ng/mL), while the other three in the inhibitor trial presented a medium ADA titer (1.0–128.0 ng/mL) considering the assay sensitivity of 1.0 ng/mL. Neutralizing ADA in vitro were found only in one determination in two participants to Explorer 4 trial and in one of participants to Explorer 5 study. No changes in PK/PD or laboratory parameters, no increased bleeding, no adverse events were

reported in PWH developing ADA compared to patients without antibodies. These data support the daily use of concizumab as prophylactic treatment in all hemophilia subjects.

The Explorer program is still ongoing, and the results of the phase 3 trials are not yet published.

Other Studies

The remaining two published reports^{21,22} analysed the pharmacokinetics and pharmacodynamics of concizumab. Yuan et al²¹ created a system PK/PD model based on the different data of pharmacokinetics and pharmacodynamics of concizumab in humans,¹⁵ rabbits¹² and monkeys.¹¹ This model may be useful to determine the correct dose regimen of concizumab for the hemophilia treatment. Kjlke et al²² performed an interesting study in vitro and ex vivo aimed at evaluating the thrombin generation (TG) of concizumab alone or together with rFVIIa, activated prothrombin complex concentrate (aPCC) or recombinant FVIII (rFVIII). These drugs were added to plasma obtained from hemophilia A patients receiving concizumab, and subsequently analysed. In case of hemophilia B the TG was assessed only for concizumab alone or together with recombinant FIX (rFIX), after being added to the plasma of patients treated with concizumab. Similar as highlighted in the previous studies, concizumab was proven to increase the thrombin generation peak following a dose-dependent model. A combination of concizumab and rFVIIa, aPCC, rFVIII (or rFIX) caused an additive effect, highlighted by the TG assay, but without a synergistic strong effect. This study showed also that 0.5 IU/mL of rFVIII (or rFIX) added to concizumab had given rise to a TG peak comparable to that obtained with 1.0 IU/mL of rFVIII (or rFIX) alone. Given these additive effects, the use of concizumab in combination with other hemostatic agents should be carefully monitored and their dosage adjusted in order not to cause serious adverse events.

Patient's Perspectives

Over the years, the unmet needs of PWH have changed. Until the discovery of cryoprecipitate by Judith Pool²³ in 1965, the greatest need for these people with coagulation disorders was to have a drug available to stop acute bleeding, a cause of disability and in the worst cases of death.

The discovery of this anti-hemophilic factor triggered the subsequent development of plasma-derived coagulation factor concentrates. With the advent of these drugs, PWH's life expectancy increases, bleeding is better controlled, and the concept of prophylaxis begins to be instilled in clinicians.

In the 80–90s, the consequences of HIV and HCV transfusion-transmitted infections have led to an increasing need for safety in the drugs used for the PWH's treatment. In this period, drugs of recombinant origin were developed that respond to the need for viral safety. Over time, thanks to the availability of many drugs with slightly different characteristics, the clinician tries to give each patient the best therapy, effective, safe and free as much as possible of the risk of developing antibodies against FVIII or FIX. The concept of tailored therapy was born.

If efficacy in treating bleeding and safety in preventing the risk of viral infections and the development of inhibitors remain among the primary needs of PWHs, others are their requests that are becoming increasingly important in the management of hemophilia.

In 2017 von Mackensen et al²⁴ highlighted how the lower frequency of infusions ranks first among the unmet needs of PWH, while the improvement in their quality of life is mentioned in fifth place. The availability of the extended half-life FVIII and FIX concentrates have undoubtedly contributed to meeting these needs, but it is the advent of new non-substitutive drugs, administered subcutaneously, which will lead to an epochal change in the treatment of PWH. Parnes et al²⁵ analyzed the data from two phase 3^{26,27} studies involving the bispecific antibody, emicizumab. In the Haven 3 trial ninety-five patients receiving the study drug completed the EmiPref survey, and 94% of them reported preferring emicizumab to their previous treatment while in the Haven 4 trial, all enrolled patients completed the EmiPref survey, with all reporting a preference for emicizumab over their prior treatment. Efficacy and ease of administration have allowed this drug to gain the preference of patients.

The thromboembolic risk, almost unknown to PWHs treated with factor concentrates, becomes of primary importance in the case of these new non-replacement therapies, especially in those that act on the coagulation system, reducing the levels of anticoagulant activity, compensating for the defect in procoagulant activity, and thus increasing the thrombin generation.^{28,29} Concizumab falls into this category. In fact, the pivotal studies, Explorer 6, Explorer 7, and Explorer 8, have been temporarily stopped in March 2020 after non-fatal thrombosis occurred in three different participants. The studies were restarted a few months later, but with a new reduced dosing regimen of drug to mitigate the thromboembolic risk, as recently presented to the “15th Annual Congress European Association of Haemophilia and Allied Disorders (EAHAD)” by Chowdary et al³⁰ and Astermark et al³¹. All three participants to clinical trials who experienced these adverse events had distinct thrombotic risk factors,³⁰ therefore maximum attention must be paid to concizumab use especially in subjects at greater risk of thromboembolic events, such as the elderly with underlying cardiovascular problems, or in case of association with other anti-hemorrhagic drugs.

Subcutaneous administration of these drugs has created great expectations especially for the treatment of younger hemophilia patients, but the exclusion of newborns (< 1 year-olds) from the pivotal studies, associated with the variability of the hemostatic system found in children of different ages, makes it difficult to define with certainty the use of these non-replacement therapies in this population of patients.³²

Certainly, both patients and clinicians expect a lot from concizumab and the other subcutaneous drugs, available as emicizumab, or still in development, but long-term studies and registries are needed to give them a valid answer.

Conclusion

The development of new subcutaneous drugs has marked a milestone in the hemophilia treatment, changing the paradigm of patient's management.

A drug as emicizumab has in fact allowed PWH-A with inhibitors to lead a life like that of those who do not have these antibodies. A weekly, bi-weekly or even monthly prophylaxis regimen has reduced the use of bypassing agents and of long and not always effective immunotolerance induction (ITI) regimens. The reduction in bleeding that led to almost complete zeroing of ABR significantly improved the quality of life of these patients. Inhibitor appearance in PWH-B is very rare, but their treatment is very difficult, in fact, attempts to eradicate the antibodies by using FIX at high doses, as occurs during ITI, can cause the development of anaphylactic reactions, even severe, in over half of the patients treated. Therefore, these patients have only one therapy available, the use of rFVIIa, a drug with a very short half-life which requires very frequent infusions. The new subcutaneous drugs that can be used in PWH-AB, which act on the coagulation system by rebalancing it, such as concizumab and the other anti-TFPIs or the antithrombin inhibitor, fitusiran, can certainly represent a future therapeutic option also for patients with FIX deficiency.

In the case of patients who do not have inhibitors against FVIII or FIX, these subcutaneous drugs may represent an important turning point in initiating primary prophylaxis in children. In fact, the initiation of prophylaxis is now delayed mainly due to their difficult venous access. Of course, large studies and registries are needed to define the safety of these drugs in infants and newborns.

Safety is also a very important topic in adults, as several cases of thrombosis in humans have been reported, not detected in animal models, and which have also led to the temporary suspension of pivotal studies, this should suggest to clinicians the need for some attention in the future use of these new drugs.

After these due considerations, we can affirm that the preliminary data on concizumab, which emerged from the first studies, are encouraging, but the outcome of the ongoing trials is awaited to define with certainty advantages and limits of this drug.

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Disclosure

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Prevalence and Predictors of Cytopenias in HIV-Infected Adults at Initiation of Antiretroviral Therapy in Mehal Meda Hospital, Central Ethiopia

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Prevalence and Predictors of Cytopenias in HIV-Infected Adults at Initiation of Antiretroviral Therapy in Mehal Meda Hospital, Central Ethiopia

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Background: Hematologic abnormalities are common complications of patients infected with HIV associated with accelerated deterioration in CD4⁺ cell counts, disease progression, poor quality of life and death. Few studies have evaluated the magnitude of cytopenias at the initiation of antiretroviral therapy (ART) in sub-Saharan Africa. The aim of this study was to determine the prevalence and predictors of cytopenias among HIV-infected adults at initiation of ART in a resource-limited setting in Ethiopia.

Methods: A cross-sectional study was conducted among HIV-infected adults initiating ART at the HIV care and treatment clinic of Mehal Meda Hospital between September 2008 and June 2019. Demographic, clinical and laboratory data of patients were collected from medical records. Anemia was defined according to WHO guidelines as hemoglobin concentration <12 g/dl for non-pregnant females and <13 g/dl for males. Leucopenia was defined as total white blood cell count <4.0 × 10³ cells/μL and thrombocytopenia as platelet count <150 × 10³ cells/μL. Logistic regression analysis was used to determine factors associated with the presence of cytopenias.

Results: Out of the total 566 patients included, 36.6% (95% CI 32.7–40.6%) had anemia, 17.1% (95% CI 14.2–20.4%) had leucopenia and 14.5% (95% CI 11.8–17.6%) had thrombocytopenia. A total of 53.2% (95% CI 49.1–57.3%) of patients had at least one form of cytopenia, 14.1% (95% CI 11.4–17.2%) had bicytopenia, and only 0.5% had pancytopenia. Factors associated with the presence of any cytopenia in multivariable analysis were male sex, advanced clinical disease stage, low CD4⁺ cell count, low BMI, and decreased renal function.

Conclusion: A substantial burden of cytopenias was detected among HIV-infected adults enrolled for care and treatment services in our setting. Patients with HIV infection should be screened for hematological abnormalities at initiation of ART because of its potential for morbidity and mortality during ART.

Keywords: hematological abnormalities, cytopenia, HIV, Ethiopia

Introduction

Hematologic abnormalities especially cytopenias (anemia, leucopenia and thrombocytopenia) are common complications of patients infected with Human Immunodeficiency Virus (HIV). These abnormalities occur mainly due to direct effects of the virus on the bone marrow, suppression of bone marrow by secondary infections or neoplasms, nutritional deficiencies or side effects of the drugs used.^{1–3} Hematologic abnormalities can occur at all stages of HIV infection, but are more prevalent in patients with advanced disease and in ART naive patients than treated patients.^{4–7} In patients with HIV, cytopenias have been associated with accelerated deterioration in CD4⁺ cell counts, disease progression, poor quality of life and subsequent death.^{8–11} Hematologic abnormalities have also been demonstrated to predict poor responses to ART, Acquired Immunodeficiency Syndrome (AIDS)-related morbidity and mortality, and have been suggested as important parameters for predicting the clinical course of patients living with HIV infection.^{12,13}

Anemia is the most common hematologic abnormality seen in HIV infection, occurring in 1.3% to 95% of patients and has been associated with impaired quality of life, poor immune recovery, progression of disease and a high risk of

hospitalization or mortality.^{14–16} It is also an independent predictor of morbidity and mortality even upon initiation of therapy.^{12,13,17} The pathogenesis of anemia in HIV patients is complex and may result from opportunistic infections, nutritional deficiencies, AIDS-associated malignancies, medications, and alteration in hematopoiesis induced by HIV itself or the drugs included in ART.^{3,8,18} Factors that are associated with anemia among HIV-infected antiretroviral-naïve patients include older age, gender, presence of opportunistic infection, advanced clinical HIV disease stages, low CD4+ cell counts, low body mass index and decreasing renal function.^{19–21}

Leucopenia is the common hematologic abnormality that occurs in patients with HIV-infection and results from a direct effect of HIV virus, autoimmune disease, neoplasm and ART drugs and opportunistic infection.³ It can occur in as many as 26.8% of patients and is associated with adverse HIV-related outcomes including both death and AIDS-related morbidity.^{6,13} Studies indicated that worsening HIV disease parameters, ie, advanced clinical stages and low CD4+ cell counts, are associated with an increased risk of leucopenia among antiretroviral-naïve patients.^{20,21} Thrombocytopenia is a frequent hematologic abnormality in HIV patients and can occur at all stages of HIV infection. It can be found in about 4–40% of HIV-infected patients and results from decreased production or accelerated destruction of platelets, with immune thrombocytopenic purpura the most common cause.^{2,10,22} Thrombocytopenia is associated with a substantial decline in CD4+ cell count, poor quality of life and increased morbidity and mortality.^{9–12,22} Advanced clinical stages and immunosuppression were the factors significantly related to thrombocytopenia among antiretroviral-naïve HIV patients.

Although previous studies have demonstrated the importance of hematological alterations at baseline as influences of treatment failure and mortality during ART among patients treated in sub-Saharan African countries,^{9,12,23} few studies have evaluated the magnitude of cytopenias at the initiation of ART among HIV patients in sub-Saharan Africa. The aim of this study was to determine the prevalence and predictors of cytopenias among adult HIV-infected patients at initiation of ART in a resource-limited setting in Ethiopia.

Methods

Study Design and Population

We performed a cross-sectional study of HIV-infected adult patients initiating ART at the HIV care and treatment clinic of Mehal Meda Hospital, Central Ethiopia between September 2008 and June 2019. Mehal Meda Hospital is found in Mehal Meda town in Menz Gera Midir Woreda North Shewa Zone of the Amhara Region, Ethiopia. Mehal Meda town is about 280 km away from the capital city Addis Ababa and 148 km from Debre Birhan city, the capital of Amhara Region. The hospital provides comprehensive health-care services including HIV/AIDS diagnosis, treatment and monitoring. Patients were included in this study if they were 18 years and older, and had full blood count results available at baseline before the initiation of ART. Patients with missing data for essential variables, and pregnant women were excluded from the study analysis.

Data Collection and Definitions

The medical records of HIV-infected patients enrolled to receive first-line ART, comprised of at least three drugs from September 2008 and June 2019 were reviewed. Patients' baseline socio-demographic (age, gender, residence, education, weight and height), clinical (WHO clinical stage of disease, CD4+ cell count and presence/history of opportunistic infection), and hematological data (hemoglobin level, total white blood cell count and platelet count) were collected. Serum creatinine results available at baseline were collected and renal function was assessed by estimated glomerular filtration rate (eGFR) calculated using the Modification of Diet in Renal Disease (MDRD) equation.²⁴ Complete blood count including hemoglobin concentration, total white blood cell count and platelet count (Sysmex KX-21N, Sysmex corporation Kobe, Japan) and CD4 cell count (BD Facscount system, Becton Dickinson, California, USA) were performed as part of a routine evaluation. Anemia was defined in accordance with the WHO guidelines,²⁵ as hemoglobin concentrations <12.0 g/dL for non-pregnant females and <13.0 g/dL for males. We further classified anemia as mild (11–11.9 g/dL for females and 11–12.9 g/dL for males), moderate (8–10.9 g/dL) and severe (<8 g/dL in both genders). Leucopenia was defined as total white blood cell count <4.0×10³ cells/μL and thrombocytopenia as platelet count

$<150 \times 10^3 / \mu\text{L}$.²⁰ Any cytopenia, bicytopenia and pancytopenia were defined as presence of at least one form of cytopenias (anemia, leucopenia or thrombocytopenia), two forms and all forms of cytopenias, respectively.

Statistical Analysis

Data were entered into an “EpiData version 3.1” and analysed with SPSS version 25 software (SPSS Inc., Chicago, IL, USA). The normal distribution of the data was tested by the Kolmogorov–Smirnov test. Comparisons of continuous variables between groups were carried out using Student’s *t*-test and chi-square (χ^2) test was used for categorical variables. A logistic regression analysis was used to identify predictors of anemia, leucopenia, thrombocytopenia and any cytopenia at the initiation of ART. Age, gender, WHO clinical stage of disease, CD4+ cell count, presence/history of opportunistic infections, body mass index (BMI), and eGFR were entered into a univariable model. Variables with *P*-value <0.25 in the univariable analysis were included in the multivariable models using forward stepwise method. Odds ratio (OR) with 95% confidence interval (CI) was used to measure the strength of statistical association. All tests were two-sided and a *P* < 0.05 was considered to be statistically significant.

Results

Patient Characteristics

A total of 566 HIV-infected adult patients who enrolled in the HIV care from September 2008 through November 2019 were included in this study. The mean age was 36.1 years (standard deviation [SD] ± 9.7) and 324 patients (67.1%) were females (Table 1). The median CD4+ cell count was 264 cells/mm³ (IQR: 192–500), and 439 patients (71.9%) had WHO clinical stage I/II at the time of enrollment. The mean BMI was 20.4 kg/m² (SD ± 3.6). The mean eGFR was 99.2 mL/min/1.73 m² (SD ± 58.2). The mean hemoglobin was 12.7 g/dl (SD ± 2.68), the median total white blood cell count was 5.7×10^3 cell/ μL (IQR: 4.5–7.5), and the median platelet count was $240 \times 10^3/\mu\text{L}$ (IQR: 176–310).

Prevalence and Factors Associated with Cytopenias

Two-hundred and seven patients (36.6%; 95% CI 32.7–40.6%) had anemia, including 92 patients (16.3%) with mild, 85 (15.0%) with moderate and 30 (5.3%) with severe anemia. Advanced clinical HIV/AIDS disease stage (WHO stage III/IV), low CD4+ cell count, history of opportunistic infections, low BMI and low eGFR were associated with anemia in the univariate analysis (Table 2). The multivariable analysis indicated that advanced clinical disease stage (AOR 2.07; 95% CI 1.37–3.18), CD4+ cell count <200 cells/mm³ (AOR 2.00; 95% CI 1.20–3.32), BMI < 18.5 kg/m² (AOR 2.89; 95% CI 1.46–5.69) and low eGFR: <60 mL/min/1.73 m² (AOR 2.41; 95% CI 1.46–3.98) and 60–89.9 mL/min/1.73 m² (AOR 1.92; 95% CI 1.27–2.89) were independently associated with anemia.

Leucopenia was present in 97 patients (17.1%; 95% CI 14.2–20.4%). In univariate analysis, several risk factors met the pre-defined criteria for inclusion in the multivariable model (age, residence, education, WHO clinical disease stage and CD4+ cell count) (Table 3). In the multivariable analysis, only CD4+ cell count <200 cells/mm³ (AOR 1.93; 95% CI 1.22–3.07) was independently associated with leucopenia. Thrombocytopenia was observed in 82 patients (14.5%; 95% CI 11.8–17.6%). The univariate analysis showed a significant association between thrombocytopenia and male sex, education, WHO clinical disease stage, CD4+ cell count and history of opportunistic infections (Table 4). In multivariable analysis, male sex (AOR 3.22; 95% CI 1.93–5.38), advanced clinical disease stage (AOR 1.72; 95% CI 1.01–2.91) and CD4+ cell count <200 cells/mm³ (AOR 2.07; 95% CI 1.08–3.97) were independently associated with thrombocytopenia.

A total of 301 patients (53.2%; 95% CI 49.1–57.3%) had at least one form of cytopenia, 80 (14.1%; 95% CI 11.4–17.2%) had bicytopenia, and only 3 (0.5%) had pancytopenia. The anemia-leucopenia combination was the most frequent bicytopenia 41 (7.3%), followed by anemia-thrombocytopenia 28 (4.9%) and leucopenia-thrombocytopenia 11 (1.9%). Factors associated with the presence of any cytopenia in multivariable regression were male sex (AOR 1.73; 95% CI 1.20–2.48), advanced clinical disease stage (AOR 1.72; 95% CI 1.10–2.70), CD4+ cell count <200 cells/mm³ (AOR 2.24; 95% CI 1.49–3.37), BMI < 18.5 kg/m² (AOR 1.50; 95% CI 1.02–2.44) and decreased renal function (eGFR: <60 mL/min/1.73 m²) (AOR 2.50; 95% CI 1.52–4.12) (Table 5).

Table I Characteristics of the Study Patients at the Initiation of ART (N = 566)

Characteristics	
Age (years), Mean (SD)	36.1 ± 9.7
Age group, n (%)	
18–29 years	156 (27.6)
30–39 years	197 (34.8)
40–49 years	165 (29.2)
≥50 years	48 (8.5)
Gender, n (%)	
Male	242 (42.8)
Female	324 (57.2)
Residence, n (%)	
Urban	356 (62.9)
Rural	210 (37.1)
Education, n (%)	
<High school	452 (79.9)
≥High school	114 (20.1)
WHO stage, n (%)	
Stage I/II	439 (77.6)
Stage III/IV	127 (22.4)
CD4+ cell (cells/mm ³), Median (IQR)	264 (192–500)
<200	160 (28.3)
≥200	406 (71.7)
Opportunistic infections, n (%)	
Yes	149 (26.3)
No	417 (73.7)
Body mass index (kg/m ²), Mean (SD)	20.4 ± 3.6
Estimated glomerular filtration rate (mL/min/1.73m ²), Mean (SD)	99.2 ± 58.2
Hemoglobin (g/dl), Mean (SD)	12.7 ± 2.7
Total white blood cell count (×10 ³ cell/μL), Median (IQR)	5.7 (4.5–7.5)
Platelet count (×10 ³ cell/μL), Median (IQR)	240 (176–305)

Discussion

In this study, the prevalence of anemia, leucopenia and thrombocytopenia among antiretroviral-naïve adult HIV-positive patients enrolled for care and treatment services in Mehal Meda Hospital, central Ethiopia were 36.6%, 17.1% and 14.5%, respectively. About 53.2% of the patients had at least one form of cytopenia (anemia and/or leucopenia and/or

Table 2 Factors Associated with the Presence of Anemia at Initiation of ART

Variables	Anemia		Crude OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
	Yes, n (%)	No, n (%)				
Age (years)				0.216		0.199
<35	97 (39.4)	149 (60.6)	1.24 (0.88–1.75)		1.27 (0.89–1.84)	
≥35	110 (34.4)	210 (65.6)	I		I	
Gender				0.252		
Male	95 (39.3)	147 (60.7)	1.22 (0.87–1.73)			
Female	112 (34.6)	212 (65.4)	I			
Residence				0.097		0.153
Urban	121 (34.0)	235 (66.0)	0.74 (0.52–1.06)		0.73 (0.59–1.35)	
Rural	86 (41.0)	124 (59.0)	I		I	
Education				0.776		
<High school	164 (36.3)	288 (63.7)	0.94 (0.62–1.44)			
≥High school	43 (37.7)	71 (62.3)	I			
WHO stage				0.001		0.001
Stage III/IV	63 (49.6)	64 (50.4)	2.02 (1.35–3.01)		2.00 (1.31–3.02)	
Stage I/II	144 (32.8)	295 (67.2)	I		I	
CD4+ cell count (cells/mm ³)				<0.001		0.001
<200	80 (50.0)	80 (50.0)	2.20 (1.51–3.19)		1.90 (1.28–2.81)	
≥200	127 (31.3)	279 (68.7)	I		I	
BMI (kg/m ²)				0.001		0.004
<18.5	76 (47.5)	84 (52.5)	1.90 (1.31–2.76)		1.78 (1.20–2.65)	
≥18.5	131 (32.3)	275 (67.7)	I		I	
Opportunistic infections				0.007		0.339
Yes	68 (45.6)	81 (54.4)	1.68 (1.15–2.56)		1.23 (0.80–1.89)	
No	139 (33.3)	275 (66.7)	I		I	
eGFR (mL/min/1.73 m ²)				<0.001		0.012
<60	56 (53.3)	49 (46.7)	2.35 (1.53–3.61)		1.80 (1.14–2.85)	
≥60	151 (32.8)	310 (67.2)	I		I	

thrombocytopenia). The main factors associated with the presence of any cytopenia were male sex, advanced clinical disease stage, low CD4+ cell count, low BMI, and decreased renal function.

Anemia was the most prevalent cytopenia in this study, occurring in 36.6% of antiretroviral-naïve adult HIV-infected patients at initiation of ART. This high prevalence of anemia, already noted by other studies from sub-Saharan Africa,^{11,14,20,21,26,27} could be explained by the more advanced immunodeficiency at enrollment as well as the higher prevalence of anemia in the general population in sub-Saharan Africa.^{28,29} The presence of anemia prior to the initiation

Table 3 Factors Associated with the Presence of Leucopenia at Initiation of ART

Variables	Leucopenia		Crude OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
	Yes, n (%)	No, n (%)				
Age (years)				0.066		0.051
<35	34 (13.8)	212 (86.2)	0.66 (0.42–1.03)		0.63 (0.40–1.01)	
≥35	63 (19.7)	257 (80.3)	I		I	
Gender				0.313		
Male	37 (15.3)	205 (84.7)	0.79 (0.51–1.24)			
Female	60 (18.5)	264 (81.5)	I			
Residence				0.249		0.210
Urban	66 (18.5)	290 (81.5)	1.31 (0.83–2.09)		1.35 (0.84–2.17)	
Rural	31 (14.8)	179 (85.2)	I		I	
Education				0.207		0.173
<High school	82 (18.1)	370 (81.9)	1.46 (0.81–2.65)		1.53 (0.83–2.84)	
≥High school	15 (13.2)	99 (86.8)	I		I	
WHO stage				0.028		0.073
Stage III/IV	30 (23.6)	97 (76.4)	1.72 (1.06–2.79)		1.57 (0.96–2.58)	
Stage I/II	67 (15.3)	372 (84.7)	I		I	
CD4+ cell count (cells/mm ³)				0.004		0.005
<200	39 (24.4)	121 (75.6)	1.93 (1.23–3.05)		1.93 (1.22–3.07)	
≥200	127 (31.3)	279 (68.7)	I		I	
BMI (kg/m ²)				0.375		
<18.5	31 (19.4)	129 (80.6)	1.24 (0.77–1.99)			
≥18.5	66 (16.3)	340 (83.7)	I			
Opportunistic infections				0.380		
Yes	29 (19.5)	120 (80.5)	1.24 (0.77–2.01)			
No	68 (16.3)	349 (83.7)	I			

of combination ART may complicate the treatment of HIV, associated with poor response to ART, treatment failure and clinical disease progression to AIDS and predict decreased survival in HIV-infected patients after ART initiation.^{9,14,23} Without intervention, anemia can lead to significant symptoms like fatigue, breathlessness, difficulty in concentration and other effects on functionality and quality of life. Targeted screening for anemia at initiation of ART and modifying the potential risk factors are primary needs to improve disease outcomes in people living with HIV.

We found that advanced clinical HIV disease stage (WHO clinical stage III/IV), low CD4+ count, low BMI and low eGFR are associated with an increased risk of anemia in antiretroviral-naïve HIV-infected patients. Worsening parameters of HIV disease, ie, WHO clinical stage III/IV and CD4+ cell count <200 cells/mm³, are significantly associated with an increased risk of anemia at ART initiation, and this was consistent with findings of previous related studies.^{16,19,30,31} We

Table 4 Factors Associated with the Presence of Thrombocytopenia at Initiation of ART

Variables	Thrombocytopenia		Crude OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
	Yes, n (%)	No, n (%)				
Age (years)				0.875		
<35	35 (14.2)	211 (85.8)	0.96 (0.60–1.55)			
≥35	47 (14.7)	273 (85.3)	I			
Gender				<0.001		<0.001
Male	56 (23.1)	186 (76.9)	3.45 (2.09–5.69)		3.22 (1.93–5.38)	
Female	26 (8.0)	298 (92.0)	I		I	
Residence				0.917		
Urban	52 (14.6)	304 (85.4)	1.03 (0.63–1.67)			
Rural	30 (14.3)	180 (85.7)	I			
Education				0.026		0.132
<High school	58 (12.8)	394 (87.2)	0.55 (0.33–0.94)		0.65 (0.37–1.14)	
≥High school	24 (21.1)	90 (78.9)	I		I	
WHO stage				0.001		0.042
Stage III/IV	30 (23.6)	97 (76.4)	2.30 (1.39–3.80)		1.72 (1.01–2.97)	
Stage I/II	52 (11.8)	387 (88.2)	I		I	
CD4+ cell count (cells/mm ³)				0.002		0.004
<200	35 (21.9)	125 (78.1)	2.14 (1.32–3.47)		2.07 (1.08–3.44)	
≥200	47 (11.6)	359 (88.4)	I		I	
BMI (kg/m ²)				0.455		
<18.5	26 (16.3)	134 (83.7)	1.21 (0.73–2.01)			
≥18.5	56 (13.8)	350 (86.2)	I			
Opportunistic infections				0.044		0.233
Yes	29 (19.5)	120 (80.5)	1.66 (1.01–2.73)		1.37 (0.82–2.31)	
No	53 (12.7)	364 (87.3)	I		I	

also found evidence that low BMI is associated with an increased risk of anemia prior to ART initiation. Low level of BMI (<18.5 kg/m²) has been previously found to have significant association with the occurrence of anemia.^{19,31} Our results also support findings that impaired renal function is associated with a higher risk of anemia among HIV patients initiating ART.³² Impaired renal function in HIV infection has a significant impact on lowering hemoglobin levels, resulting in a higher risk of anemia.³³ The multifactorial drivers of anemia in HIV-infected patients prior to initiation of ART require an integrated approach to help ameliorate anemia and its negative health effects while on ART.

The prevalence of leucopenia in our study (17.1%) was comparable with other studies of HIV-infected adults initiating ART in sub-Saharan Africa, ranging between 13.4% and 24.4%.^{20,21,26,27,34} A study from India indicated that 18.3% of HIV patients had leucopenia first at the time of presentation,³⁵ while in the Chinese study, leucopenia was reported in 33.2% of antiretroviral-naïve HIV-infected patients.³⁶ In multivariate analysis, lower CD4+ cell count was independently associated with an increased risk of having leucopenia in HIV-infected antiretroviral-naïve patients. This

Table 5 Factors Associated with the Presence of Any Cytopenia at Initiation of ART

Variables	Any Cytopenia		Crude OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
	Yes, n (%)	No, n (%)				
Age (years)				0.889		
<35	130 (52.8)	116 (47.2)	0.98 (0.70–1.36)			
≥35	171 (53.4)	149 (46.6)	I			
Gender				0.006		0.003
Male	145 (59.9)	97 (40.1)	1.61 (1.15–2.26)		1.73 (1.20–2.48)	
Female	156 (48.1)	168 (51.9)	I		I	
Residence				0.270		
Urban	183 (51.4)	173 (48.6)	0.83 (0.59–1.16)			
Rural	118 (56.2)	92 (43.8)	I			
Education				0.259		
<High school	235 (52.0)	217 (48.0)	0.79 (0.52–1.19)			
≥High school	66 (57.9)	48 (42.1)	I			
WHO stage				0.004		0.042
Stage III/IV	82 (64.6)	45 (35.4)	1.83 (1.22–2.76)		1.72 (1.10–2.40)	
Stage I/II	219 (49.9)	220 (50.1)	I		I	
CD4+ cell count (cells/mm ³)				<0.001		0.001
<200	112 (70.0)	48 (30.0)	2.68 (1.81–3.96)		2.24 (1.49–3.37)	
≥200	189 (46.6)	217 (53.4)	I		I	
BMI (kg/m ²)				0.009		0.045
<18.5	99 (61.9)	61 (38.1)	1.64 (1.13–2.38)		1.50 (1.02–2.24)	
≥18.5	202 (49.8)	204 (50.2)	I		I	
Opportunistic infections				0.001		0.164
Yes	96 (64.4)	53 (35.6)	1.87 (1.27–2.76)		1.36 (0.88–2.09)	
No	205 (49.2)	212 (50.8)	I		I	
eGFR (mL/min/1.73 m ²)				<0.001		<0.001
<60	77 (73.3)	28 (26.7)	2.91 (1.82–4.65)		2.50 (1.52–4.12)	
≥60	224 (48.6)	237 (51.4)	I		I	

was consistent with other studies that have found HIV patients with low CD4+ cell count to have a higher odds of leucopenia than those with higher CD4+ cell count at the time of antiretroviral-treatment initiation.^{20,21,26,34,36}

About 14.5% of our patients had thrombocytopenia at the time of initiation of ART. This was consistent with findings reported from Tanzania,²⁶ China³⁶ and India,³⁵ where thrombocytopenia was found in 14.4%, 15.6% and 15.8% of HIV-infected adult antiretroviral-naïve patients, respectively. This was; however, lower than the 18.7% prevalence reported in Northeast Ethiopia,²⁰ and higher than 5.9% in Northwest Ethiopia³⁷ and 8.3% in Uganda.²¹ These differences could be due to the difference in the study population, sample size, study design and variation in the definition of

thrombocytopenia. Male sex, advanced clinical disease stage and severe immunosuppression (CD4+ cell count <200 cells/mm³) were independent predictors of having thrombocytopenia in this study. This was consistent with the Ugandan study²¹ that has found males to be more likely to have thrombocytopenia than females at initiation of ART. Gender-dependent difference in platelet count, being lower in males than females, could be responsible. However, some of the studies failed to find a significant relationship between sex and thrombocytopenia.^{20,26,36,37} This study also confirms the findings of other studies that have shown an association between advanced HIV disease (WHO clinical disease stage III/IV and CD4+ cell count <200 cells/mm³) and the presence of thrombocytopenia in HIV-infected antiretroviral-naïve patients.^{20,21,26,35–37}

More than half (53.2%) of the patients had at least one form of cytopenia (anemia and/or leucopenia and/or thrombocytopenia) in this study. In the Northeast Ethiopian study,²⁰ the overall magnitude of any cytopenia was 63.4% among ART naïve HIV-infected patients. In the Ugandan study by Kyeyune et al,²¹ 65% of the patients had at least one form of cytopenia at initiation of ART. In the Nigerian study, 59.4% of treatment-naïve HIV-infected patients had cytopenia.³⁸ The factors independently associated with the presence of any cytopenia in this study were male sex, advanced clinical HIV disease stage, low CD4+ cell count, low BMI, and decreased renal function. The Ugandan study²¹ reported females were more likely to have at least one form of cytopenia than males at initiation of ART, while other studies did not show any significant association between sex and the presence of any cytopenia.^{20,26} Our finding is however in line with the findings of other related studies, which reported a significant association of advanced HIV infection (advanced clinical stages and low CD4+ cell counts) with the presence of any cytopenia.^{20,21,26} The present study revealed a significant increase in the odds of anemia with decreasing BMI, which is consistent with the Ugandan study.²¹ The association of decreased renal function with any cytopenia may be due to the combined effect of impaired renal function and HIV infection on cytopenia and specifically anemia in this population.³³ However, no studies were available to assess the possible contribution of decreased renal function to the occurrence of cytopenia.

The limitations of this study include its cross-sectional design, which makes determination of temporal relationships between cytopenias and associated factors difficult. We did not assess the various causes of cytopenia and specifically anemia in this population. Well-controlled cohort studies would be appropriate to evaluate the potential impact of cytopenias on clinical outcomes of people living with HIV in our settings.

Conclusions

We detected a substantial burden of cytopenias (anemia, leucopenia and thrombocytopenia) among HIV-infected adult patients enrolled for care and treatment services in resource-limited setting in Ethiopia. The findings indicate that patients with HIV infection should be screened for hematological abnormalities at initiation of ART, with a special attention to be focused on males, patients with advanced disease stages, low CD4+ cell count, low BMI and low eGFR, because of its potential for morbidity and mortality during ART.

Abbreviations

AIDS, acquired immunodeficiency syndrome; AOR, adjusted odds ratio; ART, antiretroviral therapy; BMI, body mass index; CD4, cluster of differentiation; CI, confidence interval; eGFR, estimated glomerular filtration rate; HIV, human immunodeficiency virus; IQR, inter quartile range; MDRD, modification of diet in renal disease; OR, odds ratio; SD, standard deviation; WHO, World Health Organization.

Data Sharing Statement

The data of this study cannot be shared publicly due to presence of sensitive (confidential) participants' information and additional data than that used in this publication. But the data are available from the corresponding author on reasonable request.

Ethics and Consent Statement

Ethical approval of the protocol was achieved from the Institutional Review Board of College of Medicine and Health Sciences, Wollo University. As only routine data were analyzed, informed consent was not required; but patient's

identifiers were removed and only code numbers were used throughout the study. This study was conducted in accordance with the declaration of Helsinki.

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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.

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Disclosure

The authors declare that they have no competing interests in this work.

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ABO and Rhesus Blood Group Distribution in Mogadishu, Somalia

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Purpose: Our aim in this study is to reveal the blood group distribution by investigating retrospectively the blood types of around 60 thousand patients and donors who applied to Mogadishu Somalia Turkey Recep Tayyip Erdoğan Training and Research Hospital between 2018 and 2021.

Patients and Methods: The blood group data of 59,442 people were included in this study. 20,414 (34.35%) of the study's participants were female, 39,023 (65.65%) were male. The blood groups of participants were determined using the slide method and the gel column technique. The frequencies and percentages of O, A, B, AB and Rh blood groups were calculated.

Results: The frequencies of ABO blood group distribution indicated that blood group O and Rh+ were predominant in Mogadishu O group: 60.30%, A group: 26.50%, B group: 11.27%, AB group: 1.93%, Rh+ group: 96.49%, Rh- group: 3.43%.

Conclusion: This is the first study about blood group distribution in Mogadishu based on a large number of blood type tests and hospital data. The findings of our study can guide the blood center administrators make decisions concerning blood stocking and supply. This study can give an idea about how much fluctuations may occur in the frequency of blood group types in emigrant populations over many years.

Keywords: blood types, ABO, Rh, Mogadishu, Somalia

Introduction

Blood is the most tested, simple to obtain and most vital body fluid. A series number of glycoprotein and glycolipid antigens are found on the surface of human red blood cells. These antigens form the blood groups, the presence or absence of each different antigen on the erythrocyte surface determines the blood group type.¹ Austrian scientists Karl Landsteiner discovered blood groups A, B and O in 1901² and Decastello and Sturli found out AB blood group 1 years later in 1902. The genetic transmission of blood groups was also explained by Decastello and Sturli.³ With the discovery of the Rhesus factor by Landsteiner and Weiner in 1940, all major blood group antigens were determined.⁴ As a result of studies carried out in many research centers, different types of these surface antigens were found. According to The International Society of Blood Transfusion, there are more than 30 blood types and approximately 700 erythrocyte antigens.¹ In newborn hemolytic illness, blood transfusion, and tissue transplantation, the ABO and Rh blood group systems are extremely important.^{5,6} A and B antigens are seen not only on the erythrocyte surface, but also in many tissues, including vascular endothelium, nerve receptors, epidermis, and mucous cells. HLA antigens and A and B antigens are the two most effective factors in tissue rejection.⁷

When the literature is searched, a few research about blood group distribution in Somalia is available. It is noteworthy that Sistonen et al conducted a blood group and genetic analysis named "Distribution of Blood Groups in the East African Somali Population"⁸ in which 1026 people participated in 1987. Our study can be accepted as an update of Sistonen et al's study. The blood types of approximately 60.000 patients and donors who applied to Mogadishu Somalia

Turkey Recep Tayyip Erdogan Training and Research Hospital between 2018 and 2021 were evaluated retrospectively in this study. The frequency and proportions of O, A, B, AB and Rh blood groups were calculated. The frequencies and proportions of all groups were also extracted by gender.

In Somalia, obstetric hemorrhages; traumas caused by terrorist attacks and traffic accidents; surgeries; chronic kidney disease; critical nutritional deficiencies; parasitosis and anemic patients are the main conditions that require blood transfusion. There is no central public or private institution that supplies blood supply nationwide in Somalia yet. Health institutions supply blood for these patients from their relatives. This method may be insufficient to supply for rare blood groups such as AB and O Rh negative. Our findings may guide the establishment of health policies on blood supply and blood stock planning of hospitals in Somalia. By establishing a public or private institution and reporting the daily blood stocks of the hospitals to this centre, The situation of blood stock can be determined for Mogadishu, this stock information can be shared with the blood center managers of the hospitals. In this way, any hospital can supply blood from other hospitals in case of emergency. In addition, for blood groups that may be difficult to supply, this center can establish a database that containing the information of people have rare blood groups. People can be encouraged to donate blood voluntarily and awareness of the importance of donating blood can be created in the public. Later, this situation can accept blood from healthy donor and deliver the blood and blood productions to hospitals.

Materials and Methods

This research is based on the retrospective analysis of blood groups of 59,442 people who applied to the Blood Center of Mogadishu Somalia Turkey Recep Tayyip Erdogan Training and Research Hospital between November 2018 and November 2021. The Hospital Blood Center, which prepares whole blood and blood products, is one of Somalia's most essential and well-tested centers.

The blood groups were determined using the slide method and the gel approach based on Yves Lapierre's red blood cell agglutination response. In the slide method, Anti-A, Anti-B and Anti-D serums containing monoclonal antibodies were mixed with whole blood that contain Ethylenediaminetetraacetic acid on the slide, and those with agglutination were accepted as positive and those without which were considered negative.⁹ In the gel column technique; If there is agglutination after the application of anti-serum specific to groups, the Gel column retains the agglutinated red blood cells. If there is no agglutination, erythrocytes pass through the gel column. If erythrocytes were on the top of the gel, it was considered positive; if erythrocytes passed the gel and remained at the bottom, it was considered negative.¹⁰

Blood group data of 64,728 people were extracted from the laboratory module of the hospital data management system. The records of blood donors, all patients, and healthy people whose blood group typing is requested from hospital polyclinics, services, and intensive care units are accessible through this module. Raw data were converted to an excel file. Multiple blood group analyses of the same patient were evaluated and transformed into single data, so duplicate data were eliminated. Finally, the blood group data of 59,442 people were included in this study. The frequencies and proportions of each O, A, B, AB, Rh negative and Rh positive blood groups were calculated separately. Then, frequencies and proportions of O Rh+, O Rh-, A Rh+, A Rh-, B Rh+, B Rh-, AB Rh+ and AB Rh- were calculated. Additionally frequencies and percentages of each blood groups were calculated according to male and female sex. Excel and Spss 22 software were used to create descriptive and other statistics for all groups and subgroups. Chi-square correlation analysis was performed to show whether or not the difference between blood groups according to genders was statistically significant.

Results

After processing these data and eliminating duplicate records, the results of 59,442 participants were used in the study. 20,414 (34.35%) of the study's participants were female, while 39,023 (65.65%) were male. First, The frequencies and proportions of the O, A, B, and AB groups were calculated, respectively O Group:60.30%, A Group:26.50%, B Group:11.27%, AB Group:1.93%, (O>A>B>AB) (Table 1). The most prevalent blood group was group O, followed by group A, group B, group AB. The most prevalent blood group in men was group O 39.8%, followed by group A 17.39%, group B 7.56%, and group AB 1.32%. In women, the O group accounted for 20.92%; group A accounted for 9.11%, group B for 3.71%, and group AB for 0.61% (O>A>B>AB). Males outnumber females nearly two-fold. When

the percentages of blood groups are examined, the proportions of women's blood group are half of men's. This is owing to the small number of females in the population. Female and male subgroups have extremely similar intra-group blood group frequencies (Table 1).

Table 2 shows the frequency distributions of Rh groups. The percentage of people who are Rh positive is 96.49%, the rate of Rh negative is 3.51%. Women have the Rh positivity rate of 96.32%, which is similar to the general average. Women have the Rh negativity rate 3.68%. In men, the Rh positivity rate is 96.57%, whereas the Rh negativity rate is 3.43%.

Table 3 shows the distribution of the ABO group of combination with the Rh group. The O Rh positive blood group is the most common, accounting for 58.51% of men and 57.86% of women, 58.08% of total. The A Rh positive blood group is ranked second. Female, males and total frequencies are 25.67%, 25.72%, and 25.7%, respectively. B Rh positive blood group ranked third with 10.37%, 11.04%, and 10.81%. When the chi-square test with Spss 22 was applied to the numbers of male and female blood groups, the difference between male and female blood groups was statistically significant at the $p < 0.01$ statistical significance level ($P: 0.002$).

Discussion

Discovery of blood types and developments in transfusion medicine have made blood transfusion safe. Molecular Genetic studies reveal new blood groups, genetic differences or similarities between human populations are determined. We think that studies on blood group distribution are very necessary, especially in African countries. Because it is critical to design health policies and to direct them effectively in a geography where people's need for health services is too great and service provision is insufficient. Blood group prevalence studies on Somali are rare. Our study is the largest and only retrospective population study designed to find out blood group frequencies in Somalia. Mogadishu Somalia Turkey Recep Tayyip Erdogan Training and Research Hospital is one of the largest hospitals that accepts patients from all states and settlements of Somalia. This feature can give a rough idea that the results of the study may reflect blood group distribution across Somalia.

Table 1 ABO Blood Groups Frequencies and Proportions

Blood Groups	Women n: 20.419			Men n: 39.023			Total n: 59.442	
	Frequencies	Percentage (for Total) %	Percentage (for Women) %	Frequencies	Percentage (for Total) %	Percentage (for Men) %	Frequencies	Percentage %
O Group	12.433	20.92	60.89	23.410	39.38	59.99	35.843	60.30
A Group	5.416	9.11	26.52	10.336	17.39	26.49	15.752	26.50
B Group	2.207	3.71	10.81	4.491	7.56	11.51	6.698	11.27
AB Group	363	0.61	1.78	786	1.32	2.01	1.149	1.93
Total	20,419	34.35	100	39.023	65.65	100	59.442	100

Note: n: The number of people included in the study.

Table 2 Rh Blood Groups Frequencies and Proportions

Rhesus Groups	Women n: 20.419		Men n: 39.023		Total n: 59.442	
	Frequencies	Percentage %	Frequencies	Percentage %	Frequencies	Percentage %
Rh+	19.668	96.32	37.685	96.57	57.353	96.49
Rh-	751	3.68	1.338	3.43	2.089	3.51
Total	20.419	100.00	39.023	100.00	59.442	100.00

Note: n: The number of people included in the study.

Table 3 ABO Rh Blood Groups Frequencies and Proportions

Blood Groups	Women n: 20,419		Men n: 39,023		Total n: 59,442	
	Frequencies	Percentage %	Frequencies	Percentage %	Frequencies	Percentage %
O Rh+	11,948	58.51	22,578	57.86	34,526	58.08
O Rh–	485	2.38	832	2.13	1,317	2.22
A Rh+	5,241	25.67	10,037	25.72	15,278	25.70
A Rh–	175	0.86	299	0.77	474	0.80
B Rh+	2,118	10.37	4,309	11.04	6,427	10.81
B Rh–	89	0.44	182	0.47	271	0.46
AB Rh+	361	1.77	761	1.95	1,122	1.89
AB Rh–	2	0.01	25	0.06	27	0.05
Total	20,419	100	39,023	100	59,442	100
Pearson Chi-Square (χ^2)=22.876 p: 0.002*						

Notes: n: The number of people included in the study. *p<0.01 denotes statistical significance level. It was determined that the difference between blood groups according to genders was statistically significant. Chi-square correlation analysis was performed from Spss 22 package software.

The studies conducted by Sistonen⁸ in 1987 and Abshir¹¹ in 2020 are based on genetic researches in a smaller population. Since the genetic study conducted in the Ethiopian Somali region by Abibakar¹² in 2019 is ethnically similar to Somali population, it was used in comparison with our own study. In these 3 studies (Sistonen, Abshir, Abibakar) the frequencies were 58.28–60–50.10% for the O group, 26.81–22–26.90% for the A group, 13.06–14–15.20% for the B group, 1.85–4–5.1% for the AB group, –88–95.6% for the Rh positive group, –12–4.4% for the Rh negative group respectively. Our results are very similar in O, A, B, AB blood groups for the frequencies of blood groups conducted by Sistonen et al.⁸ Rh positive and Rh negative frequencies were not clearly specified in the Sistonen study. When the Sistonen study is compared to our study, the O group shows a 2% increase while the B group shows a 2% drop. There is little difference between group A and group AB. These findings may indicate that blood group ratios do not remain constant throughout time (approximately 35 years), and that some blood groups may increase while others decline. It can be possible with further study to disclose that these changes fluctuate at regular intervals. If there is fluctuation, for explaining the causes of these, medical and non-medical such as socio-economic, migration and climate studies in addition to genetics may be required.

There is a significant difference between the study of Abshir¹¹ and our study when the percentage rates of Rh groups are examined (Rh positive 88–96.49%, Rh negative 12–3.51%, respectively). No significant difference was observed between other blood groups (O group 60–60.3%, A group 22–26.5%, B group 14–11.27%, AB group 4–1.93%). The findings of Abibakar¹¹ in the Ethiopian Somali region are similar to the findings of our study, except for the O and AB groups, 50.1–60.3% for the O group and 5.1–1.93% for group AB. Other proportions are 26.9–26.5% for group A, 15.2–11.27% for group B, 95.6–96.49% for Rh positive, 4.4–3.51% for Rh negative respectively (Table 4).

When several studies from the Horn of Africa and other African countries in the literature are scanned, it is discovered that there is an order of O>A>B>AB among the percentages of the groups. (Table 5). The highest proportion belongs to group O, while the lowest belongs to group AB. When group B and AB are examined, our study is one of the studies with the lowest rate in African countries. In most studies, the percentages of Rh positive and Rh negative are relatively similar.^{12–18}

Conclusion

This study revealed that the groups with the highest percentages are O Rh+ (58,08%), A Rh+ (25.7%), and the groups with the lowest ratio are A Rh–(0.80%), B Rh–(0.46%), AB Rh+(1.89%), AB Rh–(0.05%). The findings of this study can guide

Table 4 The Blood Group Studies on the Somalis

Study	Blood Group (%)					
	0 Group	A Group	B Group	AB Group	Rh+	Rh–
Sistonen ⁸ (1026), (1987), (Mogadishu, Somalia)	58.28	26.81	13.06	1.85	?	?
Abshir A. Ali ¹¹ (95) (2020) (North Eastern Somalia)	60	22	14	4	88	12
Abibakar S. ismail ¹² (4614) (2019) (Ethiopia, Somali Region)	50.10	26.90	15.20	5.10	95.6	4.4
Our Study (59,442) (2021) (Mogadishu, Somalia)	60.30	26.50	11.27	1.93	96.49	3.51

Note: n: The number of people included in the study.

Table 5 Blood Group Proportions of Some Countries

Countries	Blood Groups (%)					
	0 Group	A Group	B Group	AB Group	Rh+	Rh–
Our Study, (59,442, (2022) (Mogadishu, Somalia)	60.30	26.50	11.27	1.93	96.49	3.51
Jahanpour, ¹³ (1815), (2017) (Tanzania)	52	26	19	3	98	2
Apecu, ¹⁴ (23,504), (2016) (Uganda)	50.3	24.6	20.7	4.5	98	2
Doku, ¹⁵ (42,317), (2018) (Ghana)	50	24.3	20.7	5	93.2	6.2
Hamed, ¹⁶ (10,116), (2011) (Mauritania)	49.10	28.28	18.56	4.05	94.23	5.77
Ndoula, ¹⁷ (14,546), (2014) (Cameroon)	48.62	25.07	21.86	4.45	96.32	3.68
Legese, ¹⁸ (40,053), (2021) (Ethiopia)	41.5	29.8	23.2	5.5	91.5	8.5

Note: n: The number of people included in the study.

the blood center administrators make decisions planning blood stocking and supply. Because O Rh+ and A Rh+ group are the most common blood group, they are the easiest to supply. A Rh–, B Rh–, AB Rh+, AB Rh– are rare blood groups. Although the need is low, there may be problematic in supply.

Since our study is conducted approximately after 35 years from the study by Sistonen et al it can be seen as an update on blood group distribution data. Despite slight differences from previous data, it is clear that certain groups increased (O group, +2%), others decreased (B group, –2%), while others remained relatively stable. It may be appropriate for researchers to update the blood group distribution of population by conducting new studies at regular intervals such as 30–50 years. Alternatively, blood group distribution of individuals can be processed digitally by institutional authorities and constantly updated blood group distribution information can be accessed.

Abbreviation

RH, rhesus factor.

Ethical Considerations and Consent for Publication

The ethics committee of the Mogadishu Somalia Turkey Recep Tayyip Erdogan Training and Research Hospital gave their clearance. The permit procedures and paperwork were completed, and the permit minutes were issued with the decision dated November 22, 2021, and the number MSTH/8131. The ethics committee does not request an informed consent form for retrospective studies. As an institutional policy, the hospital management considers the approval of the ethics committee to be sufficient for publication permission.

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Disclosure

The authors report no conflicts of interest in this work.

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Prevalence and Associated Factors for Post-Caesarean Delivery Blood Transfusion in Eastern Sudan: A Cross-Sectional Study

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Background: Obstetric haemorrhage is a leading cause of maternal mortality and morbidity worldwide. Caesarean delivery (CD) is associated with significant blood loss, which may require blood transfusions. This study aimed to determine the prevalence and associated factors for post-CD transfusion.

Methods: A cross-sectional study was conducted in Gadarif maternity hospital, eastern Sudan, from March to September 2020. Sociodemographic, obstetric and clinical data, including pre- and postoperative haemoglobin levels, were collected. A multivariate logistic regression analysis was performed.

Results: A total of 539 women were enrolled in the study; the median (interquartile range) age of these women was 28.0 (8.0) years. The overall post-CD transfusion rate was 8.2%. Emergency CD (adjusted odds ratio [AOR]=2.57, 95% confidence interval [CI]=1.25–5.28) and antepartum haemorrhage (AOR=44.70, 95% CI=11.18–178.76) were associated with increased risk of post-CD blood transfusion. Preoperative haemoglobin (AOR=0.48, 95% CI=0.36–0.64) and rural residence (AOR=0.45, 95% CI=0.22–0.93) were associated with reduced risk for post-CD blood transfusion.

Conclusion: The overall prevalence of post-CD transfusion in this part of Sudan is 8.2%. Women with emergency CD, low preoperative haemoglobin levels and antepartum haemorrhage were at higher risk for post-CD transfusion. Risk identification and correction of antenatal anaemia can reduce the hazard of blood transfusion among CD women.

Keywords: caesarean delivery, blood transfusion, haemoglobin, antepartum haemorrhage, Sudan

Introduction

Postpartum haemorrhage (PPH) is a major public health problem that can lead to maternal near miss, mortality and morbidity.¹ Caesarean delivery (CD) is the main risk for both intraoperative and postpartum haemorrhage.² Blood loss of more than 1000 mL after CD is considered as a postpartum haemorrhage.³ Consequently, blood transfusion following CD is not an uncommon practice in obstetrics.^{4–6}

There is a global increase in the rate of CD, particularly in the Sub-Saharan African region.⁷ The rate of post-CD blood transfusion varies from 0.36% in developed countries⁸ to 25.2% in developing countries.⁹

There are several preoperative, intraoperative and postoperative factors such as anaemia, previous uterine scar, multiparity,^{4,5,10} operation time, adhesions^{5,6,10} and uterine atony^{10,11} were associated with post-CD blood transfusion. Moreover, placenta previa and abruption, repeated scar, prolonged second stage CD and cephalopelvic disproportion were associated with post-CD blood transfusion.^{5,12,13}

Blood transfusion is a life-saving procedure; however, it carries the risk of infections, transfusion reactions (including anaphylactic shock) and death.¹⁴ These risks necessitates the use of blood transfusion only for genuine indications. A haemoglobin level of less than 8g/dl has been recommended as a cut-off for blood transfusion.¹⁵

In the Sub-Saharan African region, reservation of two units of blood for elective CD is commonly practiced.⁵ Early detection of the risk factors for post-CD blood transfusion alerts physicians to prepare blood for timed interventions.

In Sudan, there is an increase in CD rates.¹⁶ Haemorrhage following CD has been reported as the major cause of maternal near miss and mortality in Sudan.^{17,18}

We aim to assess the prevalence of blood transfusion and its associated factors among Sudanese women who have undergone CD in Gadarif maternity hospital, eastern Sudan. Data from this study are expected to reduce maternal mortality and morbidity related to post-CD blood transfusion in Sudan. This can be achieved through risk identification and proper intervention.

Methods

A cross-sectional study was conducted at Gadarif maternity hospital, eastern Sudan, during the period of March 1 to September 30, 2020.

Inclusion Criteria

All women who underwent emergency or elective CD during the study period were included.

Exclusion Criteria

Women who transfused intraoperatively and those with chronic haemolytic anaemia were excluded.

Sample Size

Sample size (539) was calculated based on the assumption that prevalence of post-caesarean blood transfusion would be 20.0% (ratio of 4:1). This assumption was based on a similar study in the region.⁵ Moreover, assumed that 40.0% of women who required blood transfusion had preoperative anaemia while 25.0% of women who did not require blood transfused had anaemia. This sample had a 95% confidence level and a 5% confidence limit. The sample size was calculated using the OpenEpi.¹⁹

Data Collection

Two medical officers gathered sociodemographic information (age, parity, education, occupation and residence). Maternal weight and height were recorded, and body mass index (BMI) was calculated preoperatively. Preoperative data also included haemoglobin level, gestational age at time of delivery, history of uterine surgery and pregnancy complications (gestational or chronic hypertension and diabetes). Obstetric conditions such as placental abnormality (placenta previa and abruptio placenta) and history of previous scar were documented. Intraoperative data, such as indication of CD, emergency or elective CD and duration of operation, were recorded from the operation notes. The women were followed until discharge from the hospital. Operation time was calculated from skin incision to closure in minutes. Blood group and the number of transfused units were obtained from the medical records of the participants.

Definitions and Measurements

The BMI was measured at time of delivery and recorded from preoperative notes. The BMI was categorized to <25 (reference) and >25.²⁰

The term other indications of CD includes macrosomia, multiple gestations, malpresentations, post-term pregnancy with failed induction of labors and others.

Haemoglobin was estimated by an automated haematology analyser according to the manufacturer's instructions (Sysmex KX-21, Japan).

Anaemia was defined as haemoglobin <11.0 g/dL. Indications for blood transfusion were haemoglobin <8.0 g/dL with symptoms and signs suggestive of severe anaemia (such as palpitations, dizziness, shortness of breathing, severe pallor,

tachycardia and hypotension)²¹ or active bleeding (more than 1000 mL). Women with haemoglobin level of <7.0 g/dL were transfused regardless of symptoms. Post-CD blood loss was estimated by visual inspection of active vaginal bleeding associated with hemodynamic instability of the pulse and blood pressure and weighting of the perineal pads. Moreover, digital weighing scale was used, with one gram is equal to one mL of blood volume.²²

The patient was considered transfused in our study protocol if she received at least one unit of packed red cell or more after CD. All women in the study received only packed red cell.

Statistics

Data were entered into a computer, and SPSS for Windows was used for data analysis. Continuous data were checked for normality using the Shapiro–Wilk test and were found to be not normally distributed. The median (interquartile range), frequency and percentages were used to present the characteristics of the participants. Univariate analyses were performed with blood transfusion (yes, no) as the dependent factor and sociodemographic data (age, education, occupation, residence, parity, blood group, BMI) and obstetric and medical conditions (diabetes, hypertension, placenta previa and abruption, history of previous scar, gestational age, emergency or elective surgery, duration of operation and haemoglobin) as independent variables. Variables in univariate analysis were compared with Mann–Whitney test, chi-squared test and Fisher exact test where applicable.

Variables with their $P < 0.2$ were shifted to construct the multivariate analysis with backward likelihood ratio methods. The unadjusted and adjusted odds ratios and 95% confidence interval (CI) were computed to show the strength of the association. A two-sided p -value of < 0.05 was considered statistically significant.

Results

Participant's Characteristics

A total of 539 women who underwent CD during the study period were recruited. Of these, 387 (71.8%) underwent elective CD and 306 (56.8%) were due to repeated scar. The medians (IQR) of age and parity were 28.0 (8.0) years and 3.0 (2.0), respectively. In total, 298 (55.3%) women resided in rural areas. Overall, 504 (93.5%) were housewives and 389 (72.2%) had a level of education lower than secondary. The median (IQR) of BMI, gestational age and duration of operation was 25.3 (4.3) kg/m², 38.0 (1.0) weeks and 45.0 (15.0) minutes, respectively. The patients who had medical diseases and antepartum haemorrhage were 56 (10.4%) and 15 (2.8%), respectively. The median (IQR) preoperative and postoperative haemoglobin levels were 11.2 (1.7) g/dL and 10.3 (1.6) g/dL, respectively (Table 1).

Caesarean Delivery Indications

The main indication for CD among the study population was repeated scar at 306 (56.8%), followed by other/combined at 120 (22.3%), cephalopelvic disproportion at 43 (8.0%), foetal distress at 28 (5.2%), pre-eclampsia/eclampsia at 19 (3.5%), second stage CD at 18 (3.3%) and placental abnormality at 5 (0.9%) (Table 2).

Prevalence of Post-CD Blood Transfusion

Of the 539 enrolled women, 44 (8.2%) were transfused (Table 1).

Univariate Analysis of the Study

The univariate analysis showed that women who received post-CD transfusion were older, had longer operation times, had emergency CD, reported antepartum haemorrhage as an indication of their CD and were from rural areas. The preoperative haemoglobin was significantly lower among the transfused group of women. There was no significant difference in the education, occupation, parity, blood group and BMI in the women who underwent post-CD transfusion and those who had no blood transfusion (Table 3).

Table I Sociodemographic and Clinical Characteristics of the Women (Number =539) with Cesarean Delivery in Eastern Sudan, 2020

Variable		
<i>Median (interquartile range)</i>		
Age, years		28.0(8.0)
Parity		3.0(2.0)
Body mass index, kg/m ²		25.3(4.3)
Gestational age, weeks		38.0(1.0)
Preoperative hemoglobin, g/dl		11.2(1.7)
Duration of operation, minutes		45.0(15.0)
Postoperative hemoglobin, g/dl		10.3(1.6)
<i>Frequency (proportion)</i>		
Residence	Rural	298(55.3)
	Urban	241(44.7)
Education level	< than secondary level	389 (72.2)
	≥ secondary level	150 (27.8)
Employment	Housewives	504 (93.5)
	Employee	35 (6.5)
Blood group	O	243 (45.1)
	None- O	296 (54.9)
Type of caesarean	Elective	387(71.8)
	Emergency	152(28.2)
Medical diseases	Yes	56 (10.4)
	No	483 (89.6)
Antepartum haemorrhage	Yes	15 (2.8)
	No	524 (97.2)
Blood transfusion	Yes	44 (8.2)
	No	495(91.8)
Repeated scar	Yes	306(56.8)
	No	233(43.2)

Multivariate Logistic Analysis

A multivariable logistic regression analysis revealed a significant association between emergency CD (AOR=2.57, 95% CI=1.25–5.28) and antepartum haemorrhage (AOR=44.70, 95% CI=11.18–178.76) with post-CD transfusion. Preoperative haemoglobin (AOR=0.48, 95% CI=0.36–0.64) and rural residence (AOR=0.45, 95% CI=0.22–0.93) were associated with a lower risk of postpartum blood transfusion. After adjustment, there was no significant association between maternal age and operation time with post-CD transfusion (Table 4).

Table 2 Indications for Cesarean Delivery in Eastern Sudan, 2020

Indications	Frequency	Proportion
Fetal distress	28	5.2
Cephalopelvic	43	8.0
Repeated cesarean	306	56.8
Placental abnormality	5	0.9
Preeclampsia/eclampsia	19	3.5
Second stage	18	3.3
Others/combined	120	22.3

Table 3 Univariate Analysis of the Factors Associated with Blood Transfusion in Women with Cesarean Delivery in Eastern Sudan, 2020

Variable		Women Who Received Blood Transfusion (44)	Women Who Did Not Receive Blood Transfusion (495)	OR (95.0% CI)	P
<i>Median (interquartile range)</i>					
Age, years		30.0(9.75)	28.0(9.0)	1.05(1.01–1.11)	0.048
Parity		3.0(2.0)	3.0(2.0)	1.11(0.93–1.32)	0.227
Body mass index, kg/m ²		24.4(3.8)	25.3(4.5)	0.99 (0.92–1.06)	0.906
Preoperative hemoglobin, g/dl		10.0(2.0)	11.2(1.5)	0.53(0.41–0.68)	< 0.001
Gestational age, weeks		38.0(1.0)	38.0(1.0)	0.86 (0.66–1.10)	0.238
Duration of operation, minutes		50.0(15.0)	45.0(15.0)	1.03(1.01–1.06)	0.009
<i>Frequency (proportion)</i>					
Type of caesarean	Elective	22(50.0)	365(73.7)	Reference	
	Emergency	22(50.0)	130(26.3)	2.80(1.50–5.24)	0.001
Medical diseases	Yes	4 (9.1)	52 (10.5)	0.85(0.29–2.47)	0.769
	No	40 (90.9)	443 (89.5)	Reference	
Antepartum haemorrhage	Yes	10 (22.7)	5 (1.0)	28.80(9.32–89.1)	<0.001
	No	34 (77.3)	490 (99.0)	Reference	
Repeated scar	Yes	21(47.7)	285(57.6)	0.67(0.36–1.24)	0.209
	No	23 (52.3)	210(42.4)	Reference	

Discussion

This cross-sectional study was conducted at a tertiary maternity hospital in Gadarif, Eastern Sudan, to determine the prevalence of post-CD blood transfusion and its associated factors. To our knowledge, this is the first study addressing the prevalence and risk factors for transfusion among Sudanese women who underwent CD.

In this study, the prevalence of post-CD transfusion was 8.2%. This is comparable with previous studies published in southwestern Nigeria (8.68%)²³ and Dhaka, Bangladesh (8.9%).²⁴ This rate of post-CD transfusion (8.2%) in our study is

Table 4 Multivariate Analysis of the Adjusted and Non- Adjusted Factors Associated with Blood Transfusion in Women with Cesarean Delivery in Eastern Sudan, 2020

Variable		Non-Adjusted		Adjusted	
		OR (95.0% CI)	P	OR (95.0% CI)	P
Age, years		1.03(0.97–1.09)	0.414	1.03(0.97–1.09)	0.414
Preoperative hemoglobin, g/dl		0.48(0.36–0.64)	<0.001	0.48(0.36–0.64)	<0.001
Duration of operation, minutes		1.03(1.01–1.07)	0.061	1.03(1.00–1.07)	0.061
Residence	Rural	0.46(0.22–0.94)	0.035	0.45(0.22–0.93)	0.030
	Urban	Reference		Reference	
Type of caesarean	Elective	Reference		Reference	
	Emergency	2.62(1.27–5.40)	0.009	2.57(1.25–5.28)	0.010
Antepartum haemorrhage	Yes	41.15(10.19–166.20)	<0.001	44.70(11.18–178.76)	<0.001
	No	Reference		Reference	

lower than the rate in Lagos, Nigeria (20.8%).⁵ Our result for blood transfusion rate (8.2%) was higher than those reported from developed countries, eg in Australia (0.36%)⁸ and in New York, USA (3.5%).²⁵

We have previously reported that 13.6% of the admitted children in the same settings (Gadarif, eastern Sudan) had blood transfusions.²⁶ A possible explanation for this variation is the presence of different types of hereditary anaemia during childhood. Sickle cell anaemia, for example, is a common indication for recurrent transfusion in paediatrics.

In the present study, emergency CD (AOR=2.57, 95% CI=1.25–5.28) was significantly associated with the requirement for blood transfusion. This finding is consistent with published data from South Western Nigeria²⁷ and Pakistan.²⁸ However, other studies did not show this association.^{8,29} Emergency CD carries the risk of intraoperative complications and haemorrhage with an increased need for blood transfusion.

The results from this study revealed that antepartum haemorrhage was associated with increased risk (AOR=44.70) for post-CD blood transfusion. Several other studies are in accordance with our results.^{6,30–32} This is probably due to the recurrent antepartum haemorrhage of placenta previa that can lead to preoperative anaemia. The interplay between antepartum haemorrhage and antenatal anaemia increased the risk for transfusion in our study. Furthermore, invasive placenta previa and disseminated intravascular coagulopathy of abruption have been associated with massive blood loss.^{13,30}

The findings of this study indicate that each increase in preoperative haemoglobin decreased the risk of blood transfusion by 52.0% (AOR=0.48). Preoperative anaemia as a risk factor for transfusion has been reported in many studies.^{5,12,29,30} Women who had preoperative anaemia were less tolerant of any amount of blood loss during surgery. A recent meta-analysis showed that the pooled prevalence of anaemia among pregnant women in Sudan was 53%.³³ In our study, antepartum haemorrhage was suggested as another factor for preoperative anaemia. This risk factor is modifiable; healthcare practitioners can correct antenatal anaemia preoperatively to reduce the risk of post-CD transfusion.

In the current study, rural residence reduced the risk for post-CD transfusion by 55.0% (AOR=0.45). Consistent with this observation, a study from Finland reported a higher rate of postpartum transfusion among those residing in urban areas.³⁴ A possible explanation is that the majority of those residing in rural areas were referred for elective CD at our tertiary hospital. Women who resided in rural areas underwent emergency CD at peripheral hospitals near their residence. This meant that most of the women from rural areas who were included in our study were of the elective type. According to this study and other studies,^{8,35,36} the risk of transfusion among elective surgery patients is less than among emergency CD patients. However, in contrast to our results, rural residence was associated with an increased risk for blood transfusion in previous studies.^{37,38} The patients in those studies had their emergency surgery in the same study settings unlike this study.

In the univariate analysis, maternal age was higher among transfused patients than among non-transfused patients. After adjustment of this confounder, no association was detected. Results recorded from previous published studies reported advanced maternal age as a risk factor for post-CD transfusion.^{5,20,35} Advanced maternal age is associated with postpartum haemorrhage due to weak uterine contractions.³⁹ Furthermore, advanced maternal age is a risk factor for placenta previa and abruption.³¹

The current study indicates that operation time was longer among transfused patients than non-transfused patients in the univariate analysis. This finding disappears after adjustment to other confounders. Studies from Nigeria⁵ and Pakistan⁶ observed longer operation times among transfused women who underwent CD. Prolonged operation time is associated with adhesion and intraoperative complications that increase the need for blood transfusion.^{6,40}

Strengths and Limitations

The strength of the study comes from the novelty of being the first study in Sudan that addressed the prevalence and associated factors of post-CD blood transfusion. The study has enough power and adequate sample size. However, women with vaginal delivery and women who received intraoperative blood transfusion were not included in the study. Moreover, the study was conducted in one hospital, unlike multicentric studies which could produce different results.

In conclusion, the prevalence of post-CD blood transfusion among Sudanese women in Gadarif, eastern Sudan, was 8.2%. The need for transfusion was significantly associated with preoperative anaemia, antepartum haemorrhage and emergency CD. Women who resided in rural areas were found to have fewer blood transfusion requirements. Blood preparation for those at risk is of paramount importance, and correction of preoperative anaemia can assist in the reduction of transfusion rate.

Abbreviations

PPH, postpartum haemorrhage; CD, caesarean delivery; BMI, body mass index; CI, confidence interval; IQR, inter-quartile range; AOR, adjusted odds ratios.

Ethics Approval and Informed Consent

This study complies with the Declaration of Helsinki. Ethics approval was obtained from the Ethics Committee of the Faculty of Medicine of Gadarif University, Sudan (Reference number #2020.07). Written informed consent was collected from each participant.

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Disclosure

The authors report no conflicts of interest in relation to this work.

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Comorbidities, Health-Related Quality of Life, Health-care Utilization in Older Persons with Hemophilia—Hematology Utilization Group Study Part VII (HUGS VII)

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Purpose: We compare the impact of hemophilia on comorbidities, joint problems, health-related quality of life (HRQoL) and health-care utilization between two age groups: 40–49 years and ≥ 50 years.

Patients and Methods: The HUGS VII study recruited persons with hemophilia A or B age ≥ 40 years. Participants completed surveys to collect data on sociodemographic and clinical characteristics, hemophilia treatment regimen, pain, joint problems, comorbidities, HRQoL, depression and anxiety, at baseline and 6-months later. Clinical chart reviews documented hemophilic severity and treatment.

Results: The sample includes 69 males, 65.2% aged ≥ 50 years, 75.4% with hemophilia A. Individuals ≥ 50 years were more likely to have mild or moderate hemophilia (68.9% vs 41.7%, $P = 0.03$) than those 40–49 years old. Among persons with mild/moderate hemophilia, those ≥ 50 years old reported a higher rate of joint pain (83.9% vs 70.0%, $P = 0.34$ at baseline, 91.3% vs 57.1%, $P = 0.06$ at follow up) or range of motion limitation (73.3% vs 60.0%, $P = 0.43$ at baseline, 73.9% vs 28.6%, $P = 0.04$ at follow up) than the younger group. Compared to the younger group, the older group reported fewer emergency room visits (4.5% vs 21.7%, $P = 0.03$), and physical therapy visits (15.9% vs 43.5%, $P = 0.01$) at baseline. The sample depression rate was 85.7%, but the differences among the age groups were not significant. The mean covariate-adjusted EQ-5D index score was lower in older persons (0.77 vs 0.89, $P = 0.02$).

Conclusion: Older persons with hemophilia in this sample are over-represented by individuals with mild/moderate disease, potentially due to premature death among those with severe disease. Although this group included a larger proportion of individuals with mild disease than the younger group, they experienced lower quality of life, more comorbidities both of aging and of hemophilic arthropathy, and lower rates of health-care utilization.

Keywords: hemophilia A, hemophilia B, burden of hemophilia, aging in hemophilia

Introduction

Hemophilia is a rare genetic bleeding disorder, which affects one in 5000 male births, or approximately 20,000 males in the U.S.¹ While there have been great advances in care and therapeutics over the past few decades, persons with hemophilia (PWH) still suffer from increased morbidity and mortality due to bleeding in joints, muscles, and other sites. Hemophilia treatment is extremely costly with the total annual cost of care per person estimated to be \$139,102 (SD

\$304,033) in 1995 US dollars.² The treatment of hemophilia is a lifetime commitment on the part of the providers, payers, affected persons and their families.

Major clinical manifestations of hemophilia involve bleeding episodes, most often in knee, ankle and elbow joints. In addition, individuals with hemophilia are at risk of bleeding into muscles and internal organs, including the central nervous system. Repeated bleeding and bleeding-related arthropathy are major causes of acute and chronic joint pain, hemophilic morbidity and reduced every day function.³ Inadequate management of bleeding events increases the risks of serious morbidities such as disability, as well as lower health-related quality of life (HRQoL). Clinical manifestations negatively impact not only the physical functioning of persons with hemophilia, but may also affect their every day functioning, mental health, and social health.⁴

With improved hemophilia care and prophylactic treatment, the life expectancy of PWH has increased, making age-related comorbidities more common. A study conducted by Khleif found that adults who were 40 years or older and were cared for by the Gulf States Hemophilia & Thrombophilia Treatment Center, had at least one comorbid condition other than hemophilia, and the majority had between 3 and 6 comorbidities.⁵ This study reported the most common conditions were chronic hepatitis C virus (HCV), hypertension, human immunodeficiency virus (HIV), chronic arthropathy, and overweight/obesity.⁵ HCV and HIV, both blood-borne viral infections, caused high mortality rate during the 1980s and 1990s.^{6,7} Another review on aging among PWH has identified the need to develop evidence to guide their treatment and their age-related comorbidities, as well as the need for additional prospective studies to guide proper delivery of care to this special population.⁸

Since 1995, the Hematology Utilization Group Studies (HUGS) have collected data from Hemophilia Treatment Centers (HTCs) to evaluate the costs of care and disease burden in PWH.^{2,9–11} However, these studies collected data from the general hemophilia population between 2 and 64 years old, with an inadequate sample of the elderly patient population. The HUGS VII study collected data focused on individuals aged 40 years and older (with the oldest being 79 years old). This article compares the impact of hemophilia on comorbidities, joint problems, HRQoL and health-care utilization between two age groups: 40–49 years and ≥ 50 years.

Materials and Methods

Design

HUGS VII is an observational cohort study that collected data from three US HTCs that provide care to PWH in three states (Colorado, Michigan, and Washington). Institutional review board approvals were obtained from the Data Coordinating Center at the University of Southern California and the three HTCs. The study complies with Declaration of Helsinki. Participants met the following inclusion criteria: 1) age 40 years or older; 2) factor VIII or factor IX activity level $\leq 30\%$; 3) male gender (since hemophilia is an X-linked disorder); 4) received the majority of their hemophilia care at the HTC to ensure the HTC can collect participant data through the study period; 5) provided care at the recruiting HTC in the year prior to enrollment in this study; 6) speaks English; and, 7) willing to provide written informed consent. Individuals judged by the clinician to be cognitively impaired or those with any additional blood disorder were excluded.

Recruitment and Procedures

Eligible participants were identified by the site study coordinator during clinic visits or through retrospective clinical chart review. After obtaining informed consent, the study coordinator provided participants with written instructions for taking surveys at baseline (enrollment) and six months later. The coordinator administered the baseline survey. Six months after enrollment, the study coordinator sent a reminder to participants to complete the follow-up survey. Participants were given the option of completing the follow-up data via a Qualtrics online survey, or through a mailed survey or by a telephone interview conducted by the study coordinator. Most participants chose to complete the online survey, although some used the mailed or telephone surveys when more convenient, or they had no internet access. Participants received a \$10 gift card for completing the initial survey and a \$20 gift card for completing the follow-up survey. Recruitment began in April 2018 and was closed in December 2019; data collection was completed in October 2020.

At each HTC, the study coordinator abstracted clinical information from each participant's medical chart for the period of six months prior to and after enrollment. Information collected included clinical characteristics of the participant, type of factor VIII or IX products prescribed (eg, recombinant derived, or plasma derived, bypassing agents, or other products), factor VIII or IX prescription information, inhibitor status and laboratory values, other anti-hemophilic medications, range of motion, outpatient doctor visits, hospitalizations, and emergency room visits. Clotting factor infusion records were acquired from pharmaceutical records supplied by the patient's home health company or pharmaceutical benefit manager, or from HTC 340B pharmacy records. Where participants did not complete the follow-up survey, clinical chart review data was still collected.

Measures

The initial survey collected data on personal characteristics including hemophilia treatment, health-care utilization, bleeding information, pain, joint problems, health status, work productivity and activity impairment, medical conditions and comorbidities, access to care, and physical activity. Sociodemographic data was also collected, including marital status, employment, education, ethnicity, race, health insurance status and household income. The six-month follow-up survey collected similar data, with the exception of medical conditions, sociodemographics, and health insurance status.

Self-Reported Comorbidities and Complications

Self-reported comorbidities questionnaire was adapted from the National Health Nutrition Examination Survey (NHANES). Comorbidities were measured by asking "Has a doctor ever told you that you have certain conditions?" The questionnaire asked about 19 medical conditions, including high blood pressure/hypertension, osteo/rheumatoid arthritis, osteoporosis, heart attack/myocardial infarction, procedures to treat coronary artery disease, congestive heart failure, skin cancer, cancer (other than skin cancer), diabetes/high blood sugar, emphysema, asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), stroke, hip fracture, Alzheimer's disease, Parkinson's disease, depression, anxiety, hepatitis C virus (HCV) infection, and HIV/AIDS status.

Self-Reported Joint Health and Pain

Self-reported joint pain and range of motion (ROM) limitation were obtained from two multiple choice questions.⁹ General and acute pain were measured by asking participants to specify the intensity of pain using a Visual Analogue Scale (VAS) ranging from 0 (no pain) to 10 (worst pain).¹²

Health-Related Quality of Life

HRQoL was measured by the EuroQoL EQ-5D-3L.¹³ The EQ-5D-3L consists of the EQ-5D descriptive system and the EQ visual analogue scale (EQ VAS). The EQ-5D-3L descriptive system comprises five dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has three levels: no problems, some problems, and extreme problems. The VAS records the respondent's self-rated health on a vertical numeric scale ranging from 0 (the worst possible health status) to 100 (the best possible health status). The US-based valuation algorithm was used to generate a time trade-off (TTO) index score, which is anchored at 0 for death and 1 for perfect health.¹⁴

Work Productivity and Activity Impairment

The hemophilia-specific version of the Work Productivity and Activity Impairment (WPAI) questionnaire was used to measure the impact of the hemophilia on the participants' productivity.¹⁵ The WPAI yields four types of scores based on the assessment in the previous 7 days: 1) absenteeism (work time missed), 2) presenteeism (impairment while working or reduced on-the-job effectiveness), 3) work productivity loss (overall work impairment or sum of absenteeism and presenteeism), 4) activity impairment. The scores are expressed in percentages.

Anxiety and Depression

Anxiety and depression were included as one of many self-reported comorbidities in the initial survey. The seven-item Generalized Anxiety Disorder (GAD-7) and the nine-question Patient Health Questionnaire (PHQ-9) were also included in the follow-up survey. GAD-7 was used to measure anxiety,¹⁶ and PHQ-9 was used to measure depression.¹⁷ If scores

on either of these measures reach 10 or greater, it is considered as diagnostic for the respective mental health condition.^{16,17}

Health-care Utilization

The patient surveys provided self-reported data on health-care utilization, including number of outpatient visits, physical therapy visits, medical procedures related to treating bleeding events, and emergency room visits, as well as overnight stays in a hospital. Participants were asked to consider the previous 30 days for the initial survey and the previous six months for the follow-up survey regarding health-care utilization. Health-care utilization data was also abstracted from participants' clinical charts to document the utilization for a period of six-month prior and post enrollment.

Statistical Analysis

Descriptive statistics, such as frequencies and proportions (for categorical variables) and means, standard deviations, medians, and ranges (for continuous variables), describe the sample in terms of participant demographic and clinical characteristics, HRQoL, WPAI scores, and health-care utilization. Comparisons of individuals' characteristics were made between two age groups, 40–49 vs ≥ 50 years old. To compare the variables between the two age groups, we defined the clinically meaningful difference as minimum 5% difference for the categorical variables, 0.5 points difference for scores that ranged from 0 to 10, or 5 points difference for scores that ranged from 10 to 100 for the continuous variables. Since all these cut points had at least 0.2 effect size, they fit Cohen's definition for small effect size. A small effect size of this magnitude may be considered as clinically relevant.¹⁸

Generalized linear regression models were used to assess mean covariate-adjusted scores for pain and EQ-5D scores by age group. These scores were obtained from general linear regression models using least squares means, which computed for each age group appropriately adjusted for the covariate effect in the general linear models. Covariates included education, employment, marital status, prophylactic treatment use, hemophilic severity, and self-reported joint problems.

Results

Participants' Characteristics

We recruited and collected baseline data from 70 males with hemophilia aged ≥ 40 years. One participant was lost to follow-up as he was no longer receiving care at the HTC during the six-month follow-up period. Fifty-seven participants (81%) completed the follow-up survey.

The analysis sample included 69 participants with a mean age of 56.8 years (standard deviation: 11.0; ranging from 40.0 to 79.0 years old), 65.2% were 50 years of age or older, and 75.4% were diagnosed with hemophilia A. The group of individuals ≥ 50 years of age had higher rates of: marriage or partnership (71.1% vs 54.2%, $P = 0.16$), high school or more education (100% vs 91.7%, $P = 0.049$), and were more likely to be retired or not employed (66.7% vs 25.0%, $P = 0.001$) than the group 40–49 years old. The older group had a significantly lower proportion of individuals with annual family income $> \$75,000$, compared to those 40–49 years old (33.3% vs 65.2%, $P = 0.02$). The older group also had a significantly higher proportion with public health insurance (47.7% vs 18.2%, $P = 0.0004$) (Table 1).

Self-Reported Clinical Characteristics

Persons ≥ 50 years were more likely to have mild or moderate hemophilia (68.9% vs 41.7%, $P = 0.03$) than persons in the 40–49 age group (Table 2). Use of prophylaxis among those with severe hemophilia did not differ by age group.

Self-Reported Comorbidities and Complications

The most frequently reported comorbidities were HCV infection (76.5%), hypertension (47.1%), depression (23.9%), anxiety (20.9%), and diabetes (17.6%). Of those with HCV infection, 78.9% had been treated, and 82.4% of those treated had cleared the virus (Table 2). When depression and anxiety were measured by the PHQ-9 and GAD-7, 85.7% of the sample met the clinical diagnosis threshold for depression and 35.7% for anxiety, but the differences among the age

Table 1 Participants' Social Demographic Characteristics and Health Insurance from Initial Survey

Variable	Total (N=69)	Age 40–49 Years (N=24)	Age ≥50 Years (N=45)	P Value ^a
Mean (Standard Deviation) age	56.8 (11.0)	44.8 (2.9)	63.3 (7.8)	<0.0001
Race				0.65
White	58 (85.3)	19 (82.6)	39 (86.7)	
Other	10 (14.7)	4 (17.4)	6 (13.3)	
Education				0.049
Less than high school	2 (2.9)	2 (8.3)	0 (0.0)	
High school or more	67 (97.1)	22 (91.7)	45 (100.0)	
Employment				0.001
Employed	33 (47.8)	18 (75.0)	15 (33.3)	
Retired/Not employed	36 (52.2)	6 (25.0)	30 (66.7)	
Marital status				0.16
Married/with a partner	45 (65.2)	13 (54.2)	32 (71.1)	
Single/not with a partner	24 (34.8)	11 (45.8)	13 (28.9)	
Hemophilia constrained choice of career				0.49
Yes	27 (42.2)	8 (36.4)	19 (45.2)	
No	37 (57.8)	14 (63.6)	23 (54.8)	
Income				0.02
\$75,000 or less	32 (54.2)	8 (34.8)	24 (66.7)	
More than \$75,000	27 (45.8)	15 (65.2)	12 (33.3)	
Health insurance covered				0.48
Entire 12 months	65 (98.5)	22 (100.0)	43 (97.7)	
Less than 12 months	1 (1.5)	0 (0.0)	1 (2.3)	
Health insurance type				0.0004
Public	25 (37.9)	4 (18.2)	21 (47.7)	
Private	32 (48.5)	18 (81.8)	14 (31.8)	
Private & Public	9 (13.6)	0 (0.0)	9 (20.5)	
Difficulty finding insurance				0.87
Yes	14 (21.5)	5 (22.7)	9 (20.9)	
No	51 (78.5)	17 (77.3)	34 (79.1)	
Difficult to get hemophilia care				0.53
Yes	4 (6.2)	2 (8.7)	2 (4.8)	
No	61 (93.8)	21 (91.3)	40 (95.2)	

Notes: Data were presented as number (column percentage) for categorical variables, or mean (standard deviation) for continuous variables. ^a P values were calculated from chi-square tests for categorical variables and t-tests for continuous variables.

groups were not statistically significant (Table 3). The older age group had higher rates of self-reported myocardial infarction (11.9% vs 0%, $P = 0.08$) and diabetes (25.0% vs 4.2%, $P = 0.03$) than those 40–49 years of age (Table 2).

Self-Reported Joint Health and Pain

Among participants with mild/moderate hemophilia, the older age group reported a higher rate of joint pain (83.9% vs 70.0%, $P = 0.34$ at baseline, 91.3% vs 57.1%, $P = 0.06$ at follow up) than the younger group. Similarly, a greater proportion of the older group reported having range of motion limitations (73.3% vs 60.0%, $P = 0.43$ at baseline, 73.9% vs 28.6%, $P = 0.04$ at follow up). Among the study sample, rates of self-reported joint pain (91.9% vs 80.0%, $P = 0.08$) or of joint range of motion limitation (83.8% vs 73.7%, $P = 0.69$) in the follow-up survey were higher among the older

Table 2 Self-Reported Clinical Characteristics from Initial Survey

Variable	Total (N=69)	Age 40–49 Years (N=24, 34.8%)	Age ≥50 Years (N=45, 65.2%)	P Value ^a
Hemophilia type				0.59
Hemophilia A	52 (75.4)	19 (79.2)	33 (73.3)	
Hemophilia B	17 (24.6)	5 (20.8)	12 (26.7)	
Hemophilia severity				0.03
Mild/Moderate	41 (59.4)	10 (41.7)	31 (68.9)	
Severe	28 (40.6)	14 (58.3)	14 (31.1)	
Used prophylactic treatment	31 (46.3)	14 (60.9)	17 (38.6)	0.08
Used prophylactic treatment in severe hemophilia	24 (85.7)	12 (85.7)	12 (85.7)	1.00
Self-reported joint pain				0.62
No pain	9 (13.2)	3 (12.5)	6 (13.6)	
Any pain	59 (86.8)	21 (87.5)	38 (86.4)	
When bleeding	5 (7.4)	3 (12.5)	2 (4.5)	
Some of time even if not bleeding	16 (23.5)	6 (25.0)	10 (22.7)	
Most of time even not bleeding	24 (35.3)	9 (37.5)	15 (34.1)	
Severe pain	14 (20.6)	3 (12.5)	11 (25.0)	
Joint range of motion limitation				0.18
No limitation	12 (17.9)	4 (16.7)	8 (18.6)	
Any limitation	55 (82.1)	20 (83.3)	35 (81.4)	
When bleeding	9 (13.4)	6 (25.0)	3 (7.0)	
When not bleeding	36 (53.7)	12 (50.0)	24 (55.8)	
Severe limitation	10 (14.9)	2 (8.3)	8 (18.6)	
Mean number of joint bleeds (Standard deviation)	0.4 (0.8)	0.3 (0.6)	0.4 (0.9)	0.76
Mean number of non-joint bleeds (Standard deviation)	0.4 (0.9)	0.4 (0.6)	0.5 (1.0)	0.54
Self-reported Comorbidities and complications^b				
Osteo/rheumatoid arthritis	32 (48.5)	9 (39.1)	23 (53.5)	0.27
Hypertension	32 (47.1)	7 (29.2)	25 (56.8)	0.03
Depression	16 (23.9)	4 (16.7)	12 (27.9)	0.30
Anxiety	14 (20.9)	5 (20.8)	9 (20.9)	0.99
Diabetes	12 (17.6)	1 (4.2)	11 (25.0)	0.03
Skin cancer	6 (9.1)	0 (0.0)	6 (14.3)	0.05
Myocardial infarction	5 (7.6)	0 (0.0)	5 (11.9)	0.08
Emphysema/asthma/chronic bronchitis/COPD	5 (7.6)	1 (4.2)	4 (9.5)	0.43
A procedure to treat CAD	4 (6.0)	0 (0.0)	4 (9.3)	0.12
Congestive heart failure	2 (3.0)	0 (0.0)	2 (4.8)	0.28
A hip fracture	2 (3.0)	0 (0.0)	2 (4.7)	0.28
Stroke	1 (1.5)	0 (0.0)	1 (2.3)	0.45
Alzheimer disease	1 (1.5)	0 (0.0)	1 (2.3)	0.45
Parkinson disease	1 (1.5)	0 (0.0)	1 (2.3)	0.45
Osteoporosis	1 (1.8)	0 (0.0)	1 (3.0)	0.40
Hepatitis C Infection	52 (76.5)	19 (79.2)	33 (75.0)	0.70
HIV/AIDS positive	9 (13.2)	4 (16.7)	5 (11.4)	0.54

Notes: Data were presented as number (column percentage) for categorical variables or mean (Standard deviation) for continuous variables. ^aP values were calculated from chi-square tests. ^bThere is no person reported cancer.

Abbreviations: COPD, chronic obstructive pulmonary disease; CAD, coronary artery disease; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome.

Table 3 Self-Reported Clinical Characteristics from Follow-Up Survey

Variable	Total (N=57)	Age 40–49 Years (N=20, 35.1%)	Age ≥50 Years (N=37, 64.9%)	P Value ^a
Self-reported joint pain				0.08
No pain	7 (12.3)	4 (20.0)	3 (8.1)	
Any pain	50 (87.7)	16 (80.0)	34 (91.9)	
Some of time even if not bleeding	19 (33.3)	9 (45.0)	10 (27.0)	
Most of time even not bleeding	21 (36.8)	3 (15.0)	18 (48.6)	
Severe pain	10 (17.5)	4 (20.0)	6 (16.2)	
Joint range of motion limitation				0.69
No limitation	11 (19.6)	5 (26.3)	6 (16.2)	
Any limitation	45 (80.4)	14 (73.7)	31 (83.8)	
When bleeding	2 (3.6)	1 (5.3)	1 (2.7)	
When not bleeding	34 (60.7)	11 (57.9)	23 (62.2)	
Severe limitation	9 (16.1)	2 (10.5)	7 (18.9)	
Number of joint bleeds	1.5 (2.3)	1.8 (2.7)	1.3 (2.0)	0.44
Number of non-joint bleeds	0.6 (1.3)	0.4 (0.7)	0.7 (1.5)	0.30
Depression measured by PHQ-9				0.85
Mild, 5–9	8 (14.3)	3 (15.0)	5 (13.9)	
Diagnostic depression, ≥10	58 (85.7)	17 (85.0)	31 (86.1)	
Moderate, 10–14	26 (46.4)	9 (45.0)	17 (47.2)	
Moderately severe, 15–19	17 (30.4)	7 (35.0)	10 (27.8)	
Severe depression, 20–27	5 (8.9)	1 (5.0)	4 (11.1)	
Anxiety measured by GAD7				0.81
Mild, 5–9	36 (64.3)	12 (60.0)	24 (66.7)	
Diagnostic anxiety, ≥10	20 (35.7)	8 (40.0)	12 (33.3)	
Moderate, 10–14	14 (25.0)	6 (30.0)	8 (22.2)	
Severe, 15–21	6 (10.7)	2 (10.0)	4 (11.1)	

Notes: Data were presented as number (column percentage). ^aP values were calculated from chi-square tests.

group than in the younger age group in the follow-up survey (Table 3). Mean number of joint bleeds and non-joint bleeds were not significantly different between age groups, either at initial survey or at follow-up (Tables 2 and 3). Mean daily pain level in general was greater in the older age group than that in the younger age group (3.8 vs 3.0, $P = 0.19$ at initial survey, 4.1 vs 3.2, $P = 0.23$ in follow-up survey, respectively) (Table 4). Mean pain rating related to acute bleeding was similar between age groups at initial survey (2.5 vs 2.3, $P = 0.73$), but not significantly different among the age groups (3.1 vs 2.2, $P = 0.30$) at follow up.

Health-Related Quality of Life

Table 4 shows the mean EQ-5D scores for the overall sample and stratified by age group. The mean covariate-adjusted EQ-5D VAS and index scores were lower among older persons (68.79 vs 73.74, $P = 0.34$ for VAS, 0.77 vs 0.89, $P = 0.02$ for EQ-5D index score) at the initial survey (Table 4). At follow up, the mean covariate-adjusted EQ-5D VAS was significantly lower in older persons than in younger individuals (68.62 vs 82.46, $P = 0.03$) and mean covariate-adjusted EQ-5D index score was lower in older persons (0.78 vs 0.89, $P = 0.07$) (Table 4).

Work Productivity and Activity Impairment

The results of WPAI were reported for 33 participants who were employed at the time of enrollment. Although none of the results were significant, those in the older age group had lower absenteeism scores (0% vs 11.1%, $P = 0.16$), or

Table 4 Pain and Health-Related Quality of Life Measurement

Variable	Total (N=69)	Age 40–49 Years (N=24, 34.8%)	Age ≥50 Years (N=45, 65.2%)	P Value ^a
Measure from initial survey				
Satisfied bleeding treatment	8.8 (2.0)	8.7 (2.2)	8.9 (1.9)	0.71
Covariate adjusted satisfied bleeding treatment	NA	9.2 (0.8)	9.2 (0.9)	0.99
Daily pain level in general	3.5 (2.4)	3.0 (2.2)	3.8 (2.6)	0.19
Covariate adjusted daily pain level in general	NA	1.4 (0.9)	2.7 (1.1)	0.10
Pain related to acute bleeding rating	2.4 (3.2)	2.3 (3.3)	2.5 (3.1)	0.73
Covariate adjusted pain related to acute bleeding rating	NA	1.4 (1.3)	2.1 (1.5)	0.50
EQ VAS	70.54 (19.75)	73.74 (17.80)	68.79 (20.73)	0.34
Covariate adjusted EQ VAS ^b	NA	81.63 (7.66)	73.67 (8.74)	0.21
EQ-5D index score	0.74 (0.18)	0.78 (0.16)	0.72 (0.19)	0.13
Covariate adjusted EQ-5D index score ^b	NA	0.89 (0.06)	0.77 (0.07)	0.02
Measure from follow up survey				
Satisfied bleeding treatment	8.4 (2.2)	8.7 (2.0)	8.2 (2.3)	0.37
Covariate adjusted satisfied bleeding treatment	NA	9.3 (0.96)	8.6 (1.0)	0.39
Daily pain level in general	3.8 (2.6)	3.2 (2.5)	4.1 (2.6)	0.23
Covariate adjusted daily pain level in general	NA	1.8 (1.0)	2.9 (1.1)	0.19
Pain related to acute bleeding rating	2.8 (3.1)	2.2 (3.2)	3.1 (3.1)	0.30
Covariate adjusted pain related to acute bleeding rating	NA	1.4 (1.3)	2.1 (1.5)	0.50
EQ VAS	68.55 (19.78)	76.11 (15.35)	64.68 (20.84)	0.04
Covariate adjusted EQ VAS ^b	NA	82.46 (7.13)	68.62 (7.81)	0.03
EQ-5D index score	0.75 (0.20)	0.79 (0.18)	0.73 (0.21)	0.22
Covariate adjusted EQ-5D index score ^b	NA	0.89 (0.07)	0.78 (0.08)	0.07

Notes: Data were presented as mean (standard deviation), or covariate adjusted mean (standard error). Covariate adjusted mean and standard error were obtained from general linear regression models least squares means, which computed for each age group appropriately adjusted for the covariate effect in the general linear models. ^aP values were calculated from t-tests or general linear regression models type III model test. ^bCovariates include education, employment, marital status, used prophylactic treatment, hemophilia severity, self-reported joint problems.

Abbreviations: N, number; EQ VAS, EuroQol visual analogue scale; NA, not apply.

activity impairment (16.7% vs 22.2%, $P = 0.52$), but had a higher presenteeism score or work productivity loss (both were 12.1% vs 6.3%, $P = 0.32$). None of the 25 persons employed at the time of the follow-up survey had missed work. Those ≥50 years old reported higher presenteeism score or work productivity loss (both were 10.0% vs 3.8% $P = 0.32$), and activity impairment (14% vs 7.7%, $P = 0.35$) from the follow-up survey.

Health-care Utilization

As compared to those 40–49 years old, fewer proportion of participants ≥50 years old reported emergency room visits (4.5% vs 21.7%, $P = 0.03$), or physical therapy visits (15.9% vs 43.5%, $P = 0.01$) at baseline, but a higher proportion of individuals in this group reported doctor visits (69.4% vs 50.0%, $P = 0.15$) and emergency room visits (27.8% vs 15.0%,

$P = 0.28$) at follow up (Table 5). Among those who visited the emergency room, older persons had a greater mean number of visits (1.4 vs 1.0, $P < 0.0001$) based on 12 months of medical chart reviews (Table 6).

Discussion

This observational study compared a sample of US men with hemophilia of all severities in their 40's (beginning middle age) to counterparts 50 years of age or older (middle and older age adulthood) who obtained care at three HTC's. This study's aim was to describe their health status, in terms of hemophilia and aging-related comorbidities, and to examine the impact of hemophilia on joint health, quality of life, and health-care services utilization.

Table 5 Self-Reported Health-care Utilization

Variable	Initial Survey ^a				Follow Up Survey ^a			
	Total (N=69)	Age		P Value [†]	Total (N=57)	Age		P Value ^b
		40–49 Years (N=24, 34.8%)	≥50 Years (N=45, 65.2%)			40–49 Years (N=20, 35.1%)	≥50 Years (N=37, 64.9%)	
HTC visits	29 (43.9)	10 (43.5)	19 (44.2)	0.96	34 (59.6)	16 (80.0)	18 (48.6)	0.02
Doctor visits	20 (30.3)	8 (34.8)	12 (27.9)	0.56	35 (62.5)	10 (50.0)	25 (69.4)	0.15
Procedure done	13 (19.4)	3 (13.0)	10 (22.7)	0.34	13 (23.2)	3 (15.0)	10 (27.8)	0.28
Hospital stays	7 (10.6)	4 (17.4)	3 (7.0)	0.19	8 (14.3)	2 (10.0)	6 (16.7)	0.49
Emergency room visits	7 (10.4)	5 (21.7)	2 (4.5)	0.03	13 (23.2)	3 (15.0)	10 (27.8)	0.28
Physical therapy	17 (25.4)	10 (43.5)	7 (15.9)	0.01	15 (26.8)	8 (40.0)	7 (19.4)	0.1
Any other visits to a medical provider	50 (73.5)	18 (75.0)	32 (72.7)	0.84	27 (48.2)	9 (47.4)	18 (48.6)	0.93

Notes: Data were presented as number (column percentage). ^aInitial survey asked healthcare utilization during past month. Follow up survey asked healthcare utilization during past 6-months. ^bP values were calculated from chi-square tests.

Abbreviation: HTC, hemophilia treatment center.

Table 6 Mean of Health-care Utilization from Clinical Chart Review for 12 Months Period

Variable	Total (N=69)	Age 40–49 Years (N=24, 34.8%)	Age ≥50 Years (N=45, 65.2%)	P Value ^a
Number months with severe medical problems	0.1 (0.4)	0.0 (0.2)	0.1 (0.5)	0.56
Number months with a new complication diagnosis	0.5 (0.9)	0.5 (0.7)	0.6 (1.0)	0.68
Number months used pain medicine	4.8 (5.6)	4.8 (5.7)	4.8 (5.6)	0.97
Number of doctor visits	4.8 (8.0)	7.3 (11.3)	3.5 (5.1)	0.13
Number of ER >0 ^b	1.3 (0.5)	1.0 (NA)	1.4 (0.5)	<0.0001
Number of ER	0.1 (0.4)	0.0 (0.2)	0.2 (0.5)	0.17
Number of hospitalizations >0 ^b	1.3 (0.9)	1.2 (0.4)	1.4 (1.1)	0.68
Number of hospitalizations	0.2 (0.6)	0.3 (0.5)	0.2 (0.7)	0.86
Number of procedures >0 ^b	1.0 (0.0)	1.0 (NA)	1.0 (0.0)	<0.0001
Number of procedures	0.1 (0.3)	0.0 (0.2)	0.1 (0.3)	0.48

Notes: Data were presented as mean (standard deviation). ^aP values were calculated from t-tests. ^bOnly for the participants who had the specified events.

Abbreviations: N, number; ER, emergency room; NA, not apply.

The study sample included more participants in the older age group, and they were more likely to have a diagnosis of mild/moderate rather than severe hemophilia. The predominance of non-severe hemophilia among the older group may be due to premature death among those with severe disease. Especially since the older cohort was born about two decades before both viral inactivation manufacturing processes eliminated both HIV and HCV from hemophilia medications in the US, and before the regional networks of HTC's nationwide were established, which enhanced widespread access to expert team-based care.¹⁹ This is also noted in an analysis of life expectancy and cause of death in individuals with hemophilia A and B in Norway from 1986-2018, which showed that the persons with severe hemophilia died at a mean age of 53.4 ± 21.7 compared to 71.5 ± 19.6 for mild and moderate hemophilia.²⁰

Not surprisingly, the older age group included a higher proportion of people retired or not employed, and these individuals were more likely to use public health insurance. There was no statistically significant difference among age groups regarding difficulty obtaining health insurance or hemophilia care. Both age groups with severe hemophilia had similar proportions using clotting factor treatment prophylactically.

Although the older age group included a lower proportion of individuals with severe hemophilia, the proportion with self-reported joint pain and joint range of motion limitations were similar among the two age groups at the initial survey. However, a higher proportion of the older age group reported severe joint pain at baseline (25.0% vs 12.5%) and at the follow-up survey (20.0% vs 16.2%). This may reflect participants' more severe joint pain when aging, even if they had less severe hemophilia. The mean number of self-reported joint bleeds and non-joint bleeds at the initial survey were not significantly different among the two age groups. At follow-up, we observed that the mean number of joint bleeds was lower in the older age group (1.3 vs 1.8), although it was not statistically significant. This result may indicate that patients with severe disease experienced more bleeding episodes. These results parallel the findings of Mazepa et al,²¹ although the HUGS VII sample extended to moderately severe populations.

The aging hemophilia population, like the general US aging population, faces age-related cardiovascular disease and malignancy. The most frequently reported comorbidity was osteo/rheumatoid arthritis (48.5%). The older age group in our study had a frequency of 53.5% for osteo/rheumatoid arthritis, which is lower than the rate reported from the literature.²² Hemophilia has been associated with low bone mineral density, with 66.6% of persons with moderate or severe hemophilia ≥ 50 years having osteoporosis.²² A higher proportion of the older age group in HUGS VII reported age-related comorbidities, including hypertension, depression, diabetes, skin cancer, and myocardial infarction than the younger group.

Although self-reported rates of depression and anxiety at baseline were 23.9% and 20.1%, the results of the self-reported depression scale (PHQ-9) administered at the six-month follow-up survey deserve attention. While there was no statistically significant difference among the age groups, 85.7% of the overall sample ($N = 57$) had a score ≥ 10 , indicating a classification of moderate/severe depression and 35.7% of the overall sample had a score of ≥ 10 on the anxiety scale.¹⁷ The baseline survey obtained depression and anxiety measures through self-report based on whether a physician had ever mentioned that the respondent had the condition. The higher rates of possible diagnosed depression and anxiety in the follow-up may be due to persons who had mental health symptoms but had not sought care. The depression rate in our sample was much higher than that among adult men in the general US population in 2019, who reported moderate/severe symptoms of depression (score PHQ-8 ≥ 10) at a rate of 5.5%.²³ The same was true for anxiety: males in the general US population reported moderate/severe symptoms of anxiety (score GAD-7 ≥ 10) at a rate of 4.3%.²⁴

The mean daily pain level in general, and pain related to acute bleeding were both higher in the older age group than the younger group, although they were not statistically significant at either time point. However, the magnitude of differences among age groups may be clinically meaningful, as a 0.9 difference was demonstrated in the follow-up survey. The older age group reported higher pain scores and lower HRQoL. Both the mean EQ VAS and index scores at the time of the follow-up survey were similar to the scores obtained at baseline. The mean EQ VAS scores (70.54, and 68.55 at initial and follow-up, respectively) were lower than the US population norm of 80.0. The mean EQ index scores (0.74, and 0.75 for initial and follow-up, respectively) were also lower than the US population norm. Even when stratified by age group, both EQ VAS and index scores were lower than US age-specific norm scores. Reduced HRQoL scores in older persons may be related to age, but may also be associated with increased pain.^{25,26} Moreover, older PWH often

experience anxiety due to chronic pain, impaired quality of life, and reduced ability to maintain an independent lifestyle.^{27,28}

Our results demonstrated that the older age group had more physician visits and fewer emergency department visits at baseline, providing some evidence that regular care may result in lower acute care use, but this was not supported by the six-month follow-up use. However, when the analysis excluded those participants lost to follow-up, the lower emergency department utilization persisted six months later. The changes between baseline and six months later may relate to one or more bleeding events or poorer outcomes due to other comorbidities, and further analyses could be helpful.

Limitations

The study sample included 69 total participants. This small sample size limits statistical power to detect between-group differences for many study variables. As hemophilia is a rare congenital condition, it is challenging to recruit a large study sample. Focusing on clinically meaningful differences rather than statistically significant differences may be helpful for interpreting this observational sample. As the study sample includes more individuals with mild/moderate hemophilia in the older age group, it is important to adjust for severity in these analyses. Further caution should be taken related to individuals in the sample who did not complete the six-month follow-up survey.

Conclusion

The 50 years or older age group was over-represented by individuals with mild/moderate disease, likely due to premature death among those with severe disease. Although those ≥ 50 years included a larger proportion of individuals with mild hemophilia than the 40–49 year group, they experienced statistically and clinically significant lower HRQoL scores, more comorbidities related to aging and hemophilic arthropathy, and lower health-care utilization. These results, coupled with the important finding of a high rate of depression and anxiety in both age groups, highlight the need for special attention be paid to PWH. The increasing survival rates among PWH reveal a growing array of physical, social, and mental health vulnerabilities requiring the attention of clinicians, caregivers, advocates and health-care policymakers.

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Immuno-Haematological Abnormalities of HIV-Infected Patients Before and After Initiation of Highly Active Antiretroviral Therapy in the Antiretroviral Therapy Clinics of Six Health Facilities at Dessie Town, Northeast Ethiopia

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Background: In people living with the human immunodeficiency virus, haematological abnormalities have been linked to an increased risk of disease progression and mortality. Hematological parameters may have a positive or negative impact on antiretroviral therapy. The aim of this study was to assess the immuno-haematological abnormalities of HIV-infected patients before and after the initiation of highly active antiretroviral therapy in the antiretroviral therapy clinics of six health facilities in Dessie, Northeast Ethiopia.

Methods: A facility-based cross-sectional study was conducted from April to May 30, 2021, at the antiretroviral therapy clinics of six health facilities in Dessie Town. A total of 378 HIV-infected patients taking highly active antiretroviral treatment for at least 6 months by using a consecutive sampling technique were included. A well-organized questionnaire was used to collect socio-demographic and clinical information. Immune-haematological parameters were tested using a Mindray BS-300 hematology analyzer and a BD FACS count CD4 analyzer. Statistical analysis was performed using SPSS version 25 statistical software. Statistical significance was defined as a P-value of 0.05 with a 95% confidence interval.

Results: Leukopenia was found in 26.7% and 16.5%, neutropenia in 16.5% and 9.4%, lymphopenia in 20% and 3.1%, and thrombocytopenia in 25.9% and 7.1% of HIV patients before and after HAART initiation, respectively. There was a significant difference in total white blood cell, absolute neutrophil, red blood cell, hemoglobin value, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, red cell distribution width, platelet and CD4+ T cell counts in HIV patients before and after the initiation of HAART with $P < 0.05$.

Conclusion and Recommendation: Anemia, leukopenia, neutropenia, lymphopenia, and thrombocytopenia were the most common haematological abnormalities found in this study before and after HAART initiation. The prevalence of thrombocytopenia, immunosuppression, and viral load was reduced considerably after starting HAART.

Keywords: HIV, anemia, leukopenia, thrombocytopenia, HAART

Introduction

Acquired Immunodeficiency Syndrome (AIDS) is an immunological disease caused by the Human Immunodeficiency Virus (HIV), which infects cells and causes malfunctions of the immune system. It results in immune-hematological abnormalities, secondary and/or opportunistic infections, and tumors.¹ In addition, extreme weakness and progressive harm of cellular and humoral immune responses are one of the characteristics of HIV.² HIV was a global public health

issue that claimed the lives of 770,000 people living with HIV (PLWHIV) at the end of 2018. From the HIV-infected personnel, more than two-thirds of the 37.9 million people were lived in Africa.³ In Ethiopia 2017 census indicated that a higher prevalence rate of HIV was found in Amhara, Oromia, and Addis Ababa, which accounted for 16.2% (613,000/37,900,000).⁴

Hematological abnormalities and/or cytopenia are the most common HIV-AIDS consequences and the leading predictors of HIV infection in PLWHIV, which has been linked to an elevated risk of illness progression and death.^{2,5} In PLWHIV, cytopenia can be caused by the adverse effect of HIV infection targeting lymphocytes, monocytes, and macrophages, resulting in irregularities in cytokine production, which in turn affects hematopoiesis.⁶ Thus, anemia, leucopenia, neutropenia, lymphopenia, and thrombocytopenia are the common complications associated with AIDS epidemics.^{7,8}

Lower CD4 counts, immunological failure, viral load increment, advanced WHO clinical stages, HBV coinfection and side effects of highly antiretroviral therapy are all major risk factors for cytopenia in HIV patients.⁹ Therefore, management of hematological abnormalities in HIV patients and early initiation of ART is important to reduce the burden of immune-hematological problems. HIV not only affects the immune system and immunological dysfunction, but it also damages the hematopoietic system and causes cytopenia in infected people.¹⁰

Different studies indicated that 4–40% of Thrombocytopenia has been found to be associated with all stages of the HIV/AIDS disease^{11,12} and has been associated with an increased morbidity and mortality of HIV associated with risks of bleeding in various tissues.^{13–15} Platelet dysfunction includes immune-mediated platelet destruction and impaired hematopoiesis.^{13,15–17} Platelet destruction by antibodies and HIV direct infection of megakaryocytes has resulted in decreased platelet output from these progenitor cells.

Despite the fact that these hematological abnormalities have a significant impact on patient well-being, treatment and care, particularly in developing countries where access to health facilities is limited and people may only seek treatment after the disease has progressed to a serious stage and is accompanied by multiple immune-hematological abnormalities.²¹ For better treatment of HIV/AIDS patients, assessing the extent and severity of immune-hematological abnormalities is therefore very critical. The purpose of this study was therefore to determine the immune-hematological abnormalities in HIV-infected individuals presenting at selected health facilities of Dessie town, Northeastern Ethiopia, before and after commencing HAART.

Materials and Methods

Study Area

This study was conducted in the ART clinics of six health facilities at Dessie town, South Wollo zone of Amhara Regional state, Northeast Ethiopia. The town is situated in the mountainous ridge and has relatively cold weather. It has a total area of 15.08km² (5.82 sq. Mi) and 401 km far from the capital city of the county, Addis Ababa, and 471 km far from Bahir Dar, the capital city of Amhara regional state. In Dessie town, there is one comprehensive specialized hospital, one general hospital, eight health centres, three private general hospitals and five higher private clinics, which provide ART services (offer HIV/AIDS prevention, treatment, care and support to the most vulnerable population). There are around 7350 HIV patients at Dessie Comprehensive Specialized Hospital, 250 at Selam General Hospital, 900 at Borumeda General Hospital, 650 at Dessie Health Centre and 500 at Ethio and Bati General Hospitals.

Study Design and Period

Health facility-based cross-sectional study was conducted from April 1 to May 30, 2021.

Population

Source Population

All HIV positive patients who visited ART clinics of the selected health facilities were the source population.

Study Population

HIV-positive patients who visited the ART clinics during the study period and those who fulfilled the eligibility criteria were included in the study.

Inclusion and Exclusion Criteria

HIV patients having complete haematological value at the baseline (at the time of ART initiation), those who were on ART for at least six months, and those who were volunteered to participate in the study were included. Those who were severely ill due to other medical conditions, those who were on medication, those who had known cancer or haematological abnormalities, those taking vitamins and iron supplementation at the time of sampling and pregnant women were excluded from the study.

Sample Size and Sampling Technique

Based on the following assumptions, the sample size was estimated using a single population proportional formula: In the previous study, the prevalence of leukopenia was 35.9%,¹¹ marginal error was 5%, level of confidence was 95%, and the source population was 10,000 people, so the correction formula was used. Using the preceding assumptions, a minimum sample size of 304 was computed, with a 10% non-response rate, resulting in a final sample size of 334 but to increase the accuracy we included 378 participants in the study.

Data Collection and Laboratory Methods

With the aid of a structured questionnaire, relevant socio-demographic information was gathered from each participant. A skilled laboratory technician collected about 4 mL of venous blood from the forearm antecubital vein using vacuum collection in a tube containing a salt ethylene diamine tetra acetate (EDTA) anticoagulant were stored at 25–30°C for 30 minutes and analyzed within two hours of collection time. The samples stored for more than 48 hours were not processed. The Mindray 3000 plus hematology analyzer was used for complete blood count analysis,¹⁸ and the BD facscount CD4 analyzer (Becton Dickinson and Company, California, USA) was used for CD4+ T cell count.¹⁹ The result was collected within six hours and filled in the data collection format.

Operational Definitions

The WHO hematologic toxicity grading system was used to identify anemia, neutropenia, and thrombocytopenia. Anemia was defined as a hemoglobin (Hgb) concentration of less than 10.5 g/dL in all children, and severity was graded as follows:²⁰ Thrombocytopenia is described as a platelet count of less than 150,000 per milliliter of blood. Platelet count $>450,000/\text{mm}^3$ is considered thrombocytosis.²¹ White blood cell (WBC) count of less than $4000/\text{mm}^3$ is considered leukopenia. WBC count $>12,000/\text{mm}^3$ is considered leukocytosis. Lymphopenia is defined as a lymphocyte count of less than $1500/\text{mm}^3$. Neutropenia is defined as an absolute neutrophil count (ANC) of less than $1500/\text{mm}^3$ with severity levels ranging from mild to severe.²²

Data Entry and Analysis

The data was gathered utilizing a well-organized and pre-tested structured questionnaire, as well as laboratory results obtained from the hematology department and the patient's registration card. SPSS version 25 was used to enter and evaluate the data. To display descriptive statistics, tables and graphs were calculated and used. Skewness and Kurtosis normality tests were used to determine the kind of distribution. Results were compared between categorical variables using parametric tests and ANOVA for continuous variables based on the distribution of the data. A paired *t*-test was employed to compare the means of each hematological parameter before and after 6 months of HAART initiation. A P-value of less than 0.05 is deemed statistically significant.

Quality Assurance and Quality Control

Starting from the data collection period to the sample processing, the lead investigator reviewed the accuracy and completeness of the gathered data every day during the post-analytical phase. All specimens were collected according to standard operating procedure, the specimen quality was confirmed, and all laboratory procedures were performed according to the manufacturer's instructions. Quality control was carried out at the pre-analytical and analytical stages, which included the appropriate use of hematological investigations, as well as the collection, storage, and transportation

of blood specimens. Furthermore, patient samples were evaluated alongside analytical stage quality control checks, which included quality control materials (Normal, Low, and High) to detect if analytic errors had occurred.

Ethical Approval

The study protocol was evaluated and approved by the Research Ethics Review Committee (RERC 0247/2021) of Wollo University, College of Health Sciences, and ethical clearance was obtained. Official letters of cooperation were submitted to the respective health facilities, and permission was obtained. Moreover, prior to commencing the study, a written informed consent was obtained from each study participant. Study participants aged less than 18 years were asked an assent, and written consent was taken from their parents or legal guardians. Throughout the study, confidentiality and any unique data security needs were maintained and ensured. Moreover, this study was conducted in accordance with the Declaration of Helsinki.

Result

General Characteristics of the Study Participants

In this study, a total of 378 HIV-positive people were included. From the total participants, females accounted for about half of the group 191 (50.5%). The age range of study participants was from 10 to 70 years, with a mean age ($\pm$ SD) 40.09 ($\pm$ 12.24), while the median (IQR) age was 43 (35–54) years. The majority 174 (46%) of the study participants were between the 31–41 age group. Most of the study participants were urban dwellers with frequency of 289 (76.5%). About 318 (84.1%) of the participants were married. About 168 (44.4%) of the study participants were under WHO clinical stage III and half of 190 (50.5%) the study participants had <350 CD4 Cells/mm³ count (Table 1).

Immuno-Haematological Values Before and After Initiation of HAART

The mean value of total white blood cell counts before starting of HAART (baseline) and after at 6 months of receiving HAART was $5.8 \pm 2.7 \times 10^3/\mu\text{L}$, $4.7 \pm 1.9 \times 10^3/\mu\text{L}$, respectively. Similarly, the amount of Neutrophil was $2.55 \pm 1.56 \times 10^3$ and $2.45 \pm 1.53 \times 10^3/\mu\text{L}$ and lymphocyte were $2.12 \pm 1.1 \times 10^3/\mu\text{L}$ and $2.56 \pm 1.3 \times 10^3/\mu\text{L}$, respectively. The mean value of WBC, ANC and TLC before initiation of HAART were 5.8 ($4.09 - 7.2$) $\times 10^3/\mu\text{L}$, 2.55 ($1.5 - 3.4$) $\times 10^3/\mu\text{L}$ and 2.12 ($1.6 - 2.99$) $\times 10^3/\mu\text{L}$ respectively and after at 6 months of HAART initiation 4.7 ($3.5 - 6.69$) $\times 10^3/\mu\text{L}$, 2.45 ($1.4 - 2.9$) $\times 10^3/\mu\text{L}$ and 2.56 ($2.3 - 4.5$) $\times 10^3/\mu\text{L}$ respectively (Table 2).

The mean values of WBC and ANC after at least 6 months of HAART initiation were decreased with significant value ($p < 0.001$ for WBC and $p = 0.008$ for ANC), respectively, whereas the mean values of TLC after at least 6 months of HAART initiation were increased with significant value ($p < 0.001$). The mean RBC count was $4.39 \pm 1.02 \times 10^6/\mu\text{L}$ at the time before HAART initiation and $4.022 \pm 0.92 \times 10^6/\mu\text{L}$ after 6 months of taking HAART. The mean Hgb concentration was decreased and found to be 12.5 ± 2.99 g/dl before HAART initiation and 12.01 ± 2.59 g/dl after 6 months of HAART. Concerning the RBC indices, the mean values of MCV, MCH, MCHC and RDW before initiation HAART were 78.8 ± 9.2 fl, 27.6 ± 2.2 pg, 35.02 ± 1.7 g/dl and $14.55 \pm 2.76\%$, respectively, and after 6 months of HAART initiation the mean values of MCV, MCH, MCHC and RDW were 77.7 ± 9.16 fl, 26.3 ± 2.2 pg, 34.74 ± 1.65 g/dl and $13.91 \pm 2.56\%$, respectively.

The mean value of RBC ($p = 0.009$), Hgb ($p < 0.001^*$), MCV ($p = 0.005$), MCH ($p = 0.001$), and RDW ($<0.001^*$) were significantly increased after 6 months of HAART initiation as compared to the patients who were before HAART initiation and their mean values were decreased, whereas the mean value of MCHC ($p = 0.002$) was increase as compared to patients who were before HAART initiation.

The mean platelet count of the study participants before HAART initiation was $246 \pm 101 \times 10^3/\mu\text{L}$ and $275 \pm 96 \times 10^3/\mu\text{L}$ after 6 months of HAART initiation, respectively. The amount of platelet count was significantly increased with a p value $<0.001^*$.

The mean CD4 count of the study participants was 252.5 ± 114.2 (Cells/mm³) and 349.1 ± 155.7 (Cells/mm³) at the baseline and after 6 months of HAART initiation, respectively. The amount count was significantly increased with a p value $<0.001^*$. The mean viral copies of the study participants were 7523.2 ± 3114.2 Viral load copies/cell and $4523.2 \pm$

Table I Sociodemographic and Clinical Characteristics from April to May 30, 2021

Demographic and Clinical Variables		Frequency	Percentage (%)
Sex	Male	187	49.5
	Female	191	50.5
Age in years	10–16	10	2.6
	17–30	77	20.4
	31–45	174	46
	>45	117	31
Residence	Rural	89	23.5
	Urban	289	76.5
Education status	Illiterate	104	27.5
	Only read and write	37	9.8
	Primary school completed	100	26.5
	Secondary school completed	64	16.9
	College and above	73	19.3
Occupation	Employed	82	21.7
	Student	27	6.3
	Merchant	97	25.7
	House wife	28	7.4
	Farmer	16	4.2
	Daily labour	131	34.7
Marital status	Single	25	6.6
	Married	318	84.1
	Divorced	18	4.8
	Widowed	17	4.5
Current WHO clinical stage	Stage-I	127	(33.6)
	Stage-II	168	(44.4)
	Stage-III	67	(17.7)
	Stage-IV	16	(4.2)
CD4 count	<350 cells/mm ³	190	(50.3)
	>351 cells/mm ³	188	(49.7)
Cotrimoxazole prophylaxis	Yes	190	(50.3)
	No	188	(49.7)
Regimen	First line	378	100
	Second line	0	0

Abbreviations: CD, cluster of differentiation; WHO, World Health Organization.

Table 2 Haematological and Immunological Values Before and After Initiation of HAART from April to May 30, 2021

Haematological Parameter	Before Initiation of HAART (n= 378) Mean $\pm$ SD	After Six Months of HAART Initiation (n= 378) Mean $\pm$ SD	P-value
WBC ($\times 103/\mu\text{L}$)	5.8 $\pm$ 2.7	4.7 $\pm$ 1.9	<0.001*
ANC ($\times 103/\mu\text{L}$)	2.55 $\pm$ 1.56	2.45 $\pm$ 1.53	0.008
TLC ($\times 103/\mu\text{L}$)	2.12 $\pm$ 1.1	2.56 $\pm$ 1.3	<0.001*
RBC ($\times 106/\mu\text{L}$)	4.39 $\pm$ 1.02	4.022 $\pm$ 0.92	0.009
Hgb (g/dl)	12.5 $\pm$ 2.99	12.01 $\pm$ 2.59	<0.001*
HCT (%)	40.7 $\pm$ 7.6	39.5 $\pm$ 7.2	0.009
MCV (fl)	78.8 $\pm$ 9.2	77.7 $\pm$ 9.1	0.005
MCH (pg)	27.6 $\pm$ 2.2	26.3 $\pm$ 2.2	0.001
MCHC (g/dl)	35.02 $\pm$ 1.7	34.74 $\pm$ 1.65	0.002
RDW (%)	14.55 $\pm$ 2.76	13.91 $\pm$ 2.56	<0.001*
PLT ($\times 103/\mu\text{L}$)	246 $\pm$ 101	275 $\pm$ 96	<0.001*
CD4 (Cells/mm ³)	252.5 $\pm$ 114.2	349.1 $\pm$ 155.7	<0.001*
Viral load copies /cell	7523.2 $\pm$ 3114.2	4523.2 $\pm$ 1525.2	0.001

Note: *P<0.05 statistically significant association.

Abbreviations: WBC, white blood cell; ANC, absolute neutrophil count; TLC, total lymphocyte; RBC, red blood cell; Hgb, haemoglobin; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, red cell distribution width, PLT, platelets; CD, cluster of differentiation.

1525.2 Viral load copies/cell at before HAART initiation and after 6 months of HAART initiation, respectively. The decrease of platelet count was significant with a p value <0.001* (Table 2).

Prevalence of Immuno-Haematological Abnormalities

In the current study, before and after treatment of HAART, hematological abnormalities were observed. The common haematological abnormalities were leukopenia, which accounted for 26.7% and 16.5%, neutropenia, which accounted for 16.5% and 9.4%, lymphopenia, which accounted for 20% and 3.1%, and thrombocytopenia, which accounted for 25.9% and 7.1% of the study participants before and after the initiation of HAART, respectively. Concerning immunological failure of study participants, the amount of CD4 count (Cells/mm³) before and after initiation of HAART were 73% and 55%, respectively, whereas, regarding the viral copies, 48.8% and 37.6% of the study subjects were greater than 1000 copies/cell before and after initiation of HAART, respectively (Figure 1).

Discussion

The current study looked at immune-haematological abnormalities in 378 HIV-positive patients who had been on HAART for more than six months at ART facilities managed by governmental and non-governmental healthcare systems in Dessie town. Anemia, leukopenia, neutropenia, lymphopenia, thrombocytopenia, and immunosuppression linked with HAART were the most common haematological abnormalities observed in the current study. Thrombocytopenia and immunosuppression became less common once HAART was initiated. Another study found an increase in the prevalence of anemia, leukopenia, neutropenia, and lymphopenia.²³ This could be owing to the reduced blood cell counts associated with advanced HIV/AIDS. Sex, race/ethnicity, geographic location, and comorbidities like tuberculosis, hepatitis

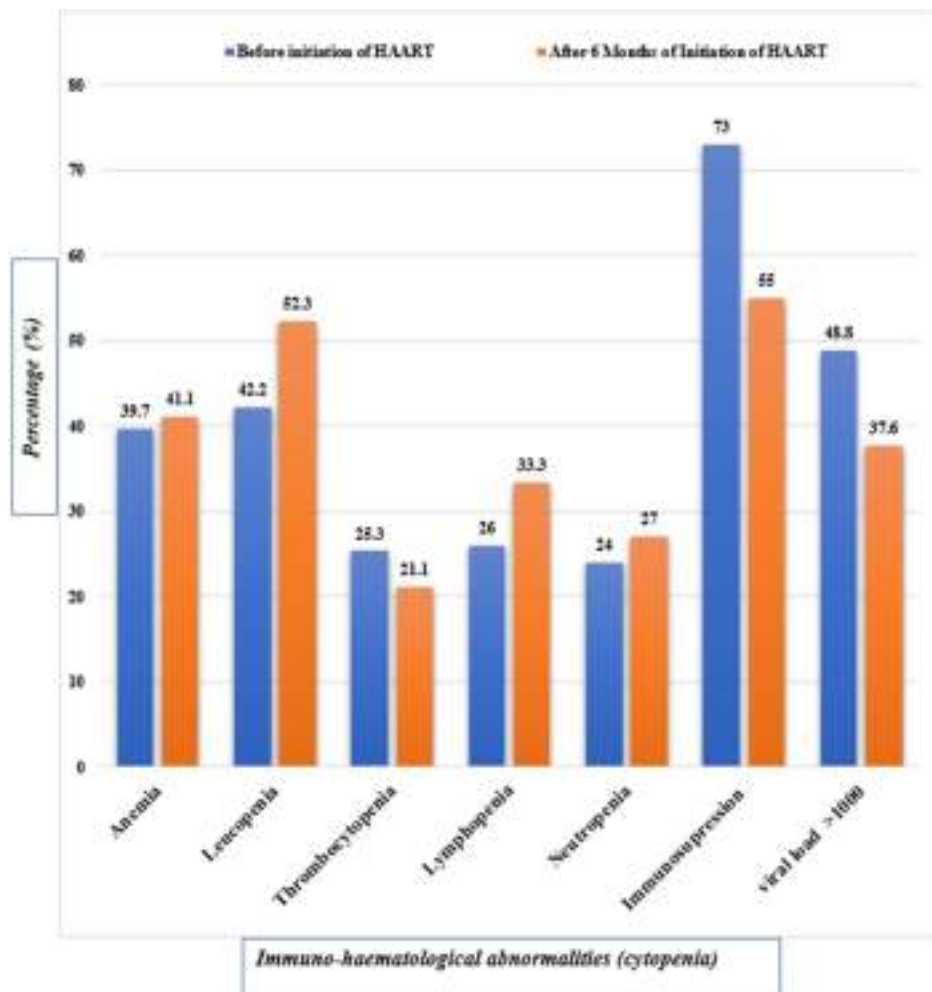


Figure 1 Immuno-haematological abnormalities among study participants. N.B Immunosuppression: Abnormal CD4 count range 50–500 cells per cubic millimeter. It means weakened immune system and may be infected with HIV and specifically below 350 cells per cubic millimeter. Viral copies: high HIV viral load generally indicates that HIV is present and replicating.

B infection, fever, and oral candidiasis have all been connected to cytopenia and an increase in the incidence of bone marrow transplantation.

Among the investigated haematological abnormalities, the mean $\pm$ SD white blood cell count was $5.8 \pm 2.7 \times 10^3/\mu\text{L}$ before HAART initiation and it was decreased to $4.7 \pm 1.9 \times 10^3/\mu\text{L}$ after 6 months of HAART initiation. This finding was supported by a similar study that reported decreased WBC count for HIV patients on HAART, conducted in Gondar, Ethiopia.^{24,25} This difference might be due to a short duration of ART intake as in the initial stage of ART initiations, WBC count will be low which gradually corrects itself over a period of time.²⁴ In the current study, the prevalence of leukopenia was 42.2% at baseline and 52.3% after six months of HAART initiation, respectively (Figure 1). The current finding is comparable with a study from Gondar, Ethiopia, which found a prevalence of 34.2% before HAART initiation and 35.9% for leukopenia after HAART.¹¹ However, the current study discovered a difference in a Nigerian study that discovered the prevalence of leukopenia before and after HAART was 6.1% and 1.7%, respectively.²⁶ This discrepancy could be explained by a difference in the defining value for leukopenia. Additionally, cancer and other diseases that wreak havoc on the bone marrow are autoimmune diseases that cause the destruction of white blood cells or bone marrow cells. Antibiotics and other medications that destroy white blood cells.

In the present study, absolute neutrophil count also presented a decrease from $2.55 \pm 1.56 \times 10^3/\mu\text{L}$ to $2.45 \pm 1.53 \times 10^3/\mu\text{L}$ before HAART initiation and after 6 months of HAART initiation, respectively. The prevalence of Neutropenia

was found to be present in 24% and 27% of patients before and after the start of HAART, respectively. It differs from a study conducted in Gondar, Ethiopia, which discovered 14.8% and 28% neutropenia before and after therapy, respectively.¹¹ This variance could be attributed to variations in the study population, sample size, research design, and clinical situations. Furthermore, certain pharmaceuticals, such as antibiotics and blood pressure medications, may suppress or limit the synthesis of granulocyte macrophage and granulocyte colony-stimulating factor, both of which are critical in granulocyte activation.

In this study, haematological parameters (the mean value) of RBC count were $4.39 \pm 1.02 (\times 10^6/\mu\text{L})$ and $4.022 \pm 0.92 (\times 10^6/\mu\text{L})$ before HAART initiation and after 6 months of HAART initiation (Table 2). These findings were comparable with a study conducted at Jimma, Ethiopia reported that a mean value of RBC count was $4.95 \pm 0.57 \times 10^6/\mu\text{L}$ and $4.14 \pm 0.07 \times 10^6/\mu\text{L}$ ²⁷ and in Addis Ababa with a mean value of RBC count ($4.41 \pm 0.71 \times 10^6/\mu\text{L}$ and $4.28 \pm 0.59 \times 10^6/\mu\text{L}$ before HAART initiation and after at least six months of receiving antiretroviral therapy, respectively.²⁸ However, a higher mean value of RBC count before HAART than after the treatment was also reported by another study.²⁹ This could be due to the unfavourable effect of Zidovudine, which is commonly indicated for RBC depletion in HAART patients, which could possibly account for the current data. The unfavourable HAART treatment of Zidovudine might suppress bone marrow production of RBC, which could possibly account for the current data.³⁰

The present study also showed that the amount of haemoglobin value was decreased after HAART initiation. The mean values of haemoglobin were $12.5 \pm 2.99 \text{ g/dl}$ and $12.01 \pm 2.59 \text{ g/dl}$ before HAART initiation and after six months of HAART initiation, respectively (Table 2). This finding is in agreement with other studies.^{25,27,31} This could be due to HAART treatment, which can cause lead poisoning, immunological dysregulation, red blood cell destruction (haemolysis), or insufficient red blood cell formation, all of which are influenced by spleen or circulatory system issues.

Anemia was found to be prevalent in 39.7% and increased to 41.1% before HAART initiation and after HAART 6 months HAART initiation. This finding is consistent with a research conducted in Europe and North America,³² Addis Ababa, Ethiopia (34.6%),³³ Jimma, Ethiopia (43.1%),²³ Brazil (37.5%),³⁴ and 46% Ghana.³⁵ The treatment of Zidovudine (ZDV) might be responsible for the increase in anemia following HAART commencement.³⁶ Immune dysregulation can increase the risk of anemia with HIV infection by the death of red blood cells or insufficient red blood cell production.³⁷ Furthermore, previous studies have shown that smoking increases hemoglobin levels and reduces the detection of anemia by lowering the oxygen carrying capacity of red blood cells.³⁸ In contrast to our study, study done showed that the prevalence of anemia in Gondar, Ethiopia, 29.7% and 11.7%,¹¹ in Addis Ababa, 41.9% and 11.4%,²⁸ Nigeria reported 57.5% and 24.3%,²⁶ and Ghana 63% and 46%³⁵ were reduced before and after the start of HAART, respectively. This gap could be attributed to a number of factors, including differences in the study population, sample size, study design, socio-demographic characteristics of study participants, and anemia definition discrepancies.

In the current study, the mean value of red cell indices (MCV, MCH, and MCHC and RDW) were decreased significantly following starting of HAART ($p < 0.001$). This was concurrent with the results of earlier investigations.³⁹ However, the current finding was different from studies done in which the amount of red cell indices (MCV, MCH, and MCHC) was increased.^{11,28,40} This might be due to failure of the bone marrow. The primary cause of anemia in patients with chronic renal disease is erythropoietin deficiency.

The amount of CD4+ T cells counts $252.5 \pm 114.2 \text{ cells}/\mu\text{L}$ were increased to $349.1 \pm 155.7 \text{ cells}/\mu\text{L}$ after 6-month initiation of HAART. This finding was in agreement with the study conducted at Addis Ababa, Ethiopia^{31,41,42} and in Maryland⁴³ stated that the mean value of CD4+ was increased after HAART initiation. This may be due to the fact that HIV infection is thought to be caused by the destruction of mature CD4+ T lymphocytes in the peripheral lymphoid system, and HIV may also inhibit the maturation of lymphoid precursors such as lymphoid-restricted T cell progenitors in the thymus and/or clonally distributed memory T cells in peripheral lymphoid organs. Increased CD4+ T cell production is linked to HAART-induced viral replication suppression.⁴⁴

The current study showed that the prevalence of immunosuppression was 73% and 55% at the baseline and after HAART initiation, respectively (Figure 1). This finding is supported by studies conducted in India where 89.2% of the cases had a CD4 count of fewer than 200 cells/l at the time of diagnosis⁴⁵ and Maharashtra where 89.2% of the cases had immunosuppression at baseline.⁴⁶ This could be due to the fact that the majority of the patients were severely

immunocompromised at the outset of the research. Furthermore, HIV's cytotoxic effect on T helper cells might cause immunosuppression, resulting in a low CD4 level. Immunosuppression is reduced after starting treatment because HAART enhances immune function by lowering HIV viral replication and increasing CD4+ T-cell numbers.⁴⁷

This study showed that platelet counts were significantly increased from $246 \pm 101 (\times 10^3/\mu\text{L})$ to $275 \pm 96 (\times 10^3/\mu\text{L})$ after 6 months of the treatment (Table 2). The prevalence of thrombocytopenia was 25.3% at baseline and 21.1% after six months of HAART initiation. This finding was comparable with studies conducted in Gondar,¹¹ Addis Ababa 25% to 5.7%,²⁸ Gondar, 4.1% and 9.1%,¹¹ Goba Ethiopia 11.4% to 4.5%,⁴⁸ Uganda 17.8% to 13.0%⁴⁹ which collectively showed that the prevalence of thrombocytopenia decreased after initiation of HAART. Another study in systematic review and meta-analysis showed that the prevalence of thrombocytopenia was 21% and 11.64% before and after initiation of highly active antiretroviral therapy (HAART), respectively.⁵⁰ This variation could be due to hematopoiesis-related disorders, opportunistic infections, and immune-suppression factors related to HIV which subsequently lead to low platelet count.⁵¹ Furthermore, there are differences in the definition of thrombocytopenia, study design, and study population size.

Conclusion and Recommendations

In this study, statistically significant variations in haematological parameters were observed from the baseline and after HAART initiation. The magnitude of hematological abnormalities, before and after initiation of HAART, was leukopenia (26.7% and 16.5%), neutropenia, (16.5% and 9.4%), lymphopenia (20% and 3.1%), and thrombocytopenia (25.9% and 7.1%), respectively. Before and after HAART, there were statistically significant differences in total WBC count, neutrophils, lymphocytes, Hgb, MCV, MCH, MCHC, and platelets. The study subjects exhibited high and low CD4 counts and viral copies before and after starting HAART, respectively, in terms of CD4 count, immunological failure, and viral copy. According to existing data, a significant proportion of HIV patients remained anemic after six months of antiretroviral therapy (ART), emphasizing the need for routine anemia screening and treatment to prevent the harmful effects of anemia.

Abbreviations

AIDS, acquired immune deficiency syndrome; ART, antiretroviral therapy; ANC, absolute neutrophil count; AZT/ZDV, Azidothymidine/Zidovudine; CBC, Complete blood count; CD4, cluster of differentiation 4; EDTA, Ethylene diamine tetra acetate; HAART, highly active antiretroviral therapy; HGB, Haemoglobin concentration; IQC, Internal quality control; LYM, Lymphocyte; MCH, Mean Corpuscular Haemoglobin; MCHC, Mean Corpuscular Haemoglobin Concentration; MCV, Mean Corpuscular Volume; NEUT, Neutrophils; PCV, Packed Cell Volume; PLT, Platelets; RBC, Red Blood Cells; SPSS, Statistical Package for Social Sciences; WBC, White Blood Cell; WHO, World Health Organization.

Data Sharing Statement

Data supplementary to the conclusions of this article are within the manuscript.

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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 state that they have no conflicts of interest in this research work.

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Acquired Hemophilia A: Current Guidance and Experience from Clinical Practice

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Abstract: In acquired hemophilia A (AHA), autoantibodies to coagulation factor VIII (FVIII) neutralize FVIII activity leading to a potentially severe bleeding diathesis that carries a high rate of morbidity and mortality. This disorder is rare and occurs mainly in adults over 60 years of age or in the postpartum period. The diagnosis should be suspected in patients with new-onset bleeding without a personal or family history of bleeding and can be confirmed via specific assays for FVIII inhibitors. Treatment involves both hemostatic therapies to decrease bleeding and immune modulation strategies to re-establish immune tolerance to FVIII. There are limited data on treatment for refractory disease, based mostly on small case series. Registry studies have informed consensus guidelines for optimal hemostatic therapies and initial immunosuppressive therapies. Additional studies are needed to evaluate novel hemostatic agents and develop biomarkers to risk-stratify treatment while limiting adverse events.

Keywords: factor VIII, autoantibodies, hemostasis, immune modulation

Introduction

As opposed to alloantibodies that occur in up to 30% of patients with severe congenital hemophilia A (HA), acquired hemophilia A (AHA) is a rare disease that arises from autoantibodies to coagulation factor VIII (FVIII). Of the coagulation factors, autoantibodies to FVIII are the most common with an incidence of approximately 1 to 1.5 per million population per year.^{1,2} The vast majority of patients are adults with a median age of 64–78 years; however, pediatric cases have been reported³ with an estimated incidence of 0.045 per million per year.⁴ The disease carries high rates of morbidity and mortality, especially in elderly patients with other comorbidities. However, owing to its rare nature, data that inform consensus guidelines on management and prognostic markers are largely limited to registries and expert opinion.

Presentation and Diagnosis

Although most cases are idiopathic, AHA has been associated with pregnancy, autoimmune disorders such as rheumatoid arthritis or systemic lupus erythematosus, malignancies, and certain medications (Table 1).^{1,2,5–10} Medications reported to be associated with FVIII antibodies include penicillins, sulfonamides, phenytoin, interferons and fludarabine.^{11,12} In a systematic review of 42 pediatric cases of acquired coagulation inhibitors, 28 were directed against FVIII and were either associated with infections, medications, and autoimmune disorders or were idiopathic in nature. The median inhibitor titer was 8.5 Bethesda units (BU)/mL (range 1.7–6500). Pediatric patients have a higher response rate than adults, and some have spontaneous resolution (eg, when associated with infection or antibiotic use).³

Irrespective of age, these patients typically present with spontaneous bleeding episodes or post-surgical hemorrhage. Mucosal bleeding events, including large hematomas or ecchymoses, severe epistaxis, gastrointestinal bleeding, and gross hematuria, are far more common than joint bleeds as seen in congenital HA. The most common presentation is subcutaneous bleeding (80%) followed by bleeding in muscular (45%), gastrointestinal (21%), genitourinary (9%) and retroperitoneal (9%) spaces.^{1,6} In post-partum AHA, soft-tissue, muscular, and vaginal bleeding are the common presentations. Transplacental

Table 1 Demographic and Clinical Characteristics of Patients with Acquired Hemophilia A in Large Registry Studies

Study	Green ⁵	Delgado ⁶	Collins ¹	EACH2 ²	Tay ⁷	Borg ⁸	Huang ⁹	GTH-AH ¹⁰
Patients (n)	215	234	172	501	25	82	65	102
Age (years)		64 (8–93)	78 (2–98)	74 (62–80)	78 (27–99)	76.7 (25–103)	64 (18–94)	74 (26–97)
Inhibitor (BU/mL)		10 (0.9–32,000)	13 (4–38)	12.8 (4.2–42.5)	11 (1.2–460)	16.1 (1–2800)	19.4 (0.74–2414)	19 (1–1449)
Male (%)	53	45	43	53	48	61	64	58
Underlying disorder								
Idiopathic	82 (43.6)	135 (57.7)	95 (63.3)	260 (51.9)	19 (79)	45 (54.8)	34 (52)	68 (67)
Malignancy	12 (6.4)	43 (18.4)	22 (14.7)	59 (11.8)	1 (4)	16 (19.5)	8 (12)	13 (13)
Autoimmune	32 (17.0)	22 (9.4)	25 (16.7)	67 (13.4)	3 (12)	12 (14.6)	4 (6)	20 (20)
Post-partum	13 (7.0)	34 (14.5)	3 (2)	42 (8.4)	2 (8)	6 (7.3)	3 (5)	5 (5)
Infection	NR	NR	NR	19 (3.8)	NR	NR	NR	NR
Dermatologic	8 (4.3)	NR	5 (3.3)	7 (1.4)	NR	NR	3 (5)	NR
Drugs	10 (5.3)	NR	NR	17 (3.4)	NR	NR	11 (17)	NR
Other	21 (16.5)	NR	NR	58 (11.6)	NR	NR	2 (3)	NR

Note: Data reported as n (%) or median (range).

Abbreviation: NR, not reported.

passage of the IgG antibodies may result in bleeding in the neonate.^{13,14} Some patients may present solely with laboratory abnormalities without bleeding. Bleeding episodes are often serious,² and the overall mortality rate approaches 20%.^{1,2} The residual FVIII activity does not always predict the risk of bleeding.

Clinicians must maintain a high index of suspicion for this diagnosis in adult patients with new onset of bruising or bleeding with a prolonged PTT. Diagnostic delays may result from lack of awareness due to the rarity of AHA and the need for centers to send out the laboratory work-up to a reference laboratory. A retrospective analysis found that delay in diagnosis of greater than one month was associated with extended periods of active bleeding and higher hemostatic factor requirements.¹⁵ There was no association with recurrence rate or survival between those patients with and without a diagnostic delay in this series. However, this may be secondary to the small number of patients in the series and potential biases such as lack of reporting of “never-diagnosed” cases.

A diagnostic algorithm based upon consensus guidelines to direct testing is presented in Figure 1 and should be considered in the setting of spontaneous bleeding without prior personal or family history of bleeding.¹⁶ Initial testing should include an activated partial thromboplastin time (aPTT) and prothrombin time (PT). AHA patients will have an

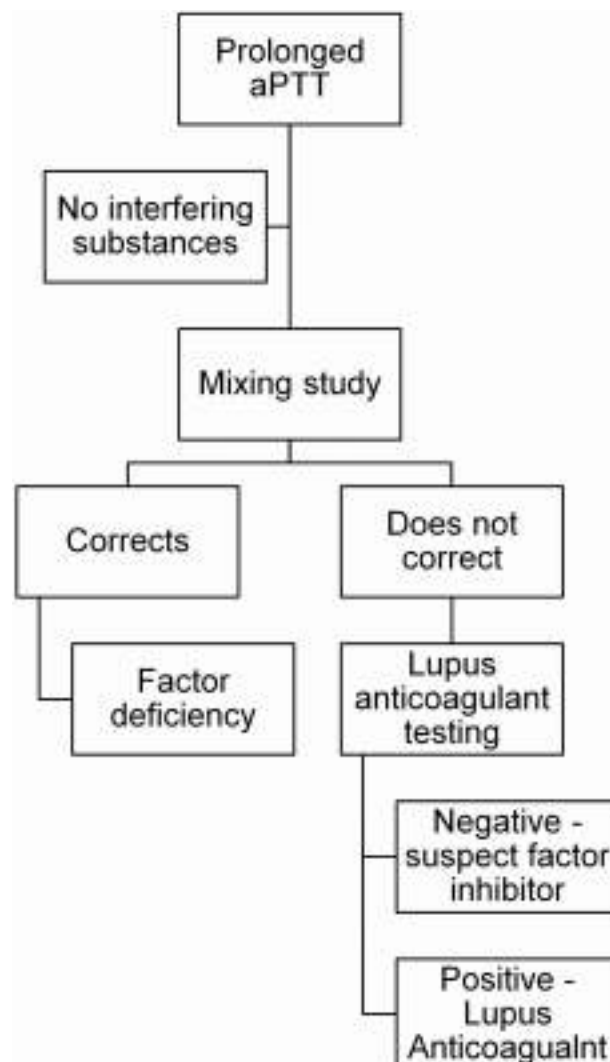


Figure 1 Laboratory testing algorithm for suspected acquired hemophilia A. After exclusion of interfering substances as a cause of prolonged aPTT, a two-hour mixing study should be done with normal pooled plasma. For samples where the aPTT corrects, a factor deficiency should be suspected, and specific factor assays conducted. For samples where the aPTT does not correct, testing for lupus anticoagulant should be done and, if negative, specific testing for a FVIII inhibitor should be conducted. Inhibitors can be measured via Bethesda assay ideally with heat inactivation.

isolated prolonged aPTT, and initial screening with a 1:1 mixing study with normal pooled plasma does not correct the prolonged clotting time. Interfering substances (eg, heparins) and lupus anticoagulants must be ruled out and FVIII activity assessed. FVIII activity is <1% in approximately 50% of cases and less than 5% in 75% of cases.¹⁶ The dilute Russell viper venom test (DRVVT) can be used for lupus anticoagulant testing and is not typically affected by FVIII inhibitors. Chromogenic FVIII activity assays are not sensitive to lupus anticoagulants.^{17–19} If FVIII is low and other tests are negative, quantification of the inhibitor titer via a Bethesda assay should then be conducted. It should be noted that patients may have concurrent lupus anticoagulants and FVIII inhibitors. Thus, identification of a lupus anticoagulant in a patient with a newly prolonged aPTT and bleeding manifestations does not entirely rule out the presence of an acquired inhibitor to FVIII.^{20,21}

The inhibitor titer is equal to the reciprocal of the plasma dilution that results in 50% inhibition of FVIII in normal plasma after incubation for 2 hours at 37°C.²² Inhibitor titers are measured in Bethesda units (BU) where 1 BU is equal to the amount of antibody that neutralizes 50% FVIII activity. Although quite accurate for type I inhibitors which display linear kinetics, autoantibodies in AHA can display type II kinetics, which have some residual FVIII activity; the Bethesda assay may underestimate the titer in the presence of type II inhibitors.^{23–25} Sensitivity and specificity of the Bethesda assay is improved by the Nijmegen modification (buffering the normal plasma) and heat inactivation of the patient's plasma prior to assessment.^{26,27} Enzyme-linked immunosorbent assays (ELISA) can be used to diagnose FVIII antibodies, but these cannot distinguish neutralizing capacity and are seen to some degree in the normal population (see below). Finally, if recombinant porcine FVIII (rpFVIII) is a therapeutic option, then a Bethesda assay specific to rpFVIII should be considered as it may help guide treatment decisions (see hemostatic therapies section).¹⁶

Pathophysiology

The precise trigger for the spontaneous production of neutralizing IgG antibodies to FVIII in patients with AHA is currently unknown. A certain percentage of the normal non-hemophilic population develop non-neutralizing IgG antibodies to FVIII, usually of low affinity and IgG₁ subclass.^{28,29} In congenital HA, longitudinal studies suggest that the alloantibody response matures from a low-affinity predominantly IgG₁ antibody to a high-affinity IgG₄, which corresponds to FVIII neutralizing activity.³⁰ Whether these non-neutralizing IgG antibodies in non-hemophilic patients are predecessors to or independent of neutralizing autoantibodies in AHA is unclear. Similar IgG subtypes are described in patients with AHA as compared to congenital HA.³¹ A recent study analyzed affinities and subtypes and noted that the most common subtypes with the highest titers and affinities were IgG₁ (88%, K_A $5.8 \times 10^{10} \text{ M}^{-1}$) and IgG₄ (98%, K_A $1.3 \times 10^{10} \text{ M}^{-1}$), though all IgG subclass antibodies and IgA (46%) or IgM (9%) antibodies were detected.³² The IgA antibodies do not correlate with FVIII neutralizing activity but do correlate with outcome (as discussed below).³² The IgG antibodies correlate with inhibitor titers³² and other studies have identified that these antibodies could be proteolytic towards FVIII.^{24,33} As in congenital HA, limited studies in AHA demonstrate that the IgG antibodies are generally polyclonal and largely bind to the A2 and C2 domains of FVIII.³⁴

As noted in Table 1, 50% or more of AHA patients do not have an underlying disorder that predisposes them to form autoantibodies. Genetic investigations have associated polymorphisms in CTLA4, non-hemophilic *F8* gene variants, and human leukocyte antigen (HLA) DRB1*16 and DQB1*0502 with AHA.^{35–38} Interestingly, these HLA types are noted to be protective towards inhibitors in congenital HA. Further, some studies have delineated FVIII-reactive CD4⁺ T cells in healthy populations and patients with congenital or acquired HA with inhibitors; albeit the frequency of these cells is lower in the healthy controls.³⁹ Thus, there is a possibility of a trigger leading to a break in tolerance that allows these CD4⁺ T cells to proliferate and lead to AHA in the right context.

Management of Acquired Hemophilia A

The initial management of AHA can be broken down into two parts: obtaining and maintaining hemostasis and re-establishing FVIII immune tolerance by eradicating the inhibitor. Given the rarity of this disease, randomized studies of the optimal management of AHA are limited. International consensus AHA guidelines, first published in 2009 and updated in 2020, provide detailed guidance based on expert experience and registry data.¹⁶ Below, we provide a brief overview of the clinical

management of AHA, highlighting important practical clinical points, and refer the reader to the International AHA guidelines for further details.¹⁶

Hemostatic Therapies

Given the rarity of the diagnosis, clinicians should refer patients to an experienced tertiary care center for management.⁴⁰ Acute bleeding is typically best managed by using a bypassing agent or rpFVIII. Human-derived recombinant factor VIII products and desmopressin (DDAVP) are generally not effective, except in cases of low inhibitor titer (<5 BU), where DDAVP may be considered for minor bleeding episodes.^{41,42} The initial product depends on institutional experience and provider preference (Table 2). Recombinant factor VIIa (rVIIa) or activated prothrombin complex concentrates (aPCC) are most frequently used. These products require frequent dosing every 2–4 hours for rVIIa or every 8–12 hours for aPCC. Neither rVIIa nor aPCC can be monitored with standard laboratory assays, and thus the frequency and intensity of dosing is based on clinical improvement in bleeding symptoms. A global consensus provides clinical and laboratory markers of appropriate hemostasis and optimal intervals for assessment of bleeding control as pertinent to each type of bleeding that may occur in patients with AHA.⁴⁰ Generally, experts suggest stabilization of hemoglobin levels, lack of transfusion requirement, and visually or clinically improved signs of bleeding as markers of appropriate hemostasis.⁴⁰ There is potential for thrombotic risks with rVIIa or aPCC, particularly with repetitive infusions of high doses. Fortunately, these events seem uncommon, with one multicenter European AHA registry (EACH2) reporting thrombotic events in 2.9% (5/174) and 4.8% (3/63) patients with AHA who received rVIIa or aPCC, respectively.⁴³ Case reports support the use of the anti-fibrinolytic agent tranexamic acid either in combination with rVIIa or aPCC.^{44–46} However, combination therapy may increase thrombotic risk, and clinicians must consider the risks and benefits for each patient.⁴⁷

More recent clinical trial data showed rpFVIII had efficacy in AHA.⁴⁸ rpFVIII is appealing as it can be monitored with one-stage clot-based FVIII assays.¹⁶ Inhibitors can be present or develop to rpFVIII, and thus current AHA guidelines suggest porcine inhibitor titers should be measured to determine the likelihood of responsiveness to this agent.¹⁶ Anti-rpFVIII titers should also be measured after initiation of therapy as there are report of inhibitors developing which may decrease the efficacy of these products.⁴² In one study, 5 of the 28 subjects treated with rpFVIII developed de novo anti-rpFVIII inhibitor between 8 and 85 days after first infusion.⁴⁸ Single institution published small series, unpublished abstract data, as well as a retrospective series report using lower than the approved doses of rpFVIII (100 IU/kg rather than 200 IU/kg) without awaiting rpFVIII titers with good hemostatic efficacy. FVIII activity (FVIII:C) monitoring can guide dosing

Table 2 Hemostatic Therapies for Management of Bleeding in Acquired Hemophilia

Agent	Dosing	Lab Monitoring	Comments
Recombinant Porcine Factor VIII	200 units/kg to achieve factor VIII 100–200% then titrate subsequent doses every 4–12 hours to maintain factor VIII trough 50% after acute bleed is controlled ⁴² Alternative: 100 units/kg with factor VIII monitoring every 2–3 hours and re-dosing based on VIII level ⁵⁰	One-stage factor VIII assay	Porcine factor VIII inhibitor titers may predict response
Recombinant Factor VIIa	70–90 mcg/kg every 2–4 hours until hemostasis is obtained and then prolong interval ⁴²	None	Recombinant VIIa can shorten INR significantly. This may cause artificially elevated INR >10 to be reported by some clinical assays. Potential thrombosis risk
Activated Prothrombin Complex Concentrate	50–100 U/kg every 8–12 hours	None	Potential thrombosis risk

Notes: Adapted from Kruse-Jarres R, Kempton CL, Baudo F, et al. Acquired hemophilia A: updated review of evidence and treatment guidance. *Am J Hematol.* 2017;92(7):695–705. with permission from © 2017 Wiley Periodicals, Inc.⁴²

when rpFVIII is used with goal of obtain FVIII:C levels initially at 100% for severe hemorrhage.¹⁶ The optimal dosing and monitoring of rpFVIII require further study.^{49–51} Martin et al described that their institutional protocol is to measure an FVIII:C 30 minutes after rpFVIII administration and then every 4 hours (immediately prior to next dose). For severe bleeds or bleeding in concerning areas (eg intracranial, neck, retroperitoneal) it is recommended to maintain FVIII:C >80% and >50% for all other bleeds.¹⁶ Doses are adjusted to maintain these goals until hemostasis has been obtained.⁵⁰

Emicizumab is a bispecific antibody binding coagulation factor IXa and X without requiring FVIII.^{52,53} Emicizumab is Food and Drug Administration (FDA) approved for patients with moderate-severe congenital HA. The drug is administered subcutaneously and has a long half-life, making it appealing for use in AHA. However, there are still questions regarding thrombotic risk with emicizumab use in AHA as patients with AHA are generally older and have more comorbidities than patients with congenital HA. There were early reports of thrombotic complications and mortality following off-label and compassionate use of emicizumab in AHA patients, but details of additional risk factors in these reports are lacking.⁵⁴ Subsequently, there is growing experience with the off-label use of the agent, which has been published or presented in abstract form.⁵⁵ A recent abstract reported a multicenter survey of US hemophilia treatment centers in which twenty-four patients with AHA were treated with emicizumab. The majority of cases obtained bleeding control and tolerated the treatment without complications, but one patient experienced a thrombotic event and two deaths occurred while on emicizumab, which were not attributed to the medication.⁵⁶ A published case series by Knoebl et al describes 12 patients with AHA and new-onset bleeding treated with emicizumab and “reduced-intensity” immunosuppression.⁵⁷ Patients were started on emicizumab at 3 mg/kg given weekly via subcutaneous injection for 2–3 weeks followed by 1.5 mg/kg every 3 weeks (with interval prolongation up to 4 weeks) and discontinuation of therapy when FVIII levels were >30%. Of note, aPCC was held or patient switched to rVIIa for 48 hours prior to initiation of emicizumab due to the association of thrombotic microangiopathy in patients with congenital HA treated with emicizumab and concurrent aPCC.⁵⁸ A rapid clinical improvement in bleeding was seen in this study at a median of 3 days (range 2–15 days). It should also be noted that as emicizumab immediately and completely corrects the aPTT, FVIII levels need to be measured by bovine chromogenic (not clot-based) assays in patients on this therapy. We currently recommend the use of emicizumab in AHA only in the clinical trial setting until more data are available regarding its safety and efficacy in AHA.

Patients with AHA are not protected from thrombotic events and may be at increased risk of thrombosis during treatment with bypassing agents. The incidence of thromboembolic events in patients receiving bypassing agents varies by study with some registries not reporting any thromboembolic events due to bypassing agents^{2,43} and others reporting an incidence of 2–10%.^{8,10,42} Current International AHA guidelines recommend initiating venous thromboembolism (VTE) prophylaxis once FVIII:C returns to normal if the patient remains hospitalized or has another indication for VTE prophylaxis.¹⁶ As AHA often occurs in the elderly where anticoagulation is often used for primary stroke prevention for atrial fibrillation and/or treatment and prevention of thrombosis, it is essential to restart appropriate anticoagulation in patients with a pre-existing indication once FVIII:C levels return to normal.¹⁶

Inhibitor Eradication

Obtaining hemostasis is the immediate priority after which strategies to eliminate the inhibitor should be promptly employed. Current guidelines recommend immunosuppressive therapy in most patients diagnosed with AHA.¹⁶ Many of the patients with AHA who develop inhibitors to FVIII are elderly, and thus clinicians must carefully consider the toxicities of therapy when choosing a regimen.

First-Line Immunosuppressant

The recommended first line of therapy is corticosteroids, typically 1 mg/kg prednisone (Table 3). Traditionally, a second agent (either rituximab or cyclophosphamide) was added in patients who failed to respond to steroids alone. However, more recent observational studies suggest higher complete remission (CR) rates when combination therapy is utilized (35.2% CR with steroid monotherapy vs 67.7–83.3% with combination therapy).⁵⁹ Current International AHA guidelines now suggest adding rituximab or cyclophosphamide to first-line therapy in patients with inhibitor titer >20 BU.¹⁶ The choice of cyclophosphamide versus rituximab depends on the comorbidities of the patient as well as provider and

Table 3 Immunosuppressant Therapies for Acquired Hemophilia A

Medication	Dosing	Cautions
First-line Therapies		
Corticosteroids	Prednisone 1 mg/kg PO daily, tapered as factor VIII increases	-Immunosuppressive, consider PCP prophylaxis with prolonged use -Agitation/mood disorders -Elevated blood glucose
Cyclophosphamide*	1.5–2 mg/kg/day PO daily for maximum of 6 weeks (alternative IV pulse every 3–4 weeks) ¹⁵	-Increased risk of secondary malignancies with prolonged use (eg, bladder and myelodysplastic syndrome) - Myelosuppressive, monitor CBC closely - Renal toxicity, ensure adequate hydration with oral dosing -Gonadal toxicity with prolonged use
Rituximab*	375 mg/m ² IV weekly x 4	-Immunosuppression, increased risk of viral infections -Hepatitis B reactivation
Second-line Therapies[#]		
Mycophenolate mofetil	1 gram per day in divided doses increased to 2 grams per day after 1 week ⁶¹	-Myelosuppression, most commonly neutropenia, which typically resolved with dose-reduction
Cyclosporine*	Initial dosing 5 mg/kg per day, adjusted to trough 200–400 ug/dL ⁶⁰	-Renal toxicity -Delirium -Hypertension
Tacrolimus*	Initial dosing 0.3 mg/kg per day, adjusted to trough 1.5 ug/dL ⁶⁰	-Renal toxicity -Delirium -Hypertension
Cyclophosphamide, vincristine, prednisone	Cyclophosphamide 7 mg/kg IV and vincristine 2 mg IV on the first day followed by cyclophosphamide 3 mg/kg daily P.O. on days 25. Cycles repeated every 3–4 weeks if inhibitor persisted ⁶²	-Myelosuppression -Renal toxicity -Neuropathy -Constipation (vincristine)
Bortezomib	1.3 mg/m ² on days 1,4,8 and 11 on 21-day cycles ⁶⁴	-Antiviral prophylaxis recommended -Neuropathy -Myelosuppressive

Notes: *Used in combination with corticosteroids. [#]Off-label use of medications. Limited data available for treatment options in patients who are refractory to steroids, cyclophosphamide, and rituximab. Adapted from Kruse-Jarres R, Kempton CL, Baudo F, et al. Acquired hemophilia A: updated review of evidence and treatment guidance. *Am J Hematol.* 2017;92(7):695–705. with permission from © 2017 Wiley Periodicals, Inc.⁴²

institutional experience. Elimination of the inhibitor is achieved with immunosuppression at a median of 5–6 weeks.⁴² While CR rates appear higher with combination therapy, a significant proportion of mortality is related to infection due to immunosuppression (outlined further in prognosis section below). Coupled with the fact that there are a percentage of patients that will have spontaneous remissions (in one series up to 35% achieved remission by 1 year) without immunosuppression, a careful patient-centered risk versus benefit analysis must be employed for each case.⁵

Second-Line Immunosuppressant

A second-line therapy should be considered if there is no response after 3–5 weeks of first-line therapy.⁴² International AHA guidelines suggest first using whichever agent (cyclophosphamide or rituximab) that was not used in the first-line setting.¹⁶ Unfortunately, there are very limited data about additional options for patients refractory to steroids, rituximab,

and cyclophosphamide. An overview of some studied regimens is outlined in Table 3. Pardos-Gea et al performed a prospective study of 11 patients with AHA treated with calcineurin inhibitors (tacrolimus or cyclosporine) and corticosteroids in the first-line setting. They reported responses in 10 out of the 11 patients with one patient experiencing a major side effect (hypertensive posterior progressive encephalopathy).⁶⁰ If calcineurin inhibitors are used, clinicians must monitor closely for renal toxicity and hypertensive complications, particularly in elderly patients with AHA.⁶⁰ Small series also suggest that mycophenolate mofetil has efficacy in AHA, although there were lower rates of initial response than prednisone and rituximab. Mycophenolate mofetil maintained remission in two patients with AHA who previously did not obtain CR with first-line therapy.⁶¹ Combining multiple immunosuppressants and inclusion of vincristine has also been reported in a case series of 6 patients with AHA, with 5 patients obtaining CR after 1–7 cycles of therapy.⁶² Case reports also describe responses to plasma-cell directed therapies, such as bortezomib, in patients with refractory AHA.^{63,64}

Immune tolerance induction (ITI) protocols with high-dose FVIII (as used in congenital HA) have been trialed in AHA,⁶⁵ but their efficacy in addition to standard immunosuppressive therapy is unclear. Current guidelines do not recommend ITI unless a patient has severe bleeding failing first-line hemostatic therapies.¹⁶

Prognosis and Response

Table 4 outlines the complete response and relapse rates as well as mortality in patients with AHA from selected registry studies. Approximately 60–80% of patients will respond to first-line immunosuppressive therapy (corticosteroids with or without either rituximab or cyclophosphamide). Relapses are not uncommon during corticosteroid withdrawal, occurring in 15% of patients in a Dutch registry.⁵⁹ After complete cessation of immunosuppressive therapy, an estimated 25% of patients will experience a relapse with a median time to relapse of 14.7 weeks (IQR 2.9–66.6 weeks).⁵⁹ Given the relapse rate, clinicians should counsel patients during weaning of immunosuppression to monitor for increased bruising or other signs of bleeding. The optimal frequency of monitoring PTT and/or FVIII levels following CR is not well defined. One of the author's practices is to monitor complete blood count and PTT and FVIII levels every 2 weeks while weaning prednisone and immediately following immunosuppression withdrawal and then monthly for at least the next 3–6 months.

Registry studies have assessed the influence of various clinical and laboratory characteristics on outcome of AHA therapy. Spontaneous remission is noted, especially in pediatric patients with AHA.³ In the prospective GTH registry study, which assessed a standardized approach of steroids alone (first line), steroids with cyclophosphamide (second line) or rituximab (refractory or relapse), predictors of response included presence of high-titer (>1:80) anti-FVIII IgA antibodies but not IgG

Table 4 Initial Complete Remission, Relapse, and Mortality Rates in Selected Registry Studies of Acquired Hemophilia A

	EACH2 Registry²	CARE Registry⁶⁷	KWARK Registry⁵⁹
Population	European prospective registry	Chinese nationwide registry	Dutch national hemophilia complication registry
Years	2003–2008	2012–2017	1992–2018
Patients (n)	501	187	143 (139 received immunosuppressive therapy)
Initial Complete Remission*	71.6% (237/331)	81.9% (127/167)	79.5% (105/132)
Steroids	NR	62.2% (23/37)	35.2%
Steroids/cyclophosphamide	NR	87.5% (56/64)	83.3%
Steroids/ rituximab	NR	90.9% (40/44)	67.7%
Relapse	NR	25.8% (40/155) after CR	15.4% during steroid withdrawal, 25% after withdrawal of immunosuppression
Mortality	26.3% (87/331)	6.7% (9/165)	38.2% (52/136)

Note: *Initial complete remission after immunosuppressive therapy.

Abbreviations: CR, complete remission; NR, not reported.

antibodies.³² In multivariable analysis, clinical predictors of poor response rates and overall survival in this study included baseline FVIII activity <1% and WHO-PS scores >2.¹⁰ A recent conference presentation displayed differential cytokine production from FVIII-stimulated peripheral blood mononuclear cells of 11 AHA patients when comparing baseline samples of healthy controls and relapsed patients but not between CR and relapsed patients.⁶⁶ However, these are preliminary findings and further studies need to be conducted to verify their predictive relevance in AHA. Additional prospective studies and biorepositories are needed to guide biomarkers of tolerance and overall response in AHA.

AHA has significant mortality, ranging from 6.7% to 38% in registry data.^{2,59} Data from multiple registries support that infection is the leading cause of mortality in this disease, likely due to the need for immunosuppression.^{2,8,10,59} For example, in the Dutch Cohort, overall mortality was 38.2% (52/132 patients) with the cause of death identified as infection in 19.2% (10/52 patients), malignancy in 13.5% (7/52) and fatal bleeding in 7.7% (4/52 patients).⁵⁹ Some studies have reported rare fatal thrombotic events in patients with AHA receiving hemostatic agents (see above).¹⁰

Conclusions

Although rare, AHA carries a high risk of morbidity and mortality and requires prompt treatment, ideally by a specialized center well versed in treating these patients. Immediate attention to control bleeding should be followed closely by therapy to re-establish FVIII immune tolerance. Initial treatment choice should involve corticosteroids with or without additional immunosuppressants based upon the risk profile of the patients. Although treatment should be initiated quickly, it can pose safety risks necessitating close monitoring of patients. Most patients will respond to initial immunosuppressive therapy with corticosteroids alone or in combination with cyclophosphamide or rituximab. Vigilance is required during discontinuation of immunosuppression to allow early identification of relapse. Additional prospective studies are necessary to determine the optimal management and identify biomarkers of response that can inform therapeutic decisions.

Disclosure

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The Role of Platelet-Selectin as a Marker of Thrombocyte Aggregation on Cerebral Sinus Venous Thrombosis

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The Role of Platelet-Selectin as a Marker of Thrombocyte Aggregation on Cerebral Sinus Venous Thrombosis

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Abstract: Cerebral sinus venous thrombosis (CVST) is a disease caused by occlusion of intracranial venous structures, including the cerebral sinuses, cortical veins, and the proximal jugular vein. Delay in diagnosis and therapy can lead to complications such as bleeding infarction and even death. Thrombosis that causes CVST is the process of forming a blood clot in a blood vessel. Thrombosis occurs when the balance between thrombogenic factors and the protective mechanisms of thrombogenesis is disturbed. Platelet function abnormalities in CVST cases can be in the form of impaired adhesion function, impaired release or secretion reactions, and impaired aggregation function. Dysfunction and disruption of endothelial structure due to inflammation causes platelet adhesion so that platelets stick together with collagen in endothelial cells. Platelet-selectin is a type 1 transmembrane protein in platelet granules and megakaryocytes and plays a role in mediating interactions between leukocytes and ligands that help the adhesion process of leukocytes and platelets so that they can be used as predictors of thrombosis in patients with CVST.

Keywords: platelet-selectin, CVST, platelet aggregation, prothrombotic

Introduction

Cerebral sinus venous thrombosis (CVST) is a disease caused by the formation of a thrombus (blood clot) in a vein in the brain.¹ CVST cases in the world are estimated to occur in three-to-four people per one million population per year. CVST occurs more often in young adults than in the elderly. This disease is more common in women than men with a ratio of 2.9:1. Hormonal disturbances during pregnancy, the puerperium, use of hormonal contraception are factors that increase the risk of CVST in women.² Impaired blood flow due to blockage of the venous system will cause increased pressure on brain tissue, because the venous system is blocked in the dural sinus area. Increased pressure on brain tissue will cause brain edema around the blocked area of the venous system. Furthermore, the capillaries and arterioles will rupture and cause cerebral hemorrhage, if the pressure increases higher, the bleeding can spread to the nearest subarachnoid space causing decreased consciousness leading to death.³ CVST is a rare cerebrovascular disease, with varying clinical symptoms and radiological features and is difficult to diagnose. Delay in the diagnosis of CVST causes severe complications, including cerebral infarction, brain parenchymal hemorrhage, brainstem herniation, and even death.¹ Thrombosis that causes CVST is the process of forming blood clots in blood vessels. Virchow revealed a triad that is the basis of thrombus formation. Virchow's triad consists of: (1) blood flow disorders resulting in stasis, (2) procoagulant and anticoagulant balance disorders that cause activation of coagulation factors, and (3) disorders of the blood vessel wall (endothelium) that cause hypercoagulation between thrombogenic factors and the protective mechanism of thrombogenesis is disturbed. Thrombogenic factors include platelet activation or its interaction with coagulation factors and natural anticoagulants. The protective mechanism of thrombogenesis is when endothelial disruption stimulates platelets to aggregate and promotes changes in coagulation factors.⁴ Thrombus or blood clots can form in veins, arteries, heart, or microcirculation. Venous thrombus mainly occurs in areas of stasis and consists of platelets and other blood components.⁴ One of the diseases caused by venous thrombosis is CVST. Thrombus can be caused by antithrombin III deficiency, protein C and S deficiency, the presence of factor V Leiden, which causes resistance to protein C activation, direct injury to the cerebral sinuses,

meningitis, and other prothrombotic disorders such as platelet aggregation. Platelet hyperaggregation can cause CVST around 22–25%.⁵ Thrombosis in CVST is generally caused by impaired platelet function and coagulation factors. Platelet function abnormalities in CVST cases can be in the form of impaired adhesion function, impaired release or secretion reactions, and impaired aggregation function. Dysfunction and disruption of endothelial structure due to inflammation causes platelet adhesion so that platelets stick together with collagen in endothelial cells. Furthermore, there will be a reaction to release platelet granules such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), norepinephrine, and others to strengthen platelet aggregates that stick together with collagen. The final process in the thrombosis process is platelet aggregation.^{6,7} The process of adhesion and secretion of platelet granules can be predicted by examination of platelet-selectin (P-selectin).

Pathogenesis of Cerebral Sinus Venous Thrombosis

The cerebral venous circulation begins in the capillary area. Blood from the brain flows through the venules, then leaves the brain through the external and internal veins, then enters the sinuses. Blood originating from the sinuses will flow into the internal jugular vein, subclavian vein, brachiocephalic vein and then into the superior vena cava.^{13–16} Circulation of veins and intracranial venous sinuses is shown in Figure 1.

The pathogenesis in patients with venous occlusion is very different from that of arterial occlusive disease. When the venous system is blocked, the drainage of blood is impaired, causing increased pressure in the brain tissue due to the blocked venous system in the dural sinuses. Increased pressure will cause brain edema in the area involved. If the tissue pressure is high, the capillaries and arterioles burst and cerebral hemorrhage occurs. Bleeding can spread to the nearby subarachnoid space.^{14,15} Perfusion of brain tissue must be maintained adequately, so that arterial blood pressure must exceed tissue pressure to allow blood to flow into the veins. When venous pressure and intracranial pressure increase, arterial perfusion becomes inadequate, resulting in true cerebral (arterial) infarction. The term “venous infarction” is very different from an arterial infarct. In venous infarcts, the cerebral edema that occurs is potentially reversible, whereas arterial infarcts are irreversible. This is because the capacity of the veins in the brain has a collateral drainage system, thus enabling the recovery of infarcts in CVST patients.^{17,18} Obstruction of the venous structures causes an increase in venous pressure, a decrease in capillary perfusion pressure, and an increase in cerebral blood volume. The mechanism of cerebral venous dilatation and venous collateral system in the early phase of CVST can still be compensated by changes in pressure, but the increase in venous pressure cannot be compensated for longer. This results in

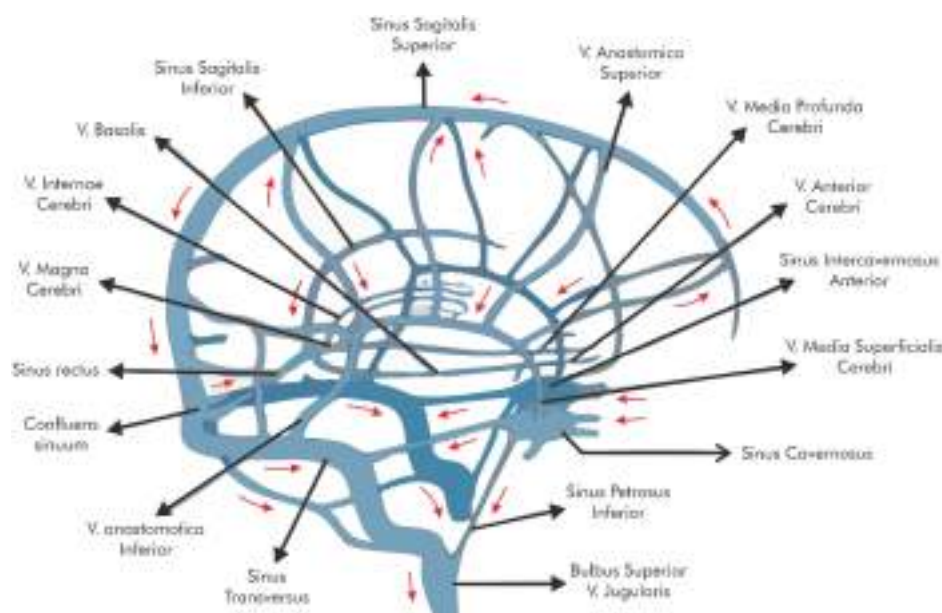


Figure 1 Intracranial venous and sinus venous circulation.

Note: Ferro CM, Canhao P. Chapter 28. Cerebral Venous Thrombosis. In: Mohr JP, Wolf PA, Grotta JC, Moskowitz MA, Mayberg MR & von Kummer R, editors. Pathophysiology, Diagnosis, and Management, 5th edition. Philadelphia: Elsevier Saunders, 2011;516–530, used with permission of Mayo Foundation for Medical Education and Research, all rights reserved.²³

decreased capillary perfusion which in turn causes blood–brain barrier disruption and vasogenic edema due to leakage of blood plasma into the interstitial tissue. Cerebral edema of a local nature and venous bleeding can occur due to rupture of capillaries or veins when intravenous pressure continues to increase. CVST patients will experience failure of the Na⁺/K⁺ ATPase pump so that water enters the cells and causes cytotoxic edema. Another effect of CVST is impaired absorption of cerebrospinal fluid. Normal absorption occurs in the arachnoid granulations, which then drain into the sagittal sinus. Thrombosis of the dural sinuses causes increased venous pressure, impaired cerebrospinal fluid absorption and increased intracranial pressure. Increased intracranial pressure is more common in the superior sagittal sinus.^{11,12} The pathogenesis of CVST can be summarized in Figure 2.

Thrombosis in CVST due to the formation of blood clots in cerebral veins. A thrombus or blood clot can form in a vein, artery, heart, or microcirculation and cause complications by causing obstruction or embolism.^{10,11} A thrombus is an abnormal clot in a vessel with or without leakage. Thrombus is divided into three types, red thrombus (coagulation thrombus), white thrombus (agglutination thrombus), and mixed thrombus. Red thrombus contains platelets and leukocytes that are evenly distributed in a mass consisting of erythrocytes and fibrin, often found in veins. White thrombus consists of fibrin and a layer of platelets, leukocytes with few erythrocytes, usually present in arteries. The most common form is a mixed thrombus of a red thrombus and a white thrombus.^{4–6} Deep blood is normally liquid but will form a clot if the platelets are activated or exposed to a surface. Virchow revealed a triad that is the basis of thrombus formation. This is known as the Virchow Triad and consists of (1) disturbances in blood flow that result in stasis, (2) disturbances in the balance of procoagulants and anticoagulants that cause activation of coagulation factors, and (3) disorders of the blood vessel wall (endothelium) that cause hypercoagulability.^{4–6} Thrombosis occurs when the balance between thrombogenic factors and impaired thrombogenic protective mechanisms. Thrombogenic factors include endothelial cell disruption, subendothelial exposure due to endothelial cell loss, platelet activation or its interaction with subendothelial collagen or von Willebrand factor, activation of coagulation, disruption of the fibrinolytic system, and stasis. The thrombogenic protective mechanism consists of antithrombin III factor released by intact endothelial cells, neutralization of active clotting factors by endothelial cell components, inhibition of active clotting factors by coagulation inhibitors, breakdown of clotting factors by protease enzymes, dilution of active clotting factors and platelet aggregates by the bloodstream, and the process of thrombolysis by the fibrinolysis system. Thrombosis is broadly due to impaired function of platelets and coagulation factors.^{4–6} Thrombus consists of fibrin, erythrocytes, leukocytes, and platelets. Arterial thrombus occurs due to rapid blood flow due to pressure from the heart's pump. Arterial thrombi are composed of platelets bound by thin fibrin. Venous thrombi form mainly in areas of stasis with slower blood flow and consist of erythrocytes, large amounts of fibrin, and platelets. One of the diseases caused by venous thrombosis is CVST.

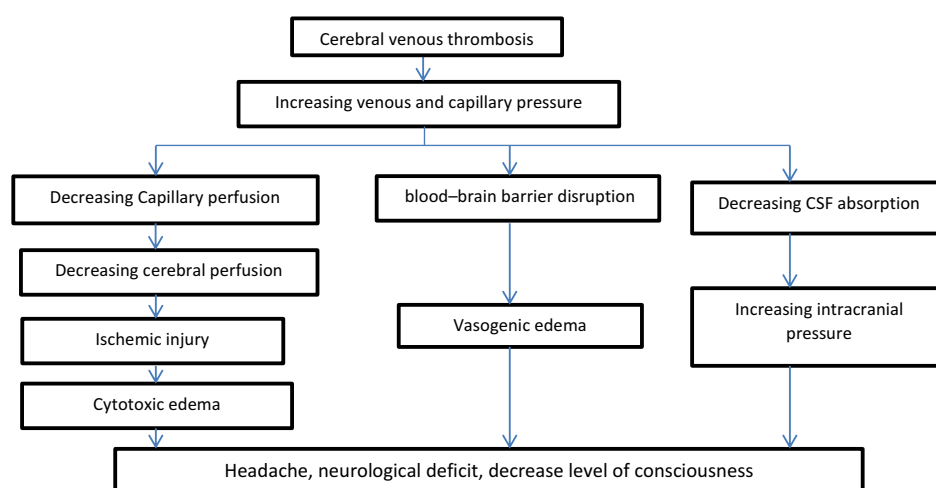


Figure 2 Pathogenesis of cerebral sinus venous thrombosis.

Note: Data from Saposnik G, Barinagarrementeria F, Brown RD, et al. Diagnosis and management of cerebral venous thrombosis: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2011;42(4):1158–1192.¹⁶

Hemostasis

Hemostasis means that blood remains in the vascular system. The components in the hemostasis mechanism are: platelets, vascular endothelium, procoagulant plasma protein factors, natural anticoagulant proteins, fibrinolytic proteins, and antifibrinolytic proteins. All these components must be available in sufficient quantities with good function and in the right place to be able to carry out the hemostasis mechanism properly. The interaction of components that can trigger the occurrence of thrombosis is referred to as prothrombotic properties. The interaction between components that inhibit excessive thrombotic process is referred to as antithrombotic properties. Hemostasis function can run normally if there is a balance between prothrombotic factors and antithrombotic factors.⁶⁻⁸ This hemostasis is played by blood vessel spasm, adhesion, platelet aggregation and active involvement of coagulation factors. These components try to keep the blood fluid and remain in the vascular system and form a temporary thrombus or hemostatic thrombus on the damaged blood vessel wall (vascular injury).^{6,7} Hemostasis and thrombosis consist of three phases: formation of platelet aggregation, formation of fibrin webs, and partial or total dissolution of aggregates by plasmin. The process of formation of initial platelet aggregation is temporary at the wound site. Platelets will bind to collagen at the site of vascular injury and are activated by thrombin which is formed in a cascade of coagulation events at the same site. Platelets can also bind to ADP released by other active platelets. Platelets will change shape, then carry out the aggregation process in the presence of fibrinogen to form a hemostatic plug or thrombus. The formation of fibrin webs or threads bound with platelet aggregates forms a stronger and more stable hemostatic plug or thrombus. Partial or total dissolution of hemostatic aggregates or thrombi by plasmin is the final phase of hemostasis.^{18,20} Hemostasis consists of primary hemostasis, secondary hemostasis and tertiary hemostasis. Primary hemostasis consists of vascular components and platelets. Primary hemostasis is the first to be involved in the process of stopping blood when bleeding occurs. This process begins with vasoconstriction of blood vessels and the formation of platelet plaques that close the wound and stop bleeding. Secondary hemostasis consists of coagulation and anticoagulation factors. The end of the secondary hemostatic mechanism is the formation of fibrin threads. Tertiary hemostasis is an advanced hemostasis mechanism played by blood. The clot or hemostatic plug that has been formed will be destroyed in the fibrinolysis system. This tertiary hemostasis aims to control so that coagulation activity is not excessive.²⁰ Primary hemostasis plays a role in the event of injury or disruption of the endothelium. Platelets will immediately perform this function by performing adhesion. Furthermore, the platelets will stick to the open wound, namely the collagen fibers. Platelets then become activated and secrete the contents of the granules. Platelet granules will attract other platelets to perform aggregation so that platelets gather around the injured area.⁶ These platelets will clump together and clog and cover the wound.²⁰ The process that occurs in primary hemostasis is shown in Figure 3.

Platelets secrete endogenous granules and dense granules, serotonin, catecholamines and the expression of GPIIb-IIIa receptors after adhesion, which ultimately leads to platelet aggregation. In the final stage there is a process of formation of

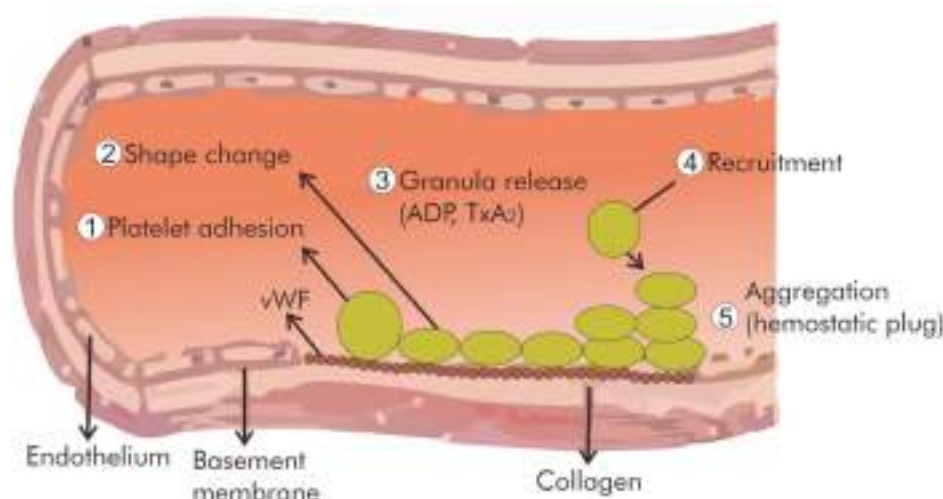


Figure 3 Component in primary hemostasis.

Note: Data from Kamisli O, Kamisli L, Kablan Y, Gonullu S, Ozcan C. The Prognostic Value of Increased Mean Platelet Volume and Platelet Distribution Width in the Early Phase of Cerebral Venous Sinus Thrombosis. *Clinn App Thrombosis Hemostasis*, 2013;19(1):29–32.⁷

a platelet plug that involves fibrinogen and von Willebrand factor called platelet aggregation.^{9,10} Light transmission aggregometry is a method used to examine platelet aggregation. This method is the gold standard for examining platelet function.¹¹ Platelet aggregation is the formation of cross-linked platelets through active GPIIb/IIIa receptors with fibrinogen bridges. Platelets in the inactive state have approximately 50–80,000 GPIIb/IIIa receptors that do not bind to fibrinogen, VWF, or other ligands. Fibrinogen serves as a scaffold for platelet aggregation via the activated form of integrin α IIb β 3 (also known as glycoprotein IIb/IIIa). Platelet aggregation via fibrinogen cross-linking provides an initial hemostatic barrier following blood vessel injury as part of the rapid primary hemostatic response.²¹ Platelet stimulation by ADP for example will cause an increase in GPIIb/IIIa molecules. This condition allows for reversible cross-linking of platelets with fibrinogen bridges.^{1,2} This process will cause a positive feedback, namely the activation of cytosolic phospholipase A2 (PLA2) which then converts platelet phospholipids into arachidonic acid. Arachidonic acid is then converted by the cyclooxygenase 1 (Cox-1) enzyme into prostaglandin G2 (PGG2) which will then be converted into prostaglandin H2 (PGH2) by the peroxidase enzyme. Furthermore, PGH2 will be converted by the enzyme thromboxane synthase into thromboxane A2 (TXA2). Thromboxane A2 is a potent platelet aggregator that will strengthen platelet aggregation to form a more stable (irreversible) aggregate. Thromboxane A2 acts on surface receptors and activates phospholipase C leading to the formation of inositol triphosphate which causes an increase in intracellular calcium. Calcium converts inactive GPIIb/IIIa receptors on the platelet membrane to a high affinity for fibrinogen which forms cross-links between platelets and causes aggregation.^{11,12}

Thrombosis

Examination of platelet abnormalities can be seen from the number, function, and basis of the abnormality. Platelet function abnormalities such as hyperaggregation are caused by impaired adhesion function, impaired release reactions, and impaired aggregation function. Impaired adhesion function can be examined by expression of cluster of differentiation 62 platelets (CD62P) or platelet-selectin (P-selectin), binding of procaspase activating compound 1 (PAC-1), fibrinogen or annexin V. Impaired reaction to release of platelet granules and compounds others can be checked with beta-thromboglobulin, platelet factor 4 or CD62P/P-selectin. Furthermore, platelet function was measured by the formation of procoagulant membranous microvesicles, platelet aggregation, formation of platelet-leukocyte conjugates, and changes in platelet volume. The GPVI was shown by several *in vitro* and *in vivo* studies to be essential for activation of the integrin for stable adhesion and subsequent signal transduction (via activation of phosphatidylinositol-3-kinase and phospholipase C γ 2) that leads to granule release, activation of the GPIIb/IIIa via inside out signaling, and platelet aggregation.²² Beta-thromboglobulin is a type of chemokine ligand (C-X-C motif). This marker is a strong chemoattractant for fibroblasts and weak for neutrophils. Beta-thromboglobulin is a stimulator of mitogenesis, extracellular matrix synthesis, glucose metabolism, and the synthesis of plasminogen activator in human fibroblasts. These markers also affect megakaryocyte maturation, and thus help in regulating platelet production. The level of beta-thromboglobulin is used to index platelet activation. These markers are measured by the enzyme-linked immunosorbent assay (ELISA) method in blood plasma or urine, and often in conjunction with platelet factor.¹⁴ Platelet factor 4 (PF4) is a minor cytokine belonging to the CXC family of chemokines also known as a chemokine (CXC motif) ligand 4 (CXCL4). These chemokines are released from platelet alpha granules that are activated during platelet aggregation, and promote blood coagulation by moderating the effects of heparin-like molecules. Platelet factor 4 is thought to play a role in wound repair and inflammation. Platelet factor 4 is a 70-amino acid protein released from 22 alpha granules from activated platelets and binds with high affinity to heparin. The main physiological role of platelet factor 4 is to neutralize heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin activity and promoting coagulation. Platelet factor 4 as a strong chemotactic for neutrophils and fibroblasts so that it plays a role in inflammation and wound repair. The chemokine PF4 is chemotactic for neutrophils, fibroblasts, and monocytes, and interacts with the splice variant of the chemokine receptor CXCR3, known as CXCR3B. The chemokine PF4 is an antigen in heparin-induced thrombocytopenia, a preferential autoimmune reaction to heparin anticoagulants.⁸ In addition to beta-thromboglobulin and PF4, we will discuss P-selectins, which are important in hemostasis in CVST.

Platelet-selectin (P-selectin)

P-selectin is the result of activation of endothelial cells and platelets.^{6,19} Experts explain that P-selectin levels can describe the process of thrombosis. André et al's research showed that P-selectin expressed by platelets was able to give a signal that made

blood component particles collect to form components of thrombus plaque. Some studies showed that P-selectin levels were significantly increased in ischemic patients compared to controls. P-selectin levels were significantly increased within 24 hours in patients with transient ischemic attack (TIA) and ischemic stroke compared with healthy control patients.^{7,8}

Platelet-selectin is a type 1 transmembrane protein in platelet granules and megakaryocyte granules. Activated platelets will experience granule fusion with the endothelial plasma membrane, causing P-selectin exposure to the activated platelet surface. Platelet-selectin also plays a role in mediating the interaction between leukocytes and a ligand that helps the process of adhesion of leukocytes and platelets, namely P-selectin glycoprotein ligand-1 (PSGL-1).¹⁹ Platelet selectin glycoprotein ligand-1 brings leukocytes and platelets to adhere to each other in damaged endothelium and eventually platelet aggregation occurs to plug the wound caused by inflammation. Platelet-selectin causes adhesion and secretion of granules, which in turn causes platelet aggregation.^{8,19} CVST patients may experience hyperaggregation of platelets. Hyperaggregation is a condition that shows the patient's blood clots more quickly. This process can be assessed by a platelet aggregation test.⁶ Platelets secrete granules (such as fibrinogen, von Willebrand factor/vWF), endogenous dense granules (such as ADP), serotonin, catecholamines and glycoprotein IIb-IIIa (GPIIb-IIIa) receptors after adhesion. In the final stage there is a process of formation of a platelet plug involving fibrinogen and von Willebrand factor called platelet aggregation.^{9,10} Stimulation of platelets by ADP will cause an increase in GPIIb/IIIa molecules. This condition allows for the reversible cross-linking of platelets with fibrinogen bridges. This process will cause positive feedback, namely the activation of cytosolic phospholipase A2 (PLA2) which then converts platelet phospholipids into arachidonic acid. Arachidonic acid is then converted by the cyclooxygenase 1 (Cox-1) enzyme into prostaglandin G2 (PGG2), which will then be converted into prostaglandin H2 (PGH2) by the peroxidase enzyme. Furthermore, PGH2 will be converted by the enzyme thromboxane synthase into thromboxane A2 (TXA2). Thromboxane A2 is a potent platelet aggregator that will strengthen platelet aggregation to form a more stable (irreversible) aggregate. Thromboxane A2 acts on surface receptors and activates phospholipase C leading to the formation of inositol triphosphate which causes an increase in intracellular calcium. Calcium converts the inactive GPIIb/IIIa receptor on the platelet membrane into a high affinity for fibrinogen, which forms cross-links between platelets and causes aggregation.¹¹ Cleanthis et al investigated that the percentage of platelet aggregation was positively correlated with P-selectin levels in study subjects with exercise.⁹ Research results Sun et al showed that the percentage of platelet aggregation was positively correlated with P-selectin levels in postpartum patients with DVT.¹⁰ Previous study showed that the mean P-selectin value in patients with chronic periodontitis was higher than the mean P-selectin value in the control group.¹¹

The Role of Platelet-selectin (P-selectin) in Cerebral Venous Thrombosis

P-selectin is produced by the endothelial cells that line the vessel walls of the circulatory system. Platelet-selectin is found in endothelial cells and platelets. Selectin is one of three families of type 1 cell surface glycoproteins consisting of E-, L- and P-selectins. These three types of selectins bind to the same sugar structure and these molecules are responsible for different targets: (1) P-selectin to secretory granules, (2) E-selectin to the plasma membrane and (3) L-selectin to the fold tip of leukocytes. The extracellular region of the P-selectin consists of three distinct domains like any other selectin type; A C-type lectin domain at the amino terminus (N-terminus), an epidermal growth factor (EGF) domain and a complement binding protein domain (same as complement regulatory protein/CRP) which has a short consensus repeat (~60 amino acids). The number of CRP repeats is the main feature that distinguishes selectin types in the extracellular region. In humans, P-selectin has nine repeats whereas E-selectin contains six and L-selectin has only two. Platelet selectins are anchored in a transmembrane region followed by a short cytoplasmic tail region. The parameter P-selectin was first identified in endothelial cells in 1989. This protein is located on chromosome 1q21-q24, with 28 ranges >50 kb and contains 17 exons in humans.⁹⁻¹¹

The major ligand for P-selectin is PSGL-1, which is expressed on almost all leukocytes. P-selectin also binds to heparin sulfate and fucoidans. Platelet-selectin glycoprotein ligand-1 is located on various hematopoietic cells such as neutrophils, eosinophils, lymphocytes, and monocytes, where it mediates the tethering and adhesion of these cells. However, PSGL-1 is not specific for P-selectin, because it can also function as a ligand for E-selectin and L-selectin. Platelet-selectin is constitutively expressed in megakaryocytes (platelet precursors) and endothelial cells. P-selectin expression is induced by two different mechanisms. The first mechanism is that P-selectin is synthesized by megakaryocytes and endothelial cells, where it is sorted into secretory granule membranes. When megakaryocytes and endothelial cells are activated by agonists such as thrombin, P-selectin is rapidly translocated to the plasma membrane from the granule. The second mechanism is an increase in P-selectin protein levels induced

by inflammatory mediators such as tumor necrosis factor- α (TNF- α), lipopolysaccharide (LPS), interleukin-4 (IL-4), and interleukin-13 (IL-13). Translocation of P-selectin to the plasma membrane in endothelial cells and activated platelets.⁸ Platelet-selectin plays an important role in the initial recruitment of leukocytes to the site of injury during inflammation. P-selectin moves from internal cell sites to the endothelial cell surface when endothelial cells are activated by molecules such as histamine or thrombin during inflammation. Platelet-selectin glycoprotein ligand-1 (PSGL-1) carries leukocytes and platelets to the wound site.^{9,11}

Platelet-selectin is critical in platelet recruitment and aggregation in areas of vascular injury. Platelet activation (via agonists such as thrombin, type II collagen and ADP) results in a “membrane reversal” in which the platelet releases dense granules and the inner wall of the granules opens on the outside of the cell. Platelet-selectin then promotes platelet aggregation through platelet-fibrin adhesion and platelet-platelet adhesion. Platelet-selectins function as cell adhesion molecules (CAM) on the surface of activated endothelial cells. This molecule coats the inner surface of blood vessels and activated platelets.^{8–10} P-selectin parameter is a marker of granule secretion and platelet activation.^{9,11} Thrombosis in CVST can occur due to abnormalities of platelets and coagulation factors.⁴ Thrombus that causes CVST is caused by platelet disorders, namely hyperaggregation of platelets around 22–25%. Thrombus caused by coagulation factors is about 38%.^{7–9} Examination of platelet abnormalities can be seen from the number, function, and basis of the abnormality. Platelet function abnormalities such as hyperaggregation are caused by impaired adhesion function, impaired release reactions, and impaired aggregation function. Impaired adhesion function can be examined by expression of cluster of differentiation 62 platelets (CD62P) or platelet-selectin (P-selectin), binding of procaspase activating compound 1 (PAC-1), fibrinogen or annexin V. Impaired reaction to release of platelet granules and compounds others can be checked with beta-thromboglobulin, platelet factor 4 or CD62P/P-selectin, procoagulant formation of membranous microvesicles, platelet aggregation, formation of platelet-leukocyte conjugates, and changes in platelet volume. The process of adhesion and secretion of platelet granules can be assessed by examining platelet-selectin levels. High platelet-selectin in plasma is the result of activation of endothelial cells and platelets which can be a predictor of thrombosis in CVST cases.^{9,11}

Conclusion

Thrombosis in CVST occurs due to the formation of blood clots in cerebral veins, which can be caused by impaired platelet function. Platelet function abnormalities in CVST cases can be in the form of impaired adhesion function, impaired release or secretion reactions, and impaired aggregation function. Platelet-selectin is critical in platelet recruitment and aggregation in areas of vascular injury. High platelet-selectin in plasma is the result of activation of endothelial cells and platelets, which can be a predictor of thrombosis in CVST cases.

Disclosure

The author reports no conflicts of interest in this work.

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Thrombocytopenia Among Pregnant Women in Southwest Ethiopia: Burden, Severity, and Predictors

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Background: Thrombocytopenia is a worldwide public health problem in pregnant women and is associated with significant maternal–fetal complications and mortality. Determination of burden, severity, and predictors of thrombocytopenia in pregnant women is imperative to develop intervention measures to mitigate their risk and public health impact, which is not well known in Ethiopia, particularly in the selected study area. Thus, this study aimed to determine the burden, severity, and predictors of thrombocytopenia among pregnant women in Wolkite University Specialized Hospital, southwest Ethiopia.

Materials and Methods: A cross-sectional study was employed among 422 pregnant women from May to August 2021. A structured questionnaire was used to collect data on socio-demographic, reproductive, and related characteristics. Venous blood samples were collected and platelet counts were determined by Sysmex XP-300 hematology analyzer. A binary logistic regression analysis was performed using SPSS version 22 software to identify independent predictors of thrombocytopenia. The p-value <0.05 was considered statistically significant.

Results: The overall burden of thrombocytopenia among pregnant women was 14.5% (95% CI: 11.4–17.8). Among them, 77%, 16.4%, and 6.6% had mild, moderate, and severe thrombocytopenia, respectively. Rural residence (AOR: 2.9, 95% CI: 1.5–5.8), increasing age (AOR: 1.9, 95% CI: 1.04–3.44), alcohol consumption (AOR: 2.48, 95% CI: 1.3–4.5), and HIV infection (AOR: 8.5, 95% CI: 1.9–18.4) were independent predictors of thrombocytopenia among the pregnant women.

Conclusion: A high burden of thrombocytopenia (14.5%) was observed among the pregnant women in this study area. Rural residence, increasing age, consumption of alcohol, and HIV infection were identified as independent predictors of thrombocytopenia. The findings of this study should be taken into consideration to conduct appropriate intervention measures on identified predictors and implement routine screening of platelet count, thrombocytopenia diagnosis, and treatments to reduce the burden of morbidity and mortality among pregnant women.

Keywords: pregnant women, thrombocytopenia, predictors, Wolkite, Ethiopia

Introduction

Thrombocytopenia is a substantial hematological abnormality among pregnant women, affecting approximately 7–11% of pregnant women around the world.^{1,2} Developing countries share a huge burden of thrombocytopenia. A study revealed 8.4% of the overall global burden of thrombocytopenia among pregnant women; with the highest burden reported from Africa (15.3%) and the lowest from Taiwan.³

Thrombocytopenia in pregnant women was defined as a blood platelet (PLT) count below $150 \times 10^9/L$,⁴ and it is categorized as mild (PLT, $100\text{--}150 \times 10^9/L$), moderate (PLT, $50\text{--}100 \times 10^9/L$), and severe thrombocytopenia (PLT, $<50 \times 10^9/L$).⁵ During pregnancy, thrombocytopenia might be associated with gestational conditions (it accounts for 70–80%), hypertensive disorders (15–20%), immune-mediated conditions (3–5%),^{6–9} chronic infections (malaria, HIV,

HBV, HCV),^{6,10} hematological malignancy,¹¹ folate deficiency, and adverse reaction from the drug.^{6,12} Furthermore, impaired platelet production or destruction, increased platelet sequestration and aggregation as well as increased platelet consumption in the peripheral tissue were the main underlying mechanisms that might contribute to the development of thrombocytopenia during pregnancy.^{6,7,12}

Thrombocytopenia is a common problem during pregnancy and is associated with several negative consequences such as excessive bleeding during or after childbirth, maternal and fetal complications,^{10,13} which may result in significant morbidity and mortality for both mother and fetus.^{3,14} Several epidemiological studies reported 19%, 15.8%, and 8.8–10.5% burden of thrombocytopenia among pregnant women in Libya,⁹ Uganda,¹⁵ and India,^{2,16,17} respectively. Furthermore, the risk of bleeding was significantly higher among severe thrombocytopenic women,^{15,18} which requires appropriate monitoring and management. The studies revealed 6.6%, 7%, 11.54%, and 10.3% prevalence of severe thrombocytopenia among pregnant women in Uganda,¹⁵ India,¹⁹ Hawassa,⁴ and Gondar,²⁰ respectively. In addition, a high burden of severe thrombocytopenia was reported in pregnant women who had bleeding problem.⁴ Therefore, assessing the burden and severity of thrombocytopenia in pregnant women is important to reduce the risk of bleeding, maternal and fetal complications as well as related mortality, which is not frequently detected and appropriately described in Ethiopia. In addition, different studies suggested that evaluation of thrombocytopenia routinely by checking the platelet count and peripheral smear in early pregnancy and third trimester would aid in the early diagnosis of thrombocytopenia.^{3,4,8} In Ethiopia, particularly in the selected study area, complete blood counts are routinely performed for all pregnant women attending their first antenatal care (ANC); however, there is no follow-up on platelet count particularly during the third trimester, labour, and delivery until severe complications were ensured. Determining severity pattern, burden, and predictors of thrombocytopenia plays a great role in the management of thrombocytopenic women and is important to develop evidence-based intervention measures, which are not well known in this study area. Therefore, this study aimed to determine thrombocytopenia burden, severity, and predictors among pregnant women in Wolkite University Specialized Hospital, southern Ethiopia.

Materials and Methods

Study Design, Area, and Period

A cross-sectional study was employed from May to August 2021 at Wolkite University Specialized Hospital in the Gurage zone, Southern Nation Nationality and Peoples Regional State (SNNPR), Ethiopia. The hospital is located 170km far away from the capital city of Ethiopia, Addis Ababa and it provides services for more than 1.2 million inhabitants living in the Gurage zone and surrounding districts. The study was conducted among pregnant women who attended ANC clinics at Wolkite University Specialized Hospital.

Sample Size Determination and Sampling Technique

A single population proportion formula was used to determine the sample size for the study by considering a 95% level of confidence, 10% non-response rate, 5% margin of error, and 50% proportion of thrombocytopenia. Finally, we got sample sizes of 422. Accordingly, pregnant women (ages range, 15–49 years) who attended the ANC clinic during the data collection period were consecutively included in the study until attaining the final sample size. Study participants who took drugs such as heparin and non-steroidal anti-inflammatory drugs like aspirin, had bleeding manifestations, splenomegaly, and chronic disease (HCV, HBV, and hypertension) were excluded from the study after reviewing their records.

Data Collection and Analysis Method

Data on socio-demographic, reproductive, and related characteristics were collected by structured and pre-tested questionnaires through interviews by a trained midwife. Four milliliters of venous blood samples were collected in an EDTA tube and platelet counts were determined by the Sysmex XP-300 hematology analyzer. Thin blood films were prepared, air-dried, and then stained with Wright stain to examine the presence of platelet aggregation and malaria parasites. Thrombocytopenia among pregnant women was defined as a platelet count (PLT) below $150 \times 10^9/L$ and thin blood films were checked for the presence of agglutination of the platelet. The severity of thrombocytopenia was categorized into mild (PLT, $100\text{--}150 \times 10^9/L$), moderate (PLT, $50\text{--}99 \times 10^9/L$), and severe thrombocytopenia ($PLT < 50 \times 10^9/L$).²⁰

Data Processing and Analysis

The data were entered and analyzed using SPSS version 22 software and presented in tables using descriptive summaries. Binary logistic regression analysis was performed to determine the presence and strength of an association between the prevalence of thrombocytopenia and independent predictors. Bivariate analysis was performed for each independent predictor to select candidate predictors for multivariate analysis by considering a p-value <0.25. Multivariate analysis was used to control the effect of confounding variables and to identify significantly associated factors with the prevalence of thrombocytopenia. The 95% CI, crude odds ratio (COR), and adjusted odds ratio (AOR) were used to see the strength and directions of associations. The p-value <0.05 was considered statistically significant.

Results

A total of 422 pregnant women were included in this study with a response rate of 100%. The mean ($\pm$ SD) age of the study participants was 29.1 $\pm$ 5.3 years (age ranges from 18 to 40). The majority of the study participants 220 (52.1%) were aged $\leq$ 29 years and rural residents 220 (52.1%). About 176 (41.7%) were in their third trimester, 129 (30.6%) in the second trimester, and 117 (27.7%) in the first trimester. Among the study participants, about 135 (32%) were illiterate (unable to read and write), 207 (49.1%) were housewives, and 126 (29.9%) had a habit of alcohol consumption during the time of the data collection (Table 1).

Table 1 Socio-Demographic, Reproductive, and Related Characteristics of Pregnant Women Attending Antenatal Care Clinics at Wolkite University Specialized Hospital, Southwest Ethiopia, May to August 2021

Variables	Categories	Frequency (Percentage)
Age in years	$\leq$ 29	220 (52.1%)
	>29	202 (47.9)
Residence	Urban	202 (47.9)
	Rural	220 (52.1)
Marital status	Singe	23 (5.5)
	Married	399 (94.5)
Educational status	Illiterate	135 (32)
	Primary	65 (15.4)
	Secondary	104 (24.6)
	Tertiary	30 (7.1)
	College/university	88 (20.9)
Occupational status	Housewife	207 (49.1)
	Merchant	91 (21.6)
	Farmer	47 (11.1)
	Government employee	77 (18.2)
Monthly income in Ethiopia Birr	$\leq$ 1500	322 (76.3)
	>1500	100 (23.7)

(Continued)

Table 1 (Continued).

Variables	Categories	Frequency (Percentage)
History of excess menstrual bleeding	No	401 (95)
	Yes	21 (5)
History of abortion	No	349 (82.7)
	Yes	73 (17.3)
ANC follow up in previous pregnancy	No	150 (35.5)
	Yes	272 (64.5)
Iron or folate supplementation	No	160 (37.9)
	Yes	262 (62.1)
Trimester	First	117 (27.7)
	Second	129 (30.6)
	Third	176 (41.7)
Number of children	No	89 (21.1)
	One	136 (32.2)
	Two	83 (19.7)
	Three	58 (13.7)
	Four or more	56 (13.3)
Alcohol consumption	No	296 (70.1)
	Yes	126 (29.9)
HIV status	Negative	413 (97.9)
	Positive	9 (2.1)

Prevalence and Severity Pattern of Thrombocytopenia Among Study Participants

The mean ($\pm$ SD) of the PLT count among the study participant was $232.07 \pm 77.48 \times 10^9/L$. The overall prevalence of thrombocytopenia among the study participant was 61 (14.5%) with 95% CI (11.4–17.8). Among thrombocytopenic study participants, 47 (77%), 10 (16.4%), and 4 (6.6%) had mild, moderate, and severe thrombocytopenia, respectively.

Independent Predictors of Thrombocytopenia Among the Study Participants

In the bivariate analysis: being a rural dweller, increasing age, educational status, history of excess menstrual bleeding, alcohol consumption, and being HIV positive were identified as candidate predictors for multivariate analysis by considering p-value <0.25 (Table 2).

A multivariate binary logistic regression model was used to identify independent predictors of thrombocytopenia among study participants. A significantly high prevalence of thrombocytopenia was observed in older study participants (18.3%) as compared to younger participants (AOR: 1.9, 95% CI: 1.04–3.44, $p=0.03$). Rural area residents were ~3 times higher odds of thrombocytopenia as compared to urban residents. Study participants who had a habit of alcohol consumption at least once per week were 2.48 times more likely to develop thrombocytopenia than their counterparts. A higher proportion of thrombocytopenia (55.6%) was found in HIV seropositive than seronegative study participants, which was significant (AOR: 8.5, 95% CI: 1.9–18.4) (Table 2).

Table 2 Predictors Associated with Thrombocytopenia Among Study Participants, 2021

Variables	Categories	Thrombocytopenia		COR (95% CI)	P-value	AOR (95% CI)	P-value
		No	Yes				
Age in years	≤29	196 (89.1)	24 (10.9)	1		1	
	>29	165 (81.7)	37 (18.3)	1.83 (1.05, 3.18)	0.032	1.9 (1.04, 3.44)	0.03
Residence	Urban	186 (92.1)	16 (7.9)	1		1	
	Rural	175 (79.5)	45 (20.5)	2.98 (1.6, 5.4)	< 0.00	2.9 (1.5, 5.8)	0.001
Educational status	Illiterate	105 (77.8)	30 (22.2)	2.5 (1.1, 5.5)	0.024	2.1 (0.9, 5)	0.07
	Primary	57 (87.7)	8 (12.3)	1.2 (0.4, 3.3)	0.68	0.8 (0.2, 2.5)	0.7
	Secondary	97 (93.3)	7 (6.7)	0.6 (0.22, 1.77)	0.36	0.6 (0.2, 1.8)	0.4
	Tertiary	23 (76.7)	7 (23.3)	2.6 (0.89, 7.9)	0.07	1.8 (0.5, 5.8)	0.3
	Higher	79 (89.8)	9 (10.2)	1		1	
History of excess menstrual bleeding	No	345 (86)	56 (14)	1		1	
	Yes	16 (76.2)	5 (23.8)	1.9 (0.6, 5.4)	0.21	3 (0.96, 9.7)	0.059
Alcohol consumption	No	263 (88.9)	33 (11.1)	1		1	
	Yes	98 (77.8)	28 (22.2)	2.27 (1.3, 3.9)	0.004	2.48 (1.3, 4.5)	0.003
HIV status	Negative	357 (86.4)	56 (13.6)	1		1	
	Positive	4 (44.4)	5 (55.6)	7.9 (2.07, 30.5)	0.002	8.5 (1.9, 18.4)	0.005

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; COR, crude odds ratio; 1, reference category.

Discussion

Platelet abnormality is a significant problem encountered in pregnant women and is associated with negative impacts on both women and the fetus.^{3,21} The present study attempted to determine thrombocytopenia burden, severity, and predictors among pregnant women at Wolkite University Specialized Hospital, southern Ethiopia. Our finding showed a 14.5% (95% CI: 11.4–17.8) burden of thrombocytopenia among pregnant women; out of them 77%, 16.4%, and 6.6% had mild, moderate, and severe thrombocytopenia, respectively. Rural residence, older age, alcohol consumption, and HIV infection were identified as independent predictors of thrombocytopenia. The burden of thrombocytopenia obtained in our study was consistent with the findings reported from Ethiopia 13.5%,⁴ Ghana 15.3%,¹¹ Uganda 15.8%,²⁵ and India 12.82%.¹⁹

Mohseni et al stated that thrombocytopenia affects around one-tenth of pregnant women and estimated 8.4% (6.9–10.1%) global burden of thrombocytopenia.³ However, our study finding was much higher than the global estimate. These warrants need to routinely evaluate thrombocytopenia in pregnant women as well as implement appropriate intervention measures to alleviate adverse outcomes. Furthermore, our finding was higher than studies reported from different parts of Ethiopia 7.7%–10.2%^{20,22,23} and Iraq 8%,⁵ but it was lower than the study reported from India 19%.²⁴ The variation in the burden of thrombocytopenia might be due to differences in sample size, hematology analyzer, and socio-demographic characteristics. In addition, our result revealed a huge burden of thrombocytopenia which should need extra attention to avoid the occurrence of adverse outcomes in pregnant women. Several literatures suggested that early evaluation of thrombocytopenia in pregnancy is required for optimal care of mother and the neonate as well as to achieve a favorable fetomaternal outcome.^{26,27}

Thrombocytopenia had a significant impact on both women and fetus outcomes. David Ruiru et al²⁵ revealed that women who had thrombocytopenia were significant immediate maternal outcomes such as placenta abruption and postpartum hemorrhage. In addition, a statistically significant association was observed between maternal thrombocytopenia and stillbirths,²⁷ among thrombocytopenic women 3.2%, 15.8%, and 24.5% had bleeding manifestations before

delivery, primary postpartum hemorrhage, and had other antepartum complications (incision site ooze, wound hematoma, episiotomy hematoma, and placental abruption), respectively.²⁷ This implies that pregnant women should be paid more attention to thrombocytopenia to avoid the occurrence of bleeding events before or after delivery.²⁸

Based on the platelet count in the current study, 77%, 16.4%, and 6.6% of pregnant women had mild, moderate, and severe thrombocytopenia, respectively. Our finding was comparable with studies reported from Ethiopia,²⁰ Ghana,¹¹ Uganda,¹⁵ and India.²⁴ The consequences of thrombocytopenia in pregnancy are mostly benign, but moderate-to-severe thrombocytopenia was associated with adverse obstetric and perinatal outcomes.²⁹ A study reported that moderate-to-severe thrombocytopenic women were at a greater risk of cesarean section, antepartum hemorrhage, postpartum hemorrhage, wound hematoma, intrauterine fetal death, preterm delivery, and intrauterine growth restriction as compared with mild thrombocytopenic pregnant women.²⁹ Therefore, it requires careful surveillance of these high-risk women for earlier detection and treatment of complications to decelerate the fetomaternal morbidity.^{26,30}

Several studies revealed that platelet count was significantly affected by different socio-demographic factors.^{20,31} Rural residence was significantly associated with thrombocytopenia in the current study. This was in line with the studies reported from different parts of the world.²⁰ The possible reasons might be a lack of information on the causes and prevention mechanism of thrombocytopenia.

Different literatures reported that platelet count is inversely associated with age.^{32–34} Older age (age > 29 years) was significantly associated with thrombocytopenia. Older study participants had nearly 2 times higher odds of thrombocytopenia than younger age groups. This finding was in agreement with studies reported from different countries.^{9,15} The probable reason for the associations might be due to stem cell reserves. The studies revealed that older individuals had a lower stem cell reserve compared to younger subjects,³⁵ and platelet hyperactivity is increased as age increase.^{31–33,35}

HIV infection is associated with several hematological abnormalities. The current study revealed that there is a statistically significant association between thrombocytopenia and HIV infection. HIV-infected participants were approximately 9 times more likely to develop thrombocytopenia compared to their non-infected counterparts. Our finding was in agreement with a previous study conducted in Kampala, western Uganda.¹⁵ The possible explanations that justify the associations might be due to decreased production,³⁶ reduced life span, increased peripheral sequestration of platelet as well as production of abnormal and dysfunctional megakaryocytes, and peripheral destruction of platelets due to cross-reactivity with HIV antibodies.^{34,36,37}

Our finding revealed a significantly higher proportion of thrombocytopenia among alcohol-consuming study participants. This might be due to the adverse effects of alcohol on platelet production and functions; it results in suppression of platelet production and impaired function.^{38,39}

Limitation of the Study

The result of our study should be interpreted in light of the following limitations; causal links between thrombocytopenia and independent predictors were not established due to the cross-sectional nature of the study design and intestinal parasites were not detected due to logistic constraints. Data on alcohol consumption with regard to type, amount, and duration was not collected.

Conclusion

A high prevalence of thrombocytopenia (14.5%) was observed among the pregnant women in this study area. Rural residence, older age, alcohol consumption, and HIV infection were identified as independent predictors of thrombocytopenia among pregnant women. The finding of this study should be taken into consideration to conduct appropriate intervention measures on identified predictors and implement routine screening of platelet count, treatments, and prevention of thrombocytopenia to reduce the burden of mortality and complications among pregnant women.

Data Sharing Statement

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

Ethical Consideration

Ethical clearance was obtained from Wolkite University Ethical Review Committee. Permission was obtained from hospital administration before commencing the study. Written informed consent was obtained from each study participant after describing the objective and procedures of the study. Every response of the participants was kept confidential and their result was also communicated to their respective physician for proper management and further investigation. The study was carried out in accordance with the Declaration of Helsinki.

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Author Contributions

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

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The authors declared that they have no competing interests in this work.

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Variation in Platelet Activation State in Pre-Donation Whole Blood: Effect of Time of Day and ABO Blood Group

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Background: Whilst there has been investigation into the effect of time of the day on platelet activation and function in healthy individuals, there is a lack of studies in the literature to examine this relationship among platelet donors.

Methods: We assessed the extent of platelet activation by percentage of platelets with surface-expressed P-selectin and flow cytometry in samples of whole blood from a group of qualified platelet donors (n = 84).

Results: The mean (SD) percentage of activated platelets in the pre-donation blood samples was $1.85 \pm 1.57\%$ (range 0.2–7.5%). In univariate analyses, the percentage of activated platelets was significantly and inversely correlated with the collection time (ie, the time of day blood samples were collected) ($r = -0.35$, $p = 0.001$) and positively correlated to mean platelet volume (MPV) ($r = 0.29$, $p = 0.008$). A weaker positive correlation was also observed with ABO blood group ($r = 0.228$, $p = 0.036$). Analysis of the collection time as a categorical variable showed a greater degree of activated platelets in samples collected between 8:00 h and 10:00 h than in samples collected during the hours of >10:00 h $\leq$ 14:00 h (2.5 ± 1.8 versus 1.1 ± 0.74 , $p < 0.001$). In the adjusted linear regression model, collection time was a significant independent predictor of platelet activation state in whole blood ($\beta = -0.26$; $p < 0.001$), as did ABO blood group ($\beta = 0.55$; $p = 0.019$).

Conclusion: Our results show that collection time is the most important predictor of platelet activation state in pre-donation whole blood among platelet donors. This work may have implications for optimizing the timing of platelet donation.

Keywords: platelet activation state, surface P-selectin, donor variability

Introduction

Platelets, are anucleate cells produced by megakaryocytes in the bone marrow that circulate in the bloodstream for about 10 days. Circulating platelets play a central role in the hemostatic response to vascular injury. They are also involved in maintaining vascular integrity and contributed to immune and inflammatory responses.^{1–3} Within an individual, circulating platelets are heterogeneous regarding their number, size, age, buoyant density, and function.^{4,5} Evidence also suggests that circulating platelets in blood from healthy individuals consist of platelet subpopulations with different degrees of activation.^{6,7} The activation state of circulating platelets can be assessed by whole-blood flow cytometry of P-selectin (CD62P) and/or the activated form of integrin α IIb β 3 (CD41/CD61) expression on the surface of platelets⁸ or by measurement of granule release markers, such as beta-thromboglobulin (β TG) and platelet factor 4 (PF4).⁹ In addition, the evaluation of P-selectin expression and the active form of α IIb β 3 integrin (detected by PAC-1 binding, a specific antibody directed against the activated form of integrin α IIb β 3) are also considered as important markers of platelet activity during storage of platelet products.¹⁰

A number of factors may influence the in vivo platelet activation state in healthy individuals. In particular, platelet activation has been shown to vary according to the time of day, with the highest measured levels during the morning

hours. For example, Musumeci et al¹¹ measured the urinary excretion of β TG in a group of healthy individuals and found higher values in the morning than in the late afternoon. The study by Jafri et al¹² reported diurnal variation in platelet activation, based on plasma levels of β TG and PF4 being higher in the early morning hours, which paralleled that of whole-blood platelet aggregation in response to epinephrine. Similarly, a significantly greater percentage of activated platelets, as assessed by P-selectin exposure,¹³ or increased agonist-induced platelet aggregation^{14–17} have been shown to occur during the early morning hours.

Platelet donors, like whole blood and plasma donors, are considered to be one of the healthiest groups of the general population.^{18,19} However, to our knowledge, the association of time of the day with estimates of the *in vivo* state of platelet activation in platelet donors has not been previously investigated. Therefore, the aim of this study was to investigate the temporal variation in platelet activation in pre-donation whole blood samples from a group of qualified platelet donors during normal daytime blood bank hours, typically between 08:00 and 14:00 hours, and whether this varied by demographic (eg, gender, age, and ABO blood group) and hematological factors (ie, platelet and red blood cell indices). As lifestyle factors are shown to influence platelet activation,^{20–23} we also examined if differences in platelet activation state could be influenced by recent (ie, past 24 hours) lifestyle behaviors.

Materials and Methods

Eighty-six platelet donors presenting at the Blood Bank in Akershus University Hospital, Norway, between April 2019 and February 2020 participated in the study. Ethical approval was obtained from the Institutional Review Board at Akershus University Hospital, Norway, and the study complied with the Declaration of Helsinki. Informed written consent was obtained from each participant prior to platelet donation. No donor had taken medications known to alter platelet function within five days of platelet donation. Information on donation time, donation history, and hematologic parameters was available in the donor database. Self-reported recent (past 24 hours) physical activity, smoking, alcohol intake, and sleep quality (see Table 1) were obtained using an anonymous questionnaire. Briefly, donors were asked whether they have had smoked (yes/no), have had physical activity (yes/no), or had consumed alcohol in the past 24 hours (yes/no). Sleep quality was assessed on a five-point scale (1, “very good”; 5, “very poor”) and was dichotomized as very good/good versus moderate. Further details of the survey and results have been described previously.¹⁹

Blood Collection Procedure

Whole-blood samples were collected from the attached sampling bags (containing 42 mL whole blood collected immediately before plateletpheresis) using standard vacutainers (BD Biosciences). For each donor, two pre-donation blood samples were collected. A blood sample was collected into a citrated tube for flow cytometric determination of platelet surface P-selectin expression. Preliminary measurements performed on blood samples from four donors who agreed to undergo a separate pre-donation venipuncture showed that the percentages of activated platelets were similar regardless of whether citrated blood samples were obtained from the sampling bags or collected from the antecubital vein after the first 3 mL of blood was discarded (data not shown). All pre-donation citrated blood samples for flow cytometric analysis were therefore collected from the attached sampling bags and analyzed within 2 hours of collection. A second sample was collected into an EDTA tube for full blood count that included platelet count and hematological parameters as a routine part of the donor testing, and for determination of platelet indices ordered for the purpose of this study (Table 1). The analyses were performed in the central laboratory at Akershus University Hospital using the Sysmex XE-5000 automated hematology analyzer.

Flow Cytometric Analysis of the Platelet Activation

For flow cytometry, 100 μ L of citrated blood (diluted 1:20 with phosphate-buffered saline [PBS] containing 0.1% bovine serum albumin [BSA], pH 7.4) was incubated at room temperature with FITC-conjugated anti-CD61 (5 μ L) and PE-conjugated anti-CD62P (10 μ L) for 15 min. Background fluorescence was assessed by labeling diluted samples with 10 μ L PE-conjugated isotype control antibody (all antibodies from Beckman Coulter, USA). After incubating, the samples were diluted to 4 mL with PBS/BSA and analyzed immediately on a FACS Canto II flow cytometer (BD Bioscience). The percentage of activated platelets (ie, CD61 and CD62P double-positive platelets) was obtained from a total of 10000

Table I Donation History, Results of Laboratory Analyses, and Percentage Distribution of Responses to Questionnaire Items by Gender and Age Group (n = 86)

Variables	Overall (n = 86)	Gender		Age Group	
		Males (n = 38)	Females (n = 48)	22–50 yr (n = 42)	51–69 yr (n = 44)
Age (years)	50.8 ± 9.7 (22–69)	52.1 ± 8.7	49.5 ± 10.4	42.6 ± 6.5	58.4 ± 4.8 [‡]
Previous donations					
Combined (number)	54 ± 41 (4–256)	61 ± 45	49 ± 37	33 ± 23	74 ± 45 [‡]
Apheresis PLTs (number)	14 ± 13 (1–43)	15 ± 13	13 ± 13	10 ± 10	18 ± 13 [†]
Donation interval (week)	10.6 ± 7.3 (2.4–42.8)	12.1 ± 7.3	9.9 ± 7.3	10.4 ± 7.3	10.8 ± 7.4
Hemoglobin (g/dl)	14.1 ± 0.1 (11.7–17.4)	15.0 ± 0.8	13.4 ± 0.8 [‡]	13.8 ± 1.0	14.4 ± 1.2*
Hematocrit (%)	43 ± 3 (36–5.1)	45 ± 2	41 ± 2 [‡]	42 ± 3	44 ± 3*
WB-PLT count (10⁹/L)	274 ± 40 (185–377)	268 ± 34	279 ± 44	275 ± 38	273 ± 42
MPV (fL)	10.5 ± 0.8 (6.7–12.8)	10.5 ± 0.75	10.5 ± 0.85	10.6 ± 0.83	10.4 ± 0.78
IPF (%)	3.0 ± 1.6 (0.7–8.6)	2.9 ± 1.4	3.1 ± 1.7	3.3 ± 1.9	2.8 ± 1.2
IPC (10⁹/L)	8.0 ± 3.9 (2.0–19.6)	7.5 ± 3.5	8.3 ± 4.2	8.7 ± 4.6	7.3 ± 3.0
PDW (%)	12.5 ± 1.5 (9.4–19.6)	12.7 ± 1.8	12.4 ± 1.3	12.6 ± 1.9	12.5 ± 1.1
P-LCR (%)	29.2 ± 5.9 (15.9–48.7)	29.4 ± 6.5	29.1 ± 5.5	29.5 ± 7.0	29.1 ± 4.7
PCT (%)	0.29 ± 0.04 (0.2–0.4)	0.28 ± 0.03	0.29 ± 0.04	0.29 ± 0.04	0.29 ± 0.04
BMI (kg/m²)	28.1 ± 4.7 (19.6–44.2)	28.2 ± 4.1	28.0 ± 5.1	28.9 ± 5.3	27.3 ± 3.9
Sleep duration (hours)	6.8 ± 0.8 (4.5–9.0)	6.6 ± 0.7	7.0 ± 0.8	6.8 ± 0.8	6.8 ± 0.8
Sleep quality n (%)					
Very good/good	73 (85)	32 (84)	41 (85)	35 (83)	38 (86)
Moderate	13 (15)	6 (16)	7 (15)	7 (17)	6 (14)
Recent HB status					
Tobacco smoking					
Yes	4 (5)	2 (5)	2 (4)	1 (2)	3 (7)
No	82 (95)	36 (95)	46 (96)	41 (98)	41 (93)
Physical activity					
Yes	38 (44)	17 (45)	21 (44)	13 (31)	25 (57)
No	48 (56)	21 (55)	27 (56)	29 (69)	19 (43)
Alcohol consumption					
Yes	9 (11)	5 (13)	4 (8)	2 (5)	7 (16)
No	77 (89)	33 (87)	44 (92)	40 (95)	37 (84)
ABO-Rh distribution					
A+/A- (n/n)	40/8	18/3	22/5	17/5	23/3
O+/O- (n/n)	31/7	14/3	17/4	15/5	16/2

Notes: Data are presented as mean ± SD (range) or number (percentage). Column percentages are given. * <0.05 ; $^{\dagger}<0.01$; $^{\ddagger}<0.001$; Mean values were compared using unpaired t-tests, categorical variables were compared using chi-square or Fisher's exact test, as appropriate.

Abbreviations: PLT, platelets; WB, whole blood; MPV, mean platelet volume; IPF, immature platelet fraction; IPC, immature platelet count (calculated by multiplying platelet count × IPF); PDW, platelet distribution width; P-LCR, platelet-large cell ratio; PCT, plateletcrit; BMI, body mass index; HB, health behavior during past 24 hours.

recorded events with the CD61+ gate. Representative FACS plots are shown in Figure 1. In one series of experiments (n = 4), these analyses were performed on pre-donation blood samples that were pre-treated with adenosine diphosphate (ADP, 10 μ M). Pre-incubation of whole-blood samples with ADP was associated with a marked increase in the percentage of activated platelets (78.7 ± 5.3% compared to 1.9 ± 2.0% in unstimulated samples), indicating that platelet activation measured in pre-donation blood samples represents basal, circulating, activation states. In a pilot study among five donors, we also compared the percentage of platelets expressing P-selectin in pre-donation blood samples that were collected into vacutainer tubes containing EDTA (known to cause platelet activation) with those collected in sodium citrate (both samples collected from the attached sampling bags). The results showed a clear increase in the percentage of P-selectin positive platelets: 36.7% ± 31.8% (range 5.7–84.2%) compared with 1.6% ± 1.6% (range 0.11–3.5%) in blood samples collected in sodium citrate.

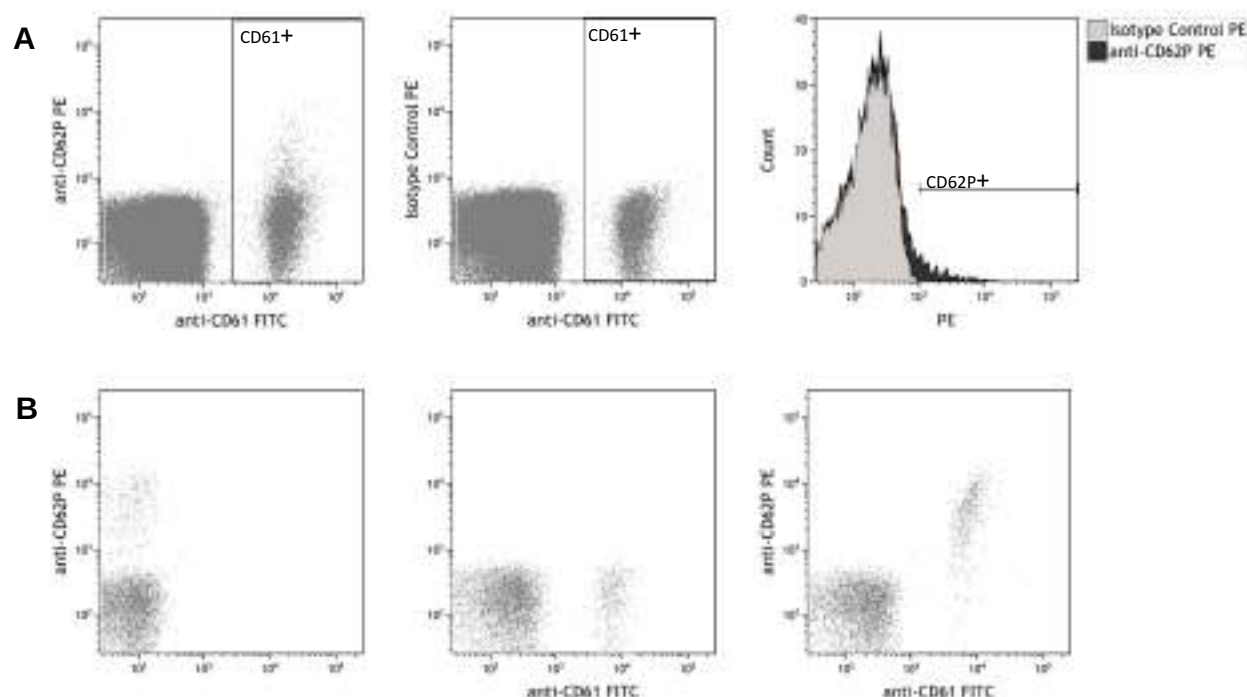


Figure 1 Representative flow cytometry plots of activated platelets in pre-donation whole blood samples. **(A)** Whole blood samples were incubated with anti-CD61 and anti-CD62P (left) or isotype control (middle), washed and analyzed by flow cytometry. Histogram overlay of CD61-positive events is shown to the right. **(B)** Flow cytometry dot plots showing whole blood samples stimulated with ADP.

Statistical Analysis

Continuous variables are summarized with mean $\pm$ standard deviation (SD) and median with interquartile range (IQR). Mean values were compared using unpaired. Categorical variables were compared using Chi-square or Fisher's exact test, as appropriate. The effect of donation time on platelet activation state was assessed either as a continuous variable or as a dichotomous variable using the median value of collection time as a cut point. Associations between platelet activation state and the other study variables were assessed using Pearson's correlation coefficients. Multivariate linear regression modeling (backward stepwise) was used to determine whether the timing of blood collection was an independent predictor of platelet activation state. Variables included were those significant at $p < 0.1$ in the univariate analysis. ABO blood group was included as a dichotomous variable (A group versus O group) in the model. The relative contribution of the independent variables in the regression analysis is presented as the standardized regression coefficient (Beta). Statistical significance was assumed at $p < 0.05$. Because the data for platelet activation were not normally distributed, they were logarithmically transformed, and p values derived from log-transformed data were used in statistical analyses. Analyses were performed using SPSS 25 software.

Results

Donor Characteristics

The donation history, pre-donation laboratory data, and the donor-reported recent (ie, past hours) lifestyle behaviors stratified by gender and age are shown in Table 1. The mean age was 50.8 ± 9.7 years with 56% of donors being female. All donors had previously made at least four donations (whole blood and/or apheresis donations combined) and had donated platelets at least once during the past six years. The mean time since the last donation was 10.6 ± 7.3 weeks. Most (>90%) donors with longer-than-average donation interval had, however, other concurrent donations, mostly plasma. There were significant differences between average values for males and females for both hemoglobin and hematocrit. Older donors had donated a higher number of both combined and platelet donations. They also had higher

values for hemoglobin and hematocrit than younger donors. No significant differences were found in other variables between the demographic subgroups. The donors in this study were ambulatory prior to platelet donation and most likely followed their usual daily activities. They had adequate sleep duration and most (85%) reported good sleep quality the previous night. Four donors had smoked ~70 minutes prior to donation and 38 reported recent physical activity within 13.0 ± 6.8 hours before donation. Nine donors had consumed alcohol the previous night.

Platelet Activation State

Eighty-six pre-donation whole-blood samples were analyzed for platelet activation. One blood sample was excluded because no anti-CD61-binding platelets could be detected, and another was excluded because of failed assay, resulting in a total of 84 samples analyzed. The percentage of activated (ie, P-selectin-expressing) platelets in pre-donation blood samples ranged from 0.2% to 7.5% ($1.85 \pm 1.57\%$; median, 1.3%; IQR, 0.8–2.3%). Twenty-seven donors (32%) had less than 1.0% activated platelets, and five donors (6%) had more than 5.0% activated platelets, indicating considerable donor variability in the levels of circulating activated platelets.

By univariate analysis, the percentage of activated platelets was significantly and inversely correlated with the time of day at which blood samples were collected (hereafter collection time, Table 2 and Figure 2) and positively correlated with mean platelet volume (MPV), a surrogate marker of platelet activation, platelet distribution width (PDW), platelet-large cell ratio (P-LCR), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and red blood cell distribution width (RDW), a measure of variability in red blood cell size (Table 2). Collection time was also marginally negatively ($p = 0.075$) associated with MPV (Table 2 and Figure 2). Platelet count, immature platelet fraction (IPF), a measure of young, reticulated platelets, and immature platelet count (IPC) showed no correlations with the percentage of activated platelets or collection time. IPF and IPC were, however, highly correlated ($r = 0.95$) and both were significantly correlated with MPV, P-LCR and PDW, as expected (Table 2). In the multivariate model with all correlates (Table 3), collection time, RDW, and ABO blood group were each independently correlated with the percentage of activated platelets, whereas MPV remained marginally significant ($p = 0.056$). The model accounted for 30% ($R^2 = 0.298$) of the variance in platelet activation in pre-donation blood.

The inverse association between platelet activation and collection time suggests that a higher proportion of circulating platelets occurs in the earlier hours of the day. To further analyze this association, collection time was dichotomized according to whether the pre-donation samples were collected between 8:00 h and 10:00 h ($n = 46$) or during $>10:00$ h $\leq 14:00$ h ($n = 38$), using the median value of collection time as the cut-off point. The mean percentage of activated platelets between 8:00 h and 10:00 h ($2.5 \pm 1.8\%$) was significantly higher than during the hours of $>10:00$ h $\leq 14:00$ h ($1.1 \pm 0.74\%$, $p < 0.001$), suggesting higher levels of circulating platelet activity at this time of day.

In univariate analyses, there were no statistically significant associations between the percentage of activated platelets in pre-donation blood and gender, age, or BMI, but an association with ABO blood group was observed (Table 3); group

Table 2 Correlation of Platelet Activation in Pre-Donation Blood Samples with Collection Time and Other Covariates ($n = 84$)

Variables	C-Time	P-Count	MPV	IPF	IPC	PDW	P-LCR	MCV	MCH	RDW
%P-selectin+	$-0.35^\dagger$	-0.03	$0.29^\dagger$	0.17	0.17	0.21^*	0.24^*	$0.33^\dagger$	0.22^*	0.28^*
C-time		-0.05	-0.19	0.02	-0.01	-0.12	-0.11	-0.16	-0.08	-0.05
P-count			-0.16	$-0.37^\dagger$	-0.10	$-0.30^\dagger$	$-0.29^\dagger$	-0.06	-0.14	-0.13
MPV				$0.54^\ddagger$	$0.60^\ddagger$	$0.81^\ddagger$	$0.86^\ddagger$	0.15	0.12	0.08
IPF					$0.95^\ddagger$	$0.62^\ddagger$	$0.63^\ddagger$	-0.01	-0.06	0.04
IPC						$0.59^\ddagger$	$0.62^\ddagger$	-0.01	-0.07	0.09
PDW							$0.96^\ddagger$	0.02	0.02	0.00
P-LCR								0.05	0.04	0.00
MCV									$0.86^\ddagger$	$0.53^\ddagger$
MCH										$0.38^\dagger$

Notes: Activated platelets were identified by P-selectin expression above PE-isotype control values. $^* < 0.05$; $^\dagger < 0.01$; $^\ddagger < 0.001$.

Abbreviations: %P-selectin+, percentage of P-selectin-expressing (ie, activated) platelets; C-time, collection time of pre-donation blood; P-count, platelet count; MCV (fL), mean corpuscular volume; MCH (pg), mean corpuscular hemoglobin; RDW (%), red blood cell distribution width. For other abbreviations, see Table 1.

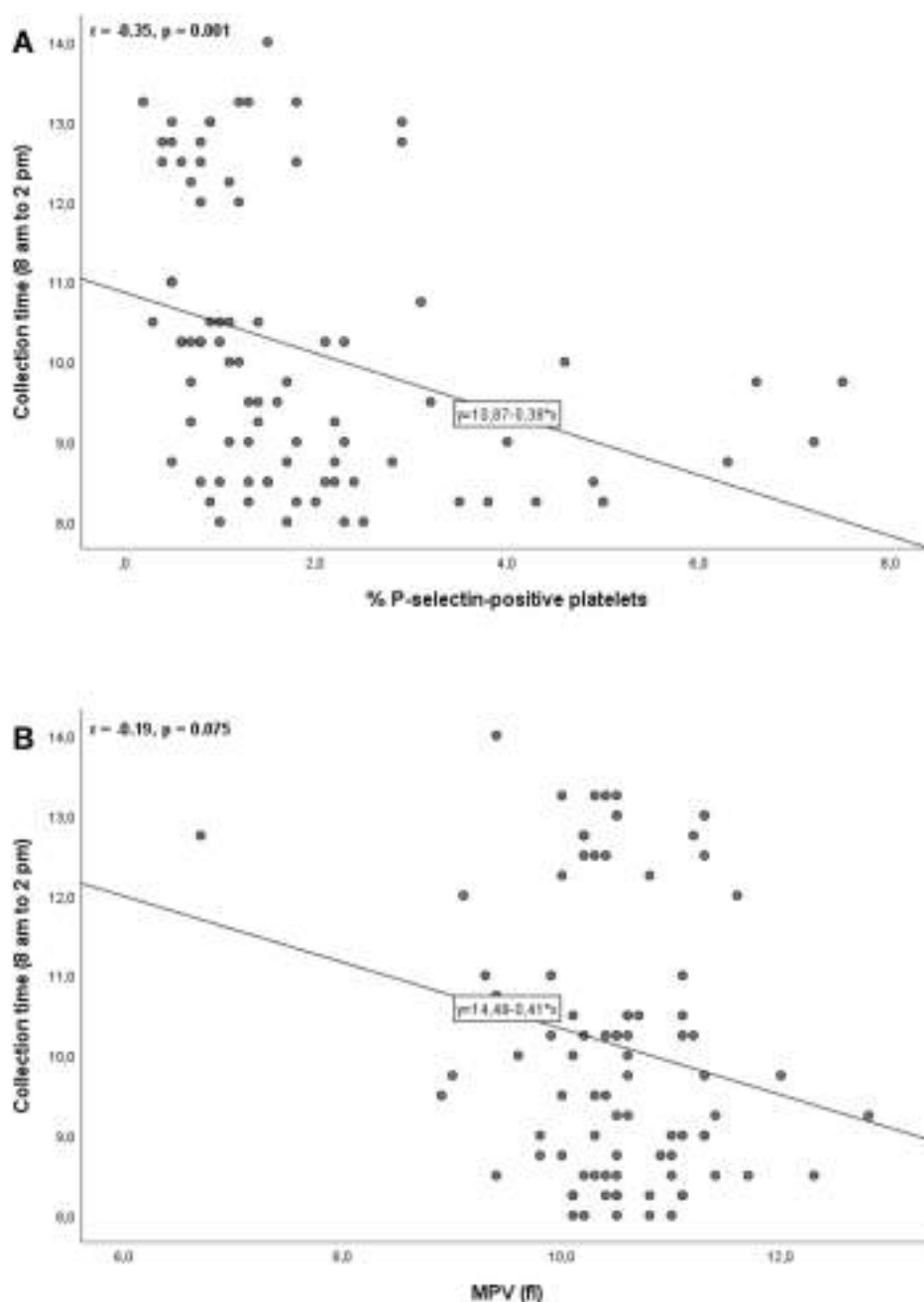


Figure 2 Relationship between (A) the percentage of P-selectin-positive platelets and (B) mean platelet volume (MPV, a surrogate marker of platelet activation) with the time of day at which blood samples were collected (collection time). The percentage of P-selectin-positive platelets, as assessed by flow cytometry, correlated inversely ($r = -0.35$), with the collection time. MPV, as measured via a Sysmex XE-5000 analyzer, was also inversely correlated with the collection time, although it did not reach statistical significance in this study.

A donors had slightly but significantly higher levels of activated platelets than did group O donors (2.1 ± 1.7 versus 1.6 ± 1.4 , $p = 0.037$). Tobacco smoking, physical activity, and alcohol consumption have been associated with increased platelet reactivity in healthy persons.^{20–22} Donors who had smoked prior to donation had a higher mean platelet activation than did non-smokers ($3.0 \pm 3.0\%$ versus $1.8 \pm 1.5\%$ in non-smokers), but this difference was not statistically significant ($p = 0.32$). The mean platelet activation did not differ significantly between donors who engaged and those who did not engage in physical activity in the past 24 hours ($1.7\% \pm 1.4\%$ versus $2.0 \pm 1.7\%$, $p = 0.48$). There was also

Table 3 Results of Univariate (Unadjusted) and Backward Multivariable Linear (Adjusted) Models for Association of Platelet Activation in Whole Blood (n = 84) to Collection Time and Other Factors

Variables	Univariate Analyses		Multivariate Analyses			
	r-value	p-value	β	Std. Error	Beta	p-value
Collection time	-0.346	0.001	-0.26	0.09	-0.29	<0.001
MPV	0.286	0.008	0.12	0.39	0.06	0.056
PDW	0.209	0.056	-0.23	0.38	-0.22	0.836
P-LCR	0.241	0.027	0.06	0.03	0.23	0.864
MCV	0.332	0.002	0.07	0.05	0.17	0.111
MCH	0.217	0.047	-0.11	0.22	-0.10	0.997
RDW	0.275	0.011	0.19	0.07	0.28	0.005
ABO group*	0.228	0.036	0.55	0.31	0.17	0.019
			Adjusted coefficient determination [R^2] = 0.298			

Notes: Data shown are correlation coefficient (r-value), regression coefficient (β) with standard error and the standardized beta coefficient. *ABO blood group was included as a categorical variable and adjusted for other factors in the model. For abbreviations, see Tables 1 and 2.

no significant difference in platelet activation between those who reported recent alcohol consumption and those who did not (1.8 ± 1.3 versus 1.9 ± 1.6 ; $p = 0.95$).

Discussion

Agreeing with previous studies in healthy donors,^{24–26} low levels of activated platelets (0.2–7.5%) were detected in unstimulated pre-donation blood samples. Our study extended these results by showing that platelet activation in pre-donation blood samples is inversely correlated with the collection time. Collection time also showed an inverse though statistically not significant correlation with another marker of platelet activation, namely MPV. The temporal variation in platelet activation was also evident when collection time was treated as a categorical variable, being significantly higher in blood samples collected between 8:00 h and 10:00 h than those collected during the hours of >10:00 h ≤14:00 h, suggesting that there may be a temporal component in the regulation of activation state of circulating platelets, consistent with previous literature.²⁷

In the adjusted regression model, collection time was shown to be independently associated with the percentage of activated platelet, as did ABO blood group and RDW (Table 3). We note, however, that the variables associated with the percentage of platelet activation together explain 30% of the variation in platelet activation, indicating that other, unmeasured, donor and/or environmental factors^{28,29} must play a major role in determining the in vivo platelet activation state.

The physiologic mechanisms that mediate diurnal variation in circulating levels of activated platelets are unclear. Using a forced desynchrony protocol in which 12 healthy volunteers (6 males and 6 females) were studied on a 20-hour recurring cycle in dim-light for 10 days, Scheer and coworkers²⁷ have shown that surface expression of platelet activation-dependent markers P-selectin and the activated form of $\alpha IIb\beta 3$ is under circadian (~24 hour) control, with peaks occurring around 09:00 h and nadir around 17:00 h. However, since platelets are devoid of nuclei and have limited *de novo* protein synthesis capacity,³⁰ they may not contain the necessary components for rhythmic expression of clock gene products.^{31,32} However, platelets may be affected by physiological factors that are not intrinsic to platelets themselves. In fact, several studies in healthy subjects have shown a diurnal variation in a number of hemostatic parameters. For example, a higher clotting tendency and a lower fibrinolytic activity have been reported during the morning hours.^{33,34} In particular, plasma levels of von Willebrand factor (VWF)^{35,36} and prothrombin fragment 1+2, markers of thrombin generation³⁷ have been shown to be higher in the morning/midday in comparison with afternoon/night, which may affect platelet activation pathways, and thereby levels of circulating activated platelets over the course of the day. Diurnal variation in other plasma factors may also have the potential to influence platelet activation pathways. For example, plasma cortisol and adrenalin levels have been shown to have somewhat

similar circadian patterns (the timing of highest values approximately at 9:00 AM and 8:00 AM, respectively),³⁸ and may likely contribute to the enhanced platelet activation in early morning hours.²⁷

Of further note is that plasma levels of VWF have been shown to be significantly higher in non-O than in group O individuals.³⁹ Our finding of higher platelet activation in blood samples from group A donors compared to group O donors could therefore be due to elevated VWF levels in these donors, which can lead to increased platelet activation via the platelet-specific GPIb-IX-V receptor. RDW, a measure of variability in red blood cell size, was the only hematologic variable that is independently associated with the percentage of activated platelets (Table 3). One possible explanation for this observation may be erythrocyte modulation of platelet activation via platelet–erythrocyte interactions. For example, Valles et al⁴⁰ have shown that supernatants of collagen-stimulated platelets after co-incubation with erythrocytes induced far more platelet activation (as measured by P-selectin exposure and PAC-1 binding) than did supernatants of collagen-stimulated platelets in the absence of erythrocytes.

Our study had some limitations. First, platelet activation in blood samples was assessed only once, whereas ≥ 4 measurements on the same day are required in order to determine whether the observed changes in platelet activation truly reflect a diurnal variation, an experimental design clearly not feasible in blood banking research. However, our results showed that there was a 2.3-fold decrease in the percentage of activated platelets in whole blood during the hours of $>10:00$ h $\leq 14:00$ h relative to the percentage of activated platelets detected in blood samples taken between 08:00 h and 10:00 h ($p < 0.001$), indicating that there is still a chance of finding a difference related to diurnal variation in platelet activation. Nevertheless, further well-designed studies which include blood sampling time points during afternoon/evening will be required to more fully characterize the effect of donation time on platelet activation states. Second, platelet activation could also be influenced by dietary composition. However, our study could not control the impact of recent food consumption on platelet activation because donors are recommended to eat/drink prior to their donation. Third, the number of donors who reported recent smoking and alcohol consumption was small, and therefore not powered to detect significant differences.

Conclusions

To our knowledge, this is the first study to investigate the association of time of day with platelet activation state, as measured by P-selectin exposure, among platelet donors during normal daytime blood bank hours. We also investigated the associations of platelet activation with a range of hematologic factors, demographic subgroups defined by age group, gender, and ABO blood group, as well as three donor-reported lifestyle behaviors. Our results showed that platelet activation was significantly and inversely associated with the collection time by univariate analysis and after adjustment for covariates. Our study also showed that blood group A was associated with higher platelet activation. This work may have implications for optimizing the timing of platelet donation, if, for example, the temporal variation in platelet activation at the time of donation can impact the final heterogeneity of the stored platelet concentrates and, potentially, transfusion outcomes. Further studies would be needed to see if the same relationship between donation time and platelet activation state, as we observed in this initial study of pre-donation blood samples, would also be reflected in platelet activation in the corresponding apheresis platelet concentrates and to determine the relative contribution of variability in platelet donation time to the total variation in platelet activation in the final platelet products.

Abbreviations

MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red blood cell distribution width; IPF, immature platelet fraction; IPC, immature platelet count.

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Disclosure

The authors declare no conflicts of interest in this work.

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High Magnitude of Neonatal Anemia Among Sick Newborns Admitted to University of Gondar Comprehensive Specialized Hospital, Northwest Ethiopia

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Introduction: Anemia is a global health problem that affects all ages, particularly children under five years. If not treated early, childhood anemia results in impaired growth, delayed cognitive development, and organ dysfunction. There is a scarcity of studies on the prevalence of anemia among hospitalized sick neonates, especially in developing countries.

Objective: This study aimed to determine the magnitude of anemia and associated factors among hospitalized sick newborns at the University of Gondar Comprehensive Specialized Hospital (UOGCSH).

Methods: An institutional-based cross-sectional study was conducted among admitted newborns from June 1 to September 30, 2020. All newborns whose gestational age (GA) ≥ 28 weeks and postnatal age ≤ 28 days, admitted to UOGCSH during the study period were included in the study. Data were collected by pediatric residents and a systematic random sampling technique was used to select the study participants. Statistical analysis was performed using SPSS version 20. Binary logistic regression was used to identify associated factors with neonatal anemia. P-value < 0.05 was considered statistically significant.

Results: During the study period, 272 newborns were enrolled. The mean hemoglobin value was 15.74 ± 4.27 gm/dL. The prevalence of neonatal anemia in the study population was 30.1% (95% CI: 24.6–35.7). Neonatal age > 7 days (AOR = 4.41, 95% CI: 1.86–10.5), maternal anemia (AOR = 9.93, 95% CI: 4.36–21.6), antepartum hemorrhage (AOR = 4.05, 95% CI: 1.54–10.7), being multiple births (AOR = 4.70, 95% CI: 1.73–12.8), subgaleal hemorrhage (AOR = 7.56, 95% CI: 1.87–30.6), and hyperbilirubinemia (AOR = 3.84, 95% CI: 1.58–9.31) were associated with neonatal anemia.

Conclusion and Recommendation: The prevalence of anemia among hospitalized newborns was high. The current study recommends that healthcare providers should screen anemia among sick newborns who had risk factors. Prevention of maternal anemia and early treatment of obstetric complications will reduce the burden of anemia in sick neonates.

Keywords: newborns, neonatal anemia, associated factors, Ethiopia

Introduction

Anemia is defined by a hemoglobin or hematocrit value that is more than two standard deviations below the mean for age.^{1,2} Anemia is a global health problem that affects all ages.^{3,4} Based on the World health organization (WHO) 2011 report, the global estimated prevalence of anemia was 47% among under-five children.^{3,5} The prevalence of anemia in newborns varies and its prevalence rate ranges from 10 to 60%; being the highest range led by African countries.^{6–9} In general, the mechanisms of anemia are blood loss, decreased RBC production, and increased destruction of RBCs.^{10,11} Anemia in the newborn period is unique to other age groups that in addition to the above mechanisms, hemorrhagic/hemolytic conditions, gestational age-dependent variation of hemoglobin, and transition period to extrauterine life contributed to neonatal anemia.^{12,13} In addition to these, heavy phlebotomy, fast-growing period of the infants, and impaired ability to increase erythropoietin (EPO) appropriately in a setting of anemia are causes of neonatal anemia, especially in preterm newborns.^{14,15}

Anemia in newborn infants and children can result in acute organ dysfunction like heart failure, respiratory distress, depressed sensorium, or multi-organ failure due to poor oxygen delivery to vital tissues.^{16–18} The long-lasting consequences of anemia could result in growth failure, impaired neurocognitive development, and increased susceptibility to infection in all stages of age due to chronic hypoxia.^{4,16,18} Because of the high prevalence and poor health outcomes of anemia in children, it is considered a contributing factor to infant and childhood mortality.^{9,19,20}

To reduce the high burden of anemia in newborns, it is wise to consider and address risk factors. The risk factors for neonatal anemia also vary depending on the settings.^{7–9,13,15,21} In previous studies, contributing factors for neonatal anemia included maternal anemia,^{7,8,22} maternal malaria attack during pregnancy,^{7,21} maternal antepartum hemorrhage (APH),^{7,23} lack of iron-folate supplements during pregnancy,²³ maternal vegetable consumption habit,⁹ septicemia in the newborn,^{7,16} prematurity¹³ and other neonatal problems including external blood loss.^{15,16}

Several studies have been conducted to measure the prevalence of anemia in newborns. However, almost all of these studies used cord blood for hemoglobin analysis at the time of delivery and did not involve hospitalized sick neonates. None of these studies included the whole period of neonatal age (1–28 days). Hence, prior studies do not represent the prevalence of anemia in sick newborns and the entire neonatal period. We believed that these two conditions and other neonatal factors could directly or indirectly affect neonatal hemoglobin levels. Therefore, the present study aimed to determine the prevalence of anemia and its associated factors among sick neonates regardless of their postnatal age and medical illness.

Methodology

Study Design, Area, and Period

An institution-based prospective cross-sectional study was conducted among sick newborns admitted to UOGCSH neonatal intensive care unit from June 1, 2020, to September 30, 2020. UOGCSH is one of the oldest academic and referral hospital in the country, which is located northwest of Ethiopia. The neonatal ward in the hospital is a level III NICU which provides the maximum care for all admitted neonates with gestational age (GA) ≥ 28 weeks. The care is provided by senior pediatricians, pediatric residents, general practitioners, and neonatal nurses. The average number of admissions is 200 patients per month. The common causes of admission are neonatal sepsis, prematurity, perinatal asphyxia, and neonatal jaundice.

Study Participants

All sick newborns admitted to the neonatal ward at UOGCSH.

Eligibility Criteria

Inclusion Criteria

All sick newborns whose GA ≥ 28 weeks and postnatal age ≤ 28 days, admitted to the neonatal ward during the study period were included in the study.

Exclusion Criteria

Those newborns whose mothers refused to participate in the study, stayed < 24 hours in the ward, admitted for a surgical procedure, and newborns who are abandoned were excluded.

Sampling Size and Sampling Techniques

The sample size was calculated by using single population proportion formula assuming the prevalence of anemia in newborns was 25%,⁹ 95% CI ($Z = 1.96$), and 5% marginal error ($w = 0.05$), adding 5% non-respondents making the final sample size of 302. A systematic random sampling technique was used to select the study participants based on their medical record numbers. The estimated number of admissions in the study period was about 800 based on the admission discharge registry. The interval k was 2.7. Every 3rd newborn was taken from the sampling frame. The first sample was the first medical record number on the first date of data collection.

Data Collection Tools and Procedure

After enrollment, data were collected with pre-tested structured questionnaires containing the following information: socio-demographic characteristics of the mother and the newborn, medical and obstetric condition of the mother, and clinical status of the newborn. Two trained pediatric residents were data collectors. The principal investigators actively supervised the data collection throughout the data collection period for data completeness. After interviewing the mother, the charts of both the mother and the newborn were reviewed for further information. The hemoglobin ordered by the treating physician at admission was used for the newborn and a recent maternal hemoglobin value was obtained from the record review.

Data Compilation and Analysis

The data were checked for completeness, then coded and entered to Epi-info version 7.2.1.0 and exported to SPSS version 20.0 for analysis. Data were reported as mean/median and SD for continuous variables and frequency and percentage for categorical variables. Bivariable and multivariable logistic regression methods were used to identify the associated factors. Multivariable logistic regression is used on the backward step if $p < 0.2$ on bivariable analysis. In multivariable logistic regression, a p -value < 0.05 was considered statistically significant. The results of these models were expressed as 95% confidence interval and odd ratios.

Operational Definition

Newborn: An infant whose age is ≤ 28 days and delivered after 28 weeks of gestational age.

Sick newborn: Newborn who was admitted to the neonatal intensive care unit (NICU) for medical treatment.

Preterm neonate: Newborn delivered before completed 37 weeks of gestational age.

Neonatal anemia: Hemoglobin < 13 gm/dL for GA ≥ 28 weeks in the first 28 days of life.

Moderate anemia: Hemoglobin value between 7 gm/dL-10 gm/dL.

Severe anemia: Hemoglobin < 7 gm/dL.

Maternal anemia: Hemoglobin < 11 gm/dL.

Ethical Considerations

The study protocol was reviewed and approved by the ethical review board of the School of Medicine, the University of Gondar in accordance with the declaration of Helsinki. The ethical review board provided an approval letter with the reference number ERB2101/2020. Written informed consent prepared in the local language (Amharic) was obtained from the parents of the study participants after the aim of the study was explained. All parents were informed of their right to withdraw from the study at any stage of the interview or to restrict their data from data analysis. All personal information of the study participants was kept confidential and their name was not mentioned in the study.

Results

Socio-Demographic Characteristics of the Study Participants

From the total of 302 randomly selected neonates, 272 newborns fulfilled the inclusion criteria and 30 were excluded (21 incomplete documentation and 9 of them did not give consent). The male to female ratio of the study participants was 1.4:1. The average GA of the newborns was 37.3 ± 2.9 weeks. One-third of the newborns were born to maternal age less than 20 years (Table 1).

Obstetric and Perinatal Profile of the Mother and Study Newborns

Most mothers had antenatal care (ANC) follow-up during pregnancy, 87% reported taking iron and folic acid for at least 1 month before delivery. More than three-fourth (79%) of the newborns were delivered by spontaneous vaginal delivery. About 87% of the newborns were singleton. Nearly one-fourth of the mothers had anemia during pregnancy while 10% of mothers had antepartum bleeding during the current gestation (Table 2).

Table 1 Socio-Demographic Characteristics of the Study Participants (N = 272) Admitted to UOGCSH, 2020

Variable	n (%)
Age at admission	
<72hr	201 (74)
3 days-7 days	24 (9)
7–28 days	47 (17)
Sex	
Male	161 (59)
Female	111 (41)
GA	
<37 weeks	82 (30)
37–42 weeks	186 (68)
>42 weeks	4 (2)
Birth weight (gram)	
1000–2499	90 (33)
2500–4000	173 (64)
>4000	9 (3)
Maternal age(years)	
< 20	93 (34)
25–35	99 (36)
>35	80 (30)
Education status	
Unable to read and write	34 (13)
Primary education	122 (45)
Secondary education	36 (13)
College and above	80 (29)
Occupation	
Employed	104 (38)
Daily laborer	9 (3)
Farmer	102 (38)
Housewife	57 (21)
Residence	
Urban	124 (46)
Rural	148 (54)

Clinical and Laboratory Parameters of the Study Participants

The commonest reason for admission to the NICU was sepsis (89%) followed by prematurity (30%) (Table 3).

The mean hemoglobin value was 15.74 ± 4.27 gm/dL. The overall prevalence of neonatal anemia was 30.1% (95% CI: 24.6–35.7), of which 1.8% had severe anemia (Figure 1). The prevalence of anemia among preterm and terms was 31.7% and 29.5%, respectively.

Factors Associated with Neonatal Anemia in the Study Participants

Multivariate logistic regression output showed that neonatal age, maternal anemia, APH, subgaleal hemorrhage, multiple births, and hyperbilirubinemia were statistically associated with neonatal anemia as shown in (Table 4).

Discussion

We found that the burden of neonatal anemia among hospitalized neonates was 30.1% and 1.8% of the hospitalized neonates had severe anemia. The WHO considers this to be a moderate public health problem which is defined as 20–39.9%.²⁴ The finding in this study is similar to the studies done in Gondar, Ethiopia, 25%,⁹ Nigeria, 28.9%,²⁰ Brazil, 32.5%,²⁵ and Bosnia, 29.3%¹³ but

Table 2 Obstetric and Perinatal Profiles of the Mother and Newborns (N = 272) Admitted at the UOGCSH, 2020

Variables	n (%)
Parity	
Primiparous	114 (42)
Multiparous	158 (58)
Fetal number	
Singleton	236 (87)
Multiple	36 (13)
Mode of delivery	
Vaginal	214 (79)
C/S	46 (17)
Instrumental assisted	12 (4)
Place of delivery	
Hospital	207 (76)
Health center	54 (20)
Home	11 (4)
ANC follow up	
Had ANC follow up	238 (87)
No ANC follow up	34 (13)
Iron & folic acid	
took $\geq$ 1 month	238 (87)
Did not take	34 (13)
Bleeding History	
Had APH	29 (11)
No APH	243 (89)
Maternal anemia	
Yes	59 (22)
No	213 (78)
HIV/AIDS	
Yes	4 (2)
No	268 (98)
Malarial attack history	
Yes	12 (4)
No	260 (96)
Maternal Malnutrition	
Yes	4 (2)
No	268 (98)

Abbreviations: APH, antepartum hemorrhage; ANC, antenatal care.

higher than the studies done in Addis Ababa, Ethiopia, 9%,²² USA, 21%,⁸ India, 19.4%,²⁶ the Netherlands, 21%,²⁷ and Iran, 11.7%.²⁸ This variation in the prevalence might be due to the differences in socio-demographic characteristics of the study populations, sample size, presence/absence of risk factors, and timing of hemoglobin determination. Most of the above studies did not assess important risk factors for neonatal anemia like maternal and obstetric conditions (APH, multiple pregnancy, HIV/AIDS, and Malaria),^{9,22} and used the cord blood for hemoglobin value, unlike the current study. In all the studies done in Ethiopia, only term neonates were included^{9,22,23} whereas only preterms were included in Bosnia.¹³ None of the studies included the entire neonatal period, which could affect the prevalence of anemia in the newborn due to physiological reduction of hemoglobin in the postnatal life.

There was no statistically significant difference in the prevalence of anemia among preterm neonates (31.7%) from term neonates (29.5%), which is similar to the study done in India.¹⁶ This lack of difference in prevalence could be

Table 3 Clinical Characteristics of the Study Participants (n = 272) Admitted to UOGCSH, 2020

Variables	n (%)
Prematurity	82 (30)
Hyperbilirubinemia	54 (20)
Perinatal Asphyxia	51 (19)
Sepsis ± meningitis	241 (89)
Acute bleeding	6 (2)
ABO/RH hemolytic diseases	15 (6)
Subgaleal hemorrhage	20 (8)
RDS	29 (11)
Severe pneumonia	17 (6)

Abbreviation: RDS, respiratory distress syndrome.

because we defined anemia as hemoglobin value less than 13 gm/dL for both preterm and term neonates which might not be the reality, especially in the first 2–3 months of postnatal life.

On the other hand, the prevalence in our study was lower than the studies in Benin, 61.1%,²⁹ Ghana, 57.3%,³⁰ and Iran, 53%.²¹ The explanations for the difference in these studies could be because newborns in these studies were born to mothers who had medical and obstetric conditions. In the study in Benin, all newborns were born to mothers who had a malaria attack during pregnancy. In Ghana; all the newborns were born to HIV-infected mothers. Finally in Iran, all the newborns were born to RH sensitized mothers. In these studies, there may be the possibility of congenital transmission of malaria and HIV causing hemolysis and bone marrow suppression resulting in a higher rate of anemia among the study newborns. RH hemolytic diseases of the newborn is the leading cause of anemia and hyperbilirubinemia in a neonate born from sensitized mothers, which could explain the higher prevalence of anemia in Iranian study.

In the current study, newborns who were born to anemic mothers during pregnancy had ten times the odds of developing neonatal anemia (AOR = 9.93, 95% CI: 4.36–21.6) than newborns born to non-anemic mothers, which was supported by studies done at St. Paul Hospital, Addis Ababa, Ethiopia²² and a study done in India.⁷ This may be due to inadequate transfer of iron, folate, and vitamins to the fetus during pregnancy, which are essential substrates for hemoglobin synthesis in the fetus.³¹ Previous

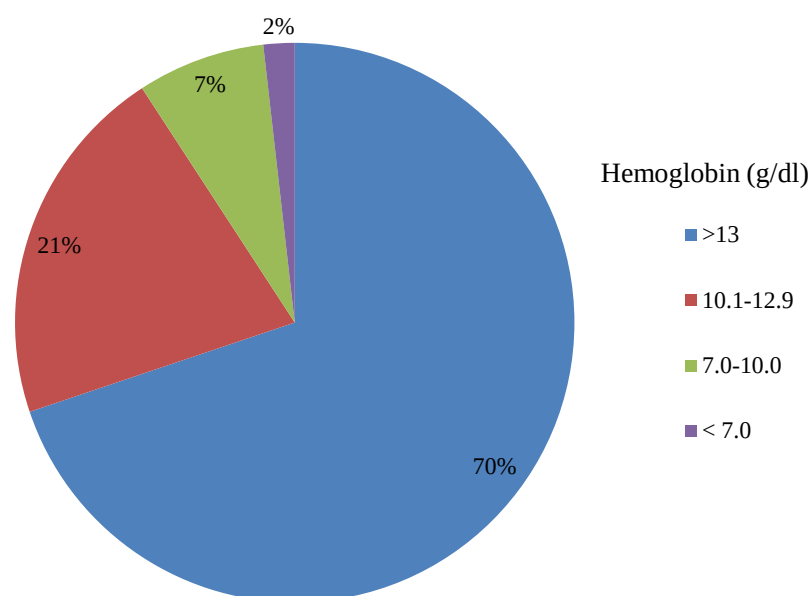


Figure 1 Hemoglobin profiles of the study participants (N = 272) admitted to UOGCSH, 2020.

Table 4 Bi-Variable and Multivariable Logistic Analysis of Factors Associated with Neonatal Anemia Among Newborns Admitted to UOGCSH, 2020 (N = 272)

Variables		Anemia	No Anemia	COR (95%, CI)	AOR (95%, CI)	P-value
Education status	Unable to read and write	10	24	1.44(0.58–3.56)	0.97(0.12–8.17)	0.982
	Grade 1–8	39	83	1.62(0.85–3.09)	1.19(0.17–8.44)	0.760
	Grade 8–12	15	21	1.62(0.85–3.10)	1.50(0.45–4.99)	0.439
	College & above	18	62	1	1	
Maternal Anemia	Yes	38	21	6.95(3.71–13.02)	9.93(4.36–21.64)	<0.001*
	NO	44	109	1	1	
Neonatal age	≤7 days	59	165	1	1	
	> 7 days	23	25	2.57(1.36–4.88)	4.41(1.86–10.47)	<0.001*
Mode of delivery	Vaginal	63	151	1.33(0.63–2.78)	1.54(0.57–4.12)	0.990
	Instrument	8	4	6.36(1.60–25.25)	5.53(0.69–43.11)	0.154
	C/S	11	35	1	1	
ANC	Yes	68	170	1.75(0.84–3.66)	0.97(0.34–2.80)	0.158
	No	14	20	1	1	
APH	Yes	16	13	3.30(1.51–7.23)	4.05(1.54–10.70)	0.006*
	No	66	177	1	1	
Fetus	Singleton	66	170	1	1	
	Multiple	16	20	2.06(1.10–4.22)	4.70(1.73–12.81)	0.002*
Subgaleal hemorrhage	Yes	12	8	3.90(1.53–9.95)	7.56(1.87–30.60)	0.007*
	No	70	182	1	1	
Hyperbilirubinemia	Yes	25	29	2.44(1.32–4.50)	3.84(1.58–9.31)	<0.001*
	No	57	161	1	1	

Notes: *p-value < 0.05.

Abbreviations: COR, crude odds ratio; AOR, adjusted odds ratio.

studies in Ethiopia investigated that, the common causes of maternal anemia in pregnant women were iron and folate deficiency, hookworm infection, malaria attack, and HIV infection.^{32–34} As per the WHO and Ethiopian obstetric national guidelines for ANC follow-up, iron and folate daily supplementation is recommended as early as the first trimester till delivery to prevent maternal anemia and low birth weight.^{35,36} Regarding the adherence of pregnant women to these protocols in the study area, a recent systematic review and meta-analysis study was done in Ethiopia which showed the overall prevalence of adherence to iron-folate supplementation was low (41%).³⁷

In addition, in this study, neonatal anemia was also strongly associated with antepartum hemorrhage (AOR = 4.05, 95% CI: 1.54–10.70) during pregnancy which was a similar finding in a study done in India^{7,38} and Scotland.³⁹ Bleeding during pregnancy and delivery might lead to a compromised placental transfer of nutrients and an ongoing fetomaternal hemorrhage in the newborn as well.

In the present study, anemia was strongly associated with multiple births, which was supported by a study done in the USA from 2011 to 2014.²⁷ Multiple pregnancies might increase twin-twin transfusion and antepartum bleeding during pregnancy, which could contribute to neonatal anemia among the study populations.

Anemia was also more often seen in newborns after the first week of life (AOR = 4.41, 95% CI: 1.86, 10.5), which is different from the study done in India.⁷ The higher magnitude of anemia after the first week of life in this study could be due to many reasons in the study population. These include either an exaggerated physiological reduction of hemoglobin in the first few months of postnatal life, repeated laboratory sampling, or bleeding from common cultural traditional malpractices (uvulectomy, circumcision) in the study population which are being done after the first week of life.

In the current study, anemia was significantly associated with hyperbilirubinemia which was a similar finding in a study done in India.⁷ Anemia and hyperbilirubinemia are related mainly by causative factors. The most common causes of hyperbilirubinemia in this study could be either due to Rh/ABO hemolytic diseases of the newborn, prematurity, subgaleal hemorrhage, or perinatal infection that directly or indirectly affect neonatal anemia.⁴⁰

Moreover, neonatal anemia in this study was also associated with subgaleal hemorrhage in the newborn (AOR = 7.56, 95% CI: 1.87–30.60). Anemia is the most common complication of subgaleal hemorrhage either due to acute bleeding in

the enclosed space (sub aponeurotic space or subperiosteal collection) or ongoing hemolysis, which could explain the high burden of anemia in our study population.⁴¹

The main strength of this study was that it is a prospective study; one of the very few studies in sick neonates admitted to the tertiary hospital across the world and assessed various contributing neonatal factors for anemia. However, it had to be interpreted with limitations. This is a cross-sectional study, which limits establishing a temporal relationship between variables that were studied. We did not identify the causes of anemia. We used the same hemoglobin cut-off value to define anemia in both term and preterm neonates to be consistent with other studies. In reality, the hemoglobin cut-off value in preterm is a bit lower than in term newborns, especially during the first 2–3 months of life.

Conclusions

The current study showed that the prevalence of neonatal anemia among hospitalized neonates was high. Maternal anemia, APH, multiple births, neonatal age, and subgaleal hemorrhage were strongly associated with neonatal anemia.

Recommendation

We recommend that healthcare providers should screen neonatal anemia among sick newborns who had risk factors. Future studies in different settings are beneficial to routinely recommend neonatal anemia screening on NICU guideline protocol. Prevention of maternal anemia and early treatment of obstetric complications will reduce the burden of anemia in newborns. Newborns with anemia should require further investigations to identify the cause and early intervention.

Abbreviations

ANC, antenatal care; AOR, adjusted odds ratio; APH, antepartum hemorrhage; COR, crude odd ratio; C/S, cesarean section; GA, gestational age; MRN, medical registration number; NICU, neonatal intensive unit; LBW, low birth weight; RDS, respiratory distress syndrome; UOGCSH, University of Gondar Comprehensive and Specialized Hospital.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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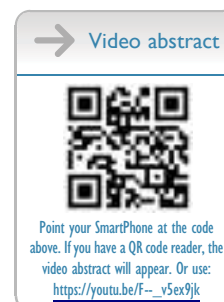
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Efficacy and Safety of Ferric Carboxymaltose in the Management of Iron Deficiency Anemia: A Multi-Center Real-World Study from India

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Background: Parenteral iron preparations, like ferric carboxymaltose (FCM), are commonly used to manage moderate-to-severe iron deficiency anemia (IDA). Real-world data on efficacy and safety of FCM is limited in India.

Methods: A retrospective, observational and real-world study was conducted to assess the efficacy and safety of FCM in adolescents and adults with IDA across 269 centers in India. Data was retrieved from medical records of patients who received FCM for management of IDA. Physicians' clinical assessment of efficacy and safety of FCM was also assessed. Data were analyzed for hematological parameters at baseline and at 4 ± 1 week for study population, and for severity of anemia.

Results: In 1800 patients with IDA, intravenous FCM resulted in a significant increase in hemoglobin (Hb) of 2.76 g/dL, serum ferritin of 35.85 μ g/L, red blood cell (RBC) count, hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) ($P < 0.001$ for all) at 4 ± 1 week as compared to baseline. In subjects with severe IDA, an increase in Hb was of 3.31 g/dL, serum ferritin increased of 35.84 μ g/L, RBC count, hematocrit and MCH improved significantly ($P < 0.001$ for all). In subjects with moderate IDA, Hb (increase of 2.63 g/dL), serum ferritin (increase of 35.92 μ g/L), RBC count, hematocrit, MCV, and MCH improved significantly ($P < 0.001$ for all). In subjects with mild IDA, only the mean Hb values at 4 weeks were significantly higher ($P < 0.001$; increased by 1.89 g/dL). Physicians rated efficacy of FCM as very good to good in 97.5% of patients. Similarly, safety of FCM was rated very good to good in 97.2% subjects.

Conclusion: FCM efficiently, safely and quickly corrects moderate-to-severe anemia in Indian patients in a short span of 4 weeks. Physicians' positive clinical impression of efficacy and safety supports clinical usage of FCM in real-world scenario.

Keywords: ferric carboxymaltose, iron deficiency anemia, pregnancy, efficacy, safety, India

Introduction

Anemia is a global public health problem affecting approximately one-third (22.8%) of the world population in 2019.¹ The 2019–2020 National Family Health Survey (NFHS-5) data shows that 57% of all Indian women and 25% of all Indian men in the age group of 15–49 years have anemia.² Anemia is a serious public health concern in India because of its high prevalence across all age groups.³ Additionally, severe anemia, defined as hemoglobin



(Hb) levels <7 g/dL, continues to be a substantial problem despite Indian guidelines and a focus on eradicating anemia.^{3,4}

Iron deficiency anemia (IDA) is the most common cause of anemia.⁵ IDA is primarily treated with iron supplementation, which can be either oral or parenteral. Oral preparations are inadequate in moderate-to-severe anemia where a faster improvement in Hb level and replenishment of iron stores is desired. Hence, parenteral iron preparations are the mainstay of treatment in moderate-to-severe anemia. However, both oral and parenteral iron preparations have side effects, which often limit their use.^{6,7} Therefore, newer intravenous (IV) iron formulations with a favorable adverse events profile as compared to traditional parenteral iron products were developed.⁷ Ferric carboxymaltose (FCM) is a third-generation parenteral iron formulation designed to overcome the limitations of existing parenteral iron preparations.⁷ FCM is a novel non-dextran IV iron agent having a very low immunogenic potential and therefore not predisposed to a high risk of anaphylactic reactions.⁸ Its properties permit the administration of large doses (maximum of 1000 mg/infusion) in a single session (15-minute infusion) without the requirement of a test dose.^{8–10}

Evidence suggests that FCM is highly effective in rapidly replenishing iron stores and correcting anemia in patients with IDA associated with a broad spectrum of anemia etiologies covering pregnancy, post-partum, gynecological causes, peri- and post-surgical, etc.^{8,11–16} However, real-world evidence (RWE) of the efficacy and safety of FCM is largely lacking, especially from India. RWE substantiates the evidence collected through clinical trials.¹⁷ The present RWE study was conducted with the objective of evaluating the efficacy and safety of intravenous FCM in the management of IDA in the adolescent and adult Indian population in a real-life scenario.

Materials and Methods

Study Design

This was a multi-center, retrospective, observational, data collection study across 269 centers in India.

Patient Characteristics

Adolescents and adults aged ≥ 14 years with IDA (hemoglobin [Hb] level between 4.0 and <12 g/dL), who provided informed consent for future use of their medical records for research, were included in the study. For subjects <18 years of age, informed consent was taken from their parent or legal guardian. The subjects were routinely treated by their physician with FCM 500/1000 mg injection in their clinical practice between January 01, 2021 and December 31, 2021. Subjects with available data on their medical records for at least two hematological parameters at baseline and/or for a minimum of 4 ± 1 week (reported as 4 weeks for simplicity) were included in the study. Subjects with a diagnosis of anemia other than IDA, severe iron deficiency with Hb <4 g/dL, known hypersensitivity to FCM or to any of its excipients, known serious hypersensitivity to other parenteral iron products, evidence of iron overload (eg, hemochromatosis/hemosiderosis), malignancy, pregnant women in the first trimester or if participant was considered unsuitable for the study by the investigator, were excluded from the study.

Sample Size

Convenience sampling¹⁸ was used as a practical method to obtain the patient sample by utilizing the medical charts available at our disposal from the 269 centers, within the stipulated time period, and of the patients meeting the study inclusion criteria.

Treatment Characteristics

Cumulative FCM dose for iron repletion was determined based on the patient's body weight and Hb level and is detailed in Table 1. FCM (Inj Orofer FCM 500/1K, Emcure Pharmaceuticals Ltd., Pune, India) was administered as part of standard care for the patient, as an IV infusion not exceeding 1000 mg iron per infusion.

Table 1 Cumulative FCM Dose for Iron Repletion

Body Weight	Cumulative FCM Dose	
	Hb <10 g/dL	Hb 10–14 g/dL
<35 kg	500 mg	500 mg
35 kg to <70 kg	1500 mg	1000 mg
≥70 kg	2000 mg	1500 mg

Notes: Maximum tolerated single dose: 1000 mg of iron (20 mL) per day. 1000 mg of iron (20 mL) not to be administered more than once a week.

Abbreviations: FCM, ferric carboxymaltose; g/dL, grams per deciliter; Hb, hemoglobin; mg, milligram; kg, kilograms.

Outcome Measures and Statistical Analysis

Data were retrieved from the medical records of the patients meeting the study inclusion criteria and anonymously filled out by trained technicians in the case record forms (CRF) designed for the study.

Physicians' clinical opinion of the efficacy and safety of FCM was also recorded in the CRF. The efficacy and safety were individually graded as very good, good, average, or poor. Efficacy was assessed clinically based on the symptomatic and hematological improvement recorded in the medical records and safety assessment was based on the adverse events/side effects of FCM recorded in the medical records.

Demographic details and hematological parameters captured in the CRFs were entered in a Microsoft Excel sheet and analyzed using descriptive statistical methods. Data were analyzed for the entire study population, and by the severity of anemia.

Categorical data were represented as frequencies and percentages. Quantitative data was described as mean $\pm$ standard deviation (SD). A paired *t*-test was carried out to compare the hematological parameters at baselines and four weeks after FCM infusion.

The following Hb values were considered as normal as per World Health Organization's (WHO's) Hb cut-off values for anemia:¹⁹ Non-pregnant women (≥ 12 g/dL); pregnant women (≥ 11 g/dL); men (≥ 13 g/dL). Anemia was categorized as mild, moderate and severe based on the World Health Organization's Hb cut-off values:¹⁹ severe anemia (Hb <8 g/dL for men/pregnant women and <7 g/dL for non-pregnant women); moderate anemia (Hb 8–10.9 g/dL for non-pregnant women and men and 7 to 9.9 g/dL for pregnant women); and mild anemia (Hb 11–11.9 g/dL for non-pregnant women; 10–10.9 for pregnant women; 11–12.9 g/dL for men).

Ethical Consideration

This study was approved by the Ripon Independent Ethics Committee. The study was registered with the Clinical Trial Registry of India (CTRI) with a wide registration number CTRI/2021/12/039065.

Results

Baseline Characteristics

Eighteen hundred patients were found to be eligible for the study; mean age was 32.37 years (range 14 to 92 years); 96.5% were females; with a mean Hb of 8 g/dL and mean serum ferritin at 42.06 μ g/L. The following comorbid conditions were identified in the study population: hypertension (2.8%), diabetes (2.3%), hookworm infestation (0.2%) and kidney disease (0.3%). The mean FCM infusion time was 18.47 minutes (range 5 to 60 minutes). The values of various hematological parameters at baseline are shown in Table 2.

Additionally, six sub-cohorts of subjects could be identified (Table 3) and the response of FCM to IDA in these sub-cohorts was also assessed.

Table 2 Patient Characteristics at Baseline

	N	Mean±SD	Median (IQR)	Range (Min-Max)
Age	1705	32.37±8.4	30(27, 35)	14 to 92
Weight	1551	57.95±9.49	58(51, 65)	30 to 98
Sex	1726	Males: 60 (3.5%)	Females: 1666 (96.5%)	–
FCM Infusion Duration (minutes)	1613	18.47±6.19	15(15, 20)	5 to 60
Baseline Hb (g/dL)	1723	8±0.98	8(7.4, 8.7)	4 to 11.5
Baseline Serum Ferritin (µg/L)	366	42.06±43.34	32.6(8.38, 60)	0.1 to 238
Baseline RBC Count (mn/mm ³)	391	3.9±1.12	3.9(3.3, 4.2)	1.7 to 12.5
Baseline Hematocrit (%)	347	31.22±6.14	31.3(26.4, 35)	16.5 to 46
Baseline MCV (fL)	371	68.94±11.55	69(62, 75)	11.3 to 102.1
Baseline MCH (pg)	363	24.07±6.16	22.61(20, 29)	2.8 to 38.8
Baseline MCHC (g/dL)	354	29.9±3.15	30(28.4, 32)	14 to 43.1
Comorbidities at baseline				
	N (%)			
Hypertension	50 (2.8%)			
Diabetes	42 (2.3%)			
Hookwork Infestation	3 (0.2%)			
Kidney Disease	6 (0.3%)			

Note: 4 weeks is 4 ±1 week.

Abbreviations: %, percentage; µg/L, micrograms per liter; FCM, ferric carboxymaltose; fL, femtoliters; g/dL, grams per deciliter; Hb, hemoglobin; IQR, interquartile range; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; min, minutes; Min-Max, minimum-maximum; mn/mm³, million per millimeter cube; N, number of participants; pg, pictograms; RBC, red blood cell; SD, standard deviation.

Table 3 All Patients' Data Set and Six Cohorts: Improvement in Hemoglobin and Serum Ferritin

	Cohort	Total Patients (N)	Improvement in Hb (g/dL) at 4 Weeks	Improvement in Ferritin (µg/L) at 4 Weeks
	All patients data set	1800	2.76*	35.85*
1.	Anemia in pregnancy	1191	2.8*	30.03*
2.	Anemia in Females (All diagnoses)	1666	2.78*	35.26*
3.	Anemia (Anemia diagnosis in men and women)	555	2.71*	58.6*
4.	Anemia in Women (Abnormal uterine bleeding + Anemia (cause not specified) + Perioperative anemia; Excluding pregnancy and postpartum anemia)	442	2.77*	62.07*
5.	Complete data set for all hematological parameters	194	3.19*	23.71*
6.	Anemia in males	60	2.5*	46.76 [#]

Notes: *P<0.001; [#]P=0.02. P value <0.05 – statistically significant difference observed in hematological parameter; Values are expressed as Mean.

Abbreviations: µg/L, micrograms per liter; g/dL, grams per deciliter; Hb, hemoglobin.

Efficacy Outcomes

The following hematological parameters improved significantly in the study population at 4 weeks ($P < 0.001$ for all): Hb increased by 2.76 g/dL; serum ferritin increased by 35.85 $\mu\text{g/L}$; similarly, there was a significant increase in red blood cell (RBC) count, hematocrit, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). There was a non-significant improvement in the mean corpuscular hemoglobin concentration (MCHC) at 4 weeks as compared to baseline ($P = 0.103$) (Table 4).

A significant improvement in Hb and ferritin was seen across the six cohorts (Table 3).

Response to FCM was also analyzed according to severity of anemia (Table 5). In subjects with severe IDA ($n = 350$), Hb increased significantly by 3.31 g/dL and serum ferritin increased significantly by 35.84 $\mu\text{g/L}$ at 4 weeks as compared to baseline ($P < 0.001$ for both); similarly, there was a significant increase in RBC count, hematocrit, and MCH ($P < 0.001$ for all). MCHC and MCV improved at 4 weeks as compared to baseline, but the difference was not statistically significant (MCHC: $P = 0.305$ and MCV: $P = 0.534$).

In subjects with moderate IDA ($n = 1300$), Hb increased significantly by 2.63 g/dL and serum ferritin increased significantly by 35.92 $\mu\text{g/L}$ at 4 weeks as compared to baseline ($P < 0.001$ for both) (Table 5); similarly, there was a significant increase in RBC count, hematocrit, MCV, and MCH ($P < 0.001$ for all). MCHC improved at 4 weeks as compared to baseline, but the difference was not statistically significant ($P = 0.17$).

In subjects with mild IDA ($n = 28$), mean Hb values at 4 weeks were significantly higher by 1.89 g/dL as compared to baseline ($P < 0.001$). The following hematological parameters improved at 4 weeks as compared to baseline, but the mean difference was not statistically significant (Table 5): serum ferritin ($P = 0.318$), RBC count ($P = 0.515$), hematocrit ($P = 0.229$), MCV ($P = 0.5$), MCH ($P = 0.188$), and MCHC ($P = 0.988$).

Safety

Adverse effects (AEs) were seen in 7.6% of the subjects (137/1800). The commonly reported AEs were as follows: nausea (3.9%), headache (3.4%), constipation (0.6%), allergic reaction (0.3%), and diarrhea (0.2%). No serious adverse events (SAEs) were reported in any of the subjects.

Physician's Efficacy and Safety Assessment Based on Data Available in Medical Records

Very good (61%) to good (36.5%) efficacy of FCM was noted in 97.5% subjects. Average efficacy was noted only in 2.5% subjects and no poor response was noted in any of subjects (Figure 1). Very good (62.2%) to good (35%) safety was reported in 97.2% subjects. Average safety was noted only in 2.8% subjects, and no poor safety issues were reported by the physicians in any of subjects (Figure 1).

Table 4 Comparing Hematological Parameters Before and After Administration of Ferric Carboxymaltose in the Whole Population

Parameter	N	At Baseline (Mean $\pm$ SD)	At 4 Weeks (Mean $\pm$ SD)	Mean Improvement $\pm$ SD
Hemoglobin (g/dL)	1678	8.01 $\pm$ 0.98	10.77 $\pm$ 1.07	2.76 $\pm$ 1.04*
Ferritin ($\mu\text{g/L}$)	312	40.88 $\pm$ 44.35	76.73 $\pm$ 65.99	35.85 $\pm$ 51.67*
RBC (mn/mm ³)	279	4.08 $\pm$ 1.21	4.67 $\pm$ 1.04	0.59 $\pm$ 1.38*
Hematocrit (%)	257	32.27 $\pm$ 6.12	35.28 $\pm$ 7.88	3 $\pm$ 6.85*
MCV (fL)	313	69.84 $\pm$ 10.77	77.33 $\pm$ 16.41	7.49 $\pm$ 19.21*
MCH (pg)	308	24.45 $\pm$ 6.23	28.21 $\pm$ 6.34	3.76 $\pm$ 7.51*
MCHC (g/dL)	302	29.76 $\pm$ 3.17	31.01 $\pm$ 13.9	1.25 $\pm$ 13.31 ^{NS}

Notes: *P value < 0.001 , Statistically significant difference; ^{NS}P value > 0.05 , Non-significant difference. 4 weeks is 4 $\pm$ 1 week.

Abbreviations: %, percentage; $\mu\text{g/L}$, micrograms per liter; fL, femtoliters; g/dL, grams per deciliter; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; mn/mm³, million per millimeter cube; N, number of participants; pg, pictograms; RBC, red blood cell; SD, standard deviation.

Table 5 Comparing Hematological Parameters Before and After Administration of Ferric Carboxymaltose by the Severity of Anemia

Severity of Anemia	Parameter	N	At Baseline (Mean $\pm$ SD)	At 4 Weeks (Mean $\pm$ SD)	Mean Improvement $\pm$ SD
Severe (N = 350)	Hemoglobin (g/dL)	350	6.83 $\pm$ 0.68	10.14 $\pm$ 1.07	3.31 $\pm$ 1.11*
	Ferritin (μg/L)	83	49.16 $\pm$ 48.16	85 $\pm$ 41.54	35.84 $\pm$ 61*
	RBC (mn/mm³)	75	3.48 $\pm$ 0.69	4.48 $\pm$ 0.7	1 $\pm$ 0.98*
	Hematocrit (%)	69	29.85 $\pm$ 7.91	34.01 $\pm$ 11.11	4.17 $\pm$ 10.08*
	MCV (fL)	83	72.73 $\pm$ 12.76	74.38 $\pm$ 20.77	1.65 $\pm$ 24.07 ^{NS}
	MCH (pg)	81	24.95 $\pm$ 6.18	28.94 $\pm$ 7.15	3.99 $\pm$ 8.09*
	MCHC (g/dL)	83	29.72 $\pm$ 3.73	30.64 $\pm$ 9.06	0.92 $\pm$ 8.16 ^{NS}
Moderate (N = 1300)	Hemoglobin (g/dL)	1300	8.28 $\pm$ 0.75	10.91 $\pm$ 0.99	2.63 $\pm$ 0.97*
	Ferritin (μg/L)	227	38.15 $\pm$ 42.69	74.06 $\pm$ 72.9	35.92 $\pm$ 48.17*
	RBC (mn/mm³)	201	4.31 $\pm$ 1.29	4.75 $\pm$ 1.14	0.44 $\pm$ 1.48*
	Hematocrit (%)	185	33.14 $\pm$ 5.08	35.74 $\pm$ 6.32	2.59 $\pm$ 5.2*
	MCV (fL)	228	68.75 $\pm$ 9.74	78.35 $\pm$ 14.49	9.6 $\pm$ 16.74*
	MCH (pg)	224	24.24 $\pm$ 6.27	27.9 $\pm$ 6.01	3.66 $\pm$ 7.34*
	MCHC (g/dL)	216	29.74 $\pm$ 2.94	31.14 $\pm$ 15.46	1.4 $\pm$ 14.91 ^{NS}
Mild (N = 28)	Hemoglobin (g/dL)	28	10.25 $\pm$ 0.38	12.14 $\pm$ 0.75	1.89 $\pm$ 0.88*
	Ferritin (μg/L)	2	8 $\pm$ 9.9	37.1 $\pm$ 32.39	29.1 $\pm$ 22.49 ^{NS}
	RBC (mn/mm³)	3	4.04 $\pm$ 0.2	4.15 $\pm$ 0.14	0.11 $\pm$ 0.25 ^{NS}
	Hematocrit (%)	3	34.5 $\pm$ 4.01	35.83 $\pm$ 3.2	1.33 $\pm$ 1.35 ^{NS}
	MCV (fL)	2	73.7 $\pm$ 17.39	83.3 $\pm$ 3.82	9.6 $\pm$ 13.58 ^{NS}
	MCH (pg)	3	26.77 $\pm$ 5.95	31.6 $\pm$ 7.3	4.83 $\pm$ 4.25 ^{NS}
	MCHC (g/dL)	3	32.07 $\pm$ 1.97	32.1 $\pm$ 1.82	0.03 $\pm$ 3.45 ^{NS}

Notes: *P value < 0.001, Statistically significant difference; ^{NS}P value > 0.05, non-significant difference. 4 weeks is 4 $\pm$ 1 week.

Abbreviations: %, percentage; μ g/L, micrograms per liter; fL, femtoliters; g/dL, grams per deciliter; Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; mn/mm³, million per millimeter cube; N, number of participants; pg, pictograms; RBC, red blood cell; SD, standard deviation.

Discussion

An expert opinion on the use of FCM in IDA found substantial evidence of the efficacy and safety of FCM in treating IDA resulting from several acute and chronic conditions.⁸

A systematic review and network meta-analysis of 21 randomized controlled trials showed that serum ferritin levels (μ g/L) improved significantly with FCM as compared to oral iron (Δ 172.8; 95% confidence interval [CI] 66.7–234.4), while Hb levels (g/dL) improved significantly with FCM as compared to intravenous ferric gluconate (Δ 0.6; 95% CI 0.2–0.9), oral iron and placebo (Δ 0.8; 95% CI 0.6–0.9 and Δ 2.1; 95% CI 1.2–3.0, respectively).⁵ FCM use is also associated with fewer side effects than other parenteral therapies.²⁰

Real-world studies on FCM are largely lacking, especially from India. Real-world studies from Turkey²¹ (N = 103 patients with IDA; $\geq$ 18 years) and Portugal²² (N = 459 iron deficient; 81.3% had IDA) reported a statistically significant increase in Hb, ferritin, hematocrit and transferrin saturation in patients treated with FCM (P < 0.05 for all [Turkey] and

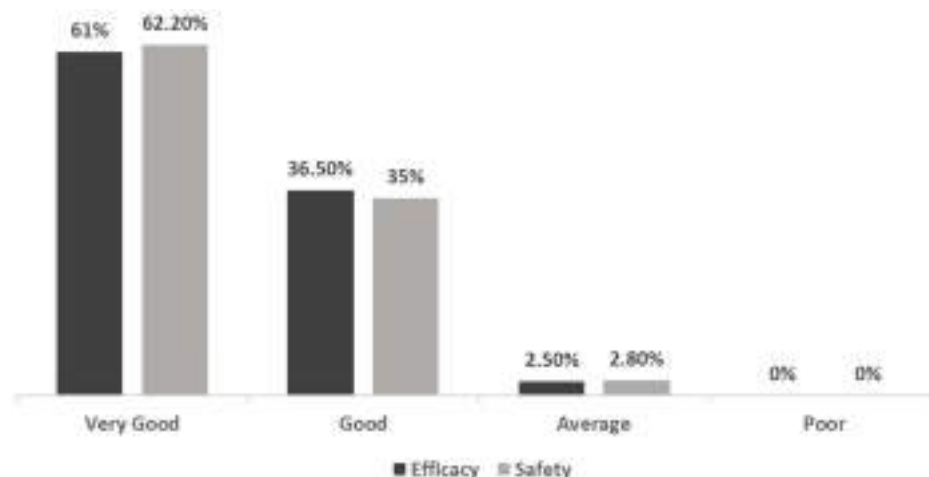


Figure 1 Physicians' assessment on efficacy and safety of ferric carboxymaltose.

Notes: Very good to good efficacy: $61 + 36.5 = 97.5\%$; very good to good safety: $62.2 + 35 = 97.2\%$.

$P < 0.001$ for all [Portugal]).^{21,22} We also reported statistically significant improvement in Hb, ferritin, hematocrit, total RBC count, MCV, and MCH between baseline and 4 weeks ($P < 0.001$ for all).

It has been seen that hematological parameters in patients with moderate-to-severe IDA improve from 6 to 12 weeks after IV iron supplementation and take longer to normalize after oral iron supplementation.^{9,21–25} Hb may, however, improve significantly within two to four weeks of IV FCM supplementation: an increase of ≥ 3 g/dL at 2 weeks^{26,27} to an increase of ≥ 3 g/dL to 5.49 g/dL at 4 weeks.^{20,28} Real-world studies have reported a significant increase in Hb levels post FCM administration; 2.6 g/dL at 4 weeks;²¹ Hb increase of ≥ 2 g/dL and ≥ 3 g/dL were attained by 41% and 20% of subjects, respectively, at 6 weeks;²² normalized Hb ≤ 1 year.²⁹ In our study, Hb improved significantly at week 4 across the study population, and irrespective of the IDA severity (increase of 1.89 g/dL in mild, 2.63 g/dL in moderate, and 3.31 g/dL in severe anemia) ($P < 0.001$ for all).

Of all the third generation intravenous iron preparations, intravenous FCM results in the highest increase in serum ferritin levels.^{5,30} Significant serum ferritin increase of 30.62 μ g/L has been reported after four weeks of FCM administration (from 8.32 ± 1.787 to 38.94 ± 6.095 μ g/L).²⁰ In our study too, we reported a significant increase in ferritin at 4 weeks by 35.85 μ g/L for the whole study population, by 35.84 μ g/L in severe anemia, and by 35.92 μ g/L in moderate anemia.

A positive response to iron therapy translating into improved hematological parameters is a true indication of IDA.²³ Hb and ferritin are the most commonly used hematological measures to assess the effect of iron supplementation. However, Hb concentration can fluctuate with posture and hydration status, while ferritin levels can change in presence of inflammatory conditions, and both the parameters can be influenced by acute exercise.²³ Therefore, assessing a battery of hematological parameters can provide a more valuable assessment of the response to therapy.

Although comprehensive laboratory tests for anemia play a crucial role in diagnosing and monitoring anemia,³¹ it is practically seen that patients often do not get the advised laboratory tests done.³² Comprehensive testing is costlier than doing only Hb.³³ This is especially true for resource-limited countries like India, where most patients bear the treatment cost out of their pockets.

Since IDA is the most common cause of anemia, and since cost is a constraint in India, many physicians in India start anemia treatment with iron supplements based on Hb values only.^{34,35} This was reflected in our study as well and Hb values were available for majority of subjects (1678/1800) in our study. Since India is a resource limited country, other laboratory tests like serum ferritin, RBC count, MCV, MCH, MCHC, and hematocrit were available for few subjects in our study (Table 4).

In such a scenario, we realized, after data collection, that laboratory tests for IDA such as iron levels, iron-binding capacity and transferrin saturation were not performed in any of the centers. This could be because these investigations

are costlier than serum ferritin. It is important to note that despite its above mentioned limitations, serum ferritin is an extremely useful and commonly used test for the diagnosis and monitoring of IDA.^{36–40}

In patients with hemodynamically stable IDA, FCM allows rapid administration of high iron doses with low immunogenicity.⁴¹ Additionally, the improved Hb and ferritin levels are sustained for six months after a single IV infusion.¹² Our study too showed that FCM significantly improved Hb, serum ferritin, RBC count, hematocrit and MCH in subjects with severe IDA ($P < 0.001$ for all).

FCM can also be administered safely in emergency department in patients with hemodynamically stable IDA, with fewer follow-up visits and better improvement in Hb levels and iron stores than those not administered FCM.⁴¹ A retrospective real-world study of 2966 adults with IDA showed that patients treated with FCM were significantly more likely to receive their full iron store repletion dose within three weeks than patients treated with ferumoxytol ($P < 0.001$) or with other parenteral iron preparations, such as iron dextran, iron sucrose, and sodium ferric gluconate in sucrose ($P < 0.001$ for all).²⁹ Patients on FCM were significantly more likely to have achieved the desired Hb levels within a year of index injection than those on ferumoxytol ($P < 0.001$). Patients on FCM required significantly fewer outpatient visits within that year than patients on ferumoxytol or with other parenteral iron preparations ($P \leq 0.001$ for all comparisons).²⁹

FCM has a good safety profile as well, with mild-to-moderate headache, nausea, dizziness, abdominal pain, diarrhea, constipation, rash and infusion-site reactions as the most commonly reported side effects.^{9,21} We too found nausea (3.9%), headache (3.4%), constipation (0.6%), allergic reaction (0.3%) and diarrhea (0.2%) as common side effects. No SAE were reported.

The study is limited by the biases associated with the retrospective study designs such as missing data, and inability to control drug dosage and frequency. Also, lack of data on transferrin saturation and serum ferritin in majority of patients is a limitation of the present study. This might be due to limited resources in the country and out-of-pocket expenses required for these particular tests. However, to the best of our knowledge, this is the largest real-world study ($N = 1800$), from India and globally, demonstrating the efficacy and safety of FCM in real-life scenarios of IDA, irrespective of age, sex, diagnosis and pregnancy status (for females). The study also showed that 98.3% of the subjects had moderate (77.5%) to severe (20.8%) anemia, which responded to FCM within 4 weeks. Health-related quality of life improvement has been reported after FCM administration, but literature on healthcare worker's assessment of efficacy and safety of FCM is lacking. Our study shows that the physicians assessed FCM to have very good to good efficacy and safety in 97.5% and 97.2% of the subjects, respectively.

Conclusions

IDA is very common in India. This large real-world study adds to the growing body of evidence that FCM efficiently, safely and quickly corrects moderate-to-severe anemia in Indian patients in a short span of 4 weeks, and may be useful in treating mild anemia if indicated and if patient can afford the cost. Physicians' positive clinical opinion in favor of efficacy and safety supports usage of FCM in daily clinical practice.

Drug Trial Registration

Clinical Trial Registry of India (CTRI) with a wide registration number CTRI/2021/12/039065 and available at <http://ctri.nic.in/Clinicaltrials/pmaindet2.php?trialid=63378&EncHid=&userName=CTRI/2021/12/039065>.

Data Transparency

All data and materials as well as software application or custom code used in the current study support the data presented and claims made in the current study and comply with the field standards of scientific writing, credibility, authenticity and personal honesty.

Data Sharing Statement

Analyzed data are available as tables and figures. Individual de-identified participant data cannot be made available. This data will be available on request if really required after contacting the corresponding author.

Ethics Approval and Informed Consent

This study was approved by the Ripon Independent Ethics Committee. This study complies with the Declaration of Helsinki. Written informed consent was obtained from all individual participants included in the study. For subjects <18 years of age, informed consent was taken from their parent or legal guardian.

Consent for Publication

No specific data or photograph of any kind of a study participant is used in this study.

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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.

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Disclosure

Dr. Ajinkya Rodge and Dr. Onkar C Swami are full-time employees of Emcure Pharmaceuticals Ltd., which actively markets Ferric Carboxymaltose. The authors report no other conflicts of interest in this work.

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Baseline Thrombocytopenia and Disease Severity Among COVID-19 Patients, Tibebe Ghion Specialized Hospital COVID-19 Treatment Center, Northwest Ethiopia

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Background: Thrombocytopenia and platelet indices in COVID-19 patients were important for prompt treatment and management of the disease. Therefore, the main objective of this study was to assess the prevalence of thrombocytopenia, platelet indices, and its association with disease severity among COVID-19 patients at the Tibebe Ghion Specialized Hospital, COVID-19 treatment center, Northwest Ethiopia.

Methods: A cross-sectional study was conducted among 117 conveniently recruited COVID-19 patients from March to June 2021. Socio-demographic and clinical data were collected using a structured questionnaire and checklist, respectively. The platelet parameters were analyzed by the Mindray-BC 5800 automated hematological analyzer. ANOVA and Kruskal–Wallis tests were used to compare the difference between parametric and non-parametric continuous variables, respectively. Binary logistic regression was used to identify the factors associated with thrombocytopenia. A P-value < 0.05 was defined as statistically significant for all statistical tests.

Results: Among COVID-19 patients, 45, 43 and 29 were mild, moderate and severe, respectively. 65.8% of the patients were males and 34.2% were alcohol drinkers with a mean age of 50.6 ± 15.4 . Moreover, 44.4% of the patients had co-morbidity. Thrombocytopenia was presented in 23.9% of the patients. It was 4.57 (95% CI: 1.30–16.07) and 6.10 (95% CI: 1.54–24.08) times more likely in the moderate and severe cases compared to mild cases, respectively. Disease severity was also associated with PDW (P-value = 0.001).

Conclusion: Even though thrombocytopenia was not presented in most moderate and severe COVID-19 patients, thrombocytopenia and PDW were associated with disease severity.

Keywords: COVID-19, Ethiopia, risk stratification, severity, thrombocytopenia

Background

Severe acquired respiratory syndrome corona virus-2 (SARS-CoV-2) occurred in Wuhan, China in December 2019.¹ The virus has genomic similarity to the severe acute respiratory syndrome-related coronavirus and is named SARS-CoV-2 which was finally dubbed coronavirus disease 2019 (COVID-19) by the World Health Organization.^{2,3}

Hemostasis is a complex process that keeps blood in a fluid state. It includes the clot formation and fibrinolysis.⁴ The imbalance of hemostasis cascades cause excessive clot formation or bleeding. In the majority of COVID-19 patients, abnormal hemostasis is common and associated disease severity and prognosis.^{5–7} Hypercoagulability is a usual

pathobiological presentation in COVID-19 patients.⁸ Therefore, it is essential to assess the hemostasis system including the platelets (PLT) parameter in COVID-19 patients for proper disease management.⁹

Thrombocytopenia in COVID-19 patients is caused by a variety of mechanisms. One of the postulated mechanisms is that coronaviruses may infect bone marrow cells, resulting in abnormal hematopoiesis and finally causes a low platelet production. The other possible mechanism may be due to viral infection and inflammation that results in lung damage. This leads to activating platelets in the lungs, resulting in aggregation and formation of micro-thrombi, which increases platelet consumption. Autoimmunity may be another potential player that induces antiphospholipid antibody production which was verified in various viral infections. COVID-19 may raise levels of auto-antibodies and immune complexes, causing the immune system to specifically destroy platelets.¹⁰

The management of critically ill patients has been incredibly hard because of the complexity and the insufficient evidence on the pathogenesis of COVID-19.¹¹ Accordingly, laboratory data like PLT parameters are needed. They play an important role in the clinical decision, treatment, and management of the diseases to improve the clinical care and ensure prompt treatment.^{12,13} It gives medical professionals the evidence-based data. Therefore, the main objective of the study was to assess the prevalence of thrombocytopenia and its association with COVID-19 severity.

Materials and Methods

Study Design, Setting and Population

A prospective cross-sectional study was conducted from March 2021 to June 2021 on COVID-19 patients attending Tibebe Ghion Specialized Hospital COVID-19 treatment center, northwest, Ethiopia. The Hospital is found in Bahir Dar town which is 565 km away from Addis Ababa, the capital city of Ethiopia. The hospital is providing different medical services to more than 5 million people in the region.¹⁴ All real-time reverse transcription polymerization chain reaction (RT-qPCR) confirmed COVID-19 patients were the source of populations and the study populations were all RT-qPCR confirmed COVID-19 patients during the study period. But, COVID-19 patients those unable to give biological samples, those who had a predetermined platelet abnormality and those on antithrombotic therapy before the baseline platelet determination were excluded from the study.

Sample Size Determination and Sampling Technique

The sample size was determined by using a census method.¹⁵ Therefore, the entire COVID-19 cases that fulfilled the inclusion criteria within the study period were recruited. A total of 117 COVID-19 patients (45 milds, 43 moderates, and 29 severe cases) were included by the convenient sampling technique.

Operational Definitions

Thrombocytopenia

Thrombocytopenia was defined as a platelet count $< 150 \times 10^3$ cells/ μ L.¹⁶

Alcohol Drinkers

Men who consume more than 4 drinks/day or more than 14 drinks/week or women who consume more than 3 drinks/day or more than 7 drinks/week.^{17,18}

Mild Cases

Mild clinical symptoms and no pneumonia manifestation found on imaging.¹⁹

Moderate Cases

Patients have symptoms like fever and respiratory tract symptoms and pneumonia manifestation can be seen in imaging.¹⁹

Severe Cases

Include those with any of the following; respiratory distress, respiratory rate ≥ 30 breaths/min; oxygen saturation $\leq 93\%$ at rest; and partial pressure of oxygen ≤ 300 . Patients with greater than 50% lesion progression within 24 to 48 hours in

pulmonary imaging should be treated as severe cases if a respiratory failure occurs and mechanical ventilation is required, shock, and complications from another organ failure that require monitoring and treatment in the ICU.¹⁹

Data Collection Procedures

Socio-Demographic and Clinical Data Collection

A pretested structured questionnaire was used as a tool for the collection of socio-demographic data. Relevant socio-demographic data (gender, age, residence, current marital status, educational status, occupation, alcohol use) and past and current medical history, including the history of chronic disease, were collected by trained nurses. A clinical data and clinical examination of patient's vital signs like body temperature, blood pressure, pulse rate, respiratory rate, and oxygen saturation percentage were also measured and recorded on a data collection checklist.

To assure the quality of the data, the questionnaire was prepared in English, translated to the Amharic language, and then translated back to English to check for consistency. It was also pre-tested on 5% of COVID-19 patients at Debre-Markos University's COVID-19 treatment center for its accuracy before actual data collection. The appropriate one-day training was given to all data collectors about the objective of the study, confidentiality issues, study participants' rights, consenting, and techniques of the interview before data collection. Socio-demographic and clinical data were collected by trained and experienced nurses under the close supervision of investigators. Vital sign measurements (blood pressure, temperature, pulse rate and respiratory rate) were measured two times and the mean was taken as the actual measurement. Every collected data was checked for its completeness daily by the investigators.

Blood Sample Collection and Analysis

After getting written informed consent from the study participants, 3 mL venous blood was collected aseptically from the patients by an experienced medical laboratory technologist from their antecubital vein by using a vacutainer collection system in an ethylene diamine tetraacetic acid anti-coagulated test tube (BD Vacutainer® K2-EDTA tube, Plymouth, England). The blood specimen and anticoagulant were mixed gently. Then the collected blood was checked for proper labeling, hemolysis, and clotting pre-analytically.

Then after the PLT count was determined at Amhara Public Health Institute Laboratory by using the Mindray BC-5800 automated hematological analyzer (Mindray, China). The hematological analyzer uses the impedance principle in which a constant electric current is passed through an isotonic solution. Then it measures the changes in electrical resistance that occur when blood cells pass through the detection aperture.²⁰

To maintain the quality of the laboratory results, pre-analytical, analytical, and post-analytical precautions of quality and the standard operating procedure (SOP) were strictly followed. All manufacturer instructions and SOP were strictly followed. Every specimen collection procedure was performed aseptically to avoid contamination for both the patient and the specimen, and the collected blood sample was checked for appropriate labeling, hemolysis and clotting pre-analytically. Preventive maintenance of the instruments was done daily, weekly, and monthly by laboratory personnel. All reagents and materials were checked for their expiration date, leakage, and signs of deterioration. The quality of the complete blood count (CBC) results were maintained by running commercially prepared three-level quality control (low, normal, and high) reagents before running the patient's sample. The quality control result was compared with a specific limit or range of quality control material. The patient specimen was run after the quality control result passed. The quality was also checked by background reading.

Safety Management

Data collectors were provided training on COVID-19 prevention, including when to get tested, use of personal protective equipment, and cleaning. Conditions of entry were displayed for any customers or visitors on entry points. Hand sanitizer was provided at multiple locations throughout the workplace. Detergent/ disinfectant surface wipes were provided to clean workstations and equipment used. Bathrooms were stocked with hand soap and paper towels and had posters with instructions on how to wash your hands. There was frequent cleaning of touched areas and surfaces.

Disinfectant solutions were maintained at an appropriate strength and used per the manufacturer's instructions. Data collectors were wearing gloves when cleaning and washing hands thoroughly with soap and water before and after each

activity. In general, the use of the required personal protective equipment like a particulate respirator, mask, gown and gloves was ensured when contacting the patient and blood sample collection from the patient and also whenever it is required in the process. All intervention procedures were done by sterilized and safe maneuvers.

Data Management and Statistical Analysis

The data were checked for completeness, cleaned, sorted, and categorized daily before being entered into EpiData version 4.6 and exported to IBM SPSS version 25 for analysis. Socio-demographic and clinical characteristics of study participants were summarized by using descriptive statistics and then presented in tables and text. Categorical variables were given as frequency rates and percentages; continuous variables were expressed as mean and standard deviation (SD) (parametric data) or median and Interquartile range (IQR) (non-parametric data). The Shapiro–Wilk test was used to verify the normality of the distribution of continuous variables. Moreover, binary logistic regression was used to identify the factors associated with thrombocytopenia. In all statistical tests, a p -value < 0.05 was considered statistically significant.

Ethical Considerations

The study was conducted after it was reviewed and approved by the Ethical Review Committee of the School of Biomedical and Laboratory Sciences, the University of Gondar with a reference number of *SBMLS/2743/13*. A permission letter to conduct the study was also obtained from Tibebe Ghion Specialized Referral Hospital's chief executive officer before the commencement of the study, and the study was as per the principles of the declaration of Helsinki II. Written informed consent was also obtained from each study participant and any participant who was not willing to participate in the study was not forced to participate. They were also informed that all the data obtained would be kept confidential by using codes instead of any personal identifiers and used only for the study purpose. Moreover, all laboratory test results were communicated to their physicians for prompt patient management.

Results

Socio-Demographic and Behavioral Characteristics

A total of 117 COVID-19 patients were enrolled in this study. Of the included participants, 77 (65.8%) were male and 94 (80.3%) were urban residents. Education-wise, 49 (41.9%) have attained university or college, while the majority of the participants were government employees, 42 (35.9%), followed by merchants, 27 (23.1%). Of the study participants, 102 (87.2%) were married and 40 (34.2%) had alcohol consumption habits. Most of the patients were between the age of 41 and 60 years old (36.8%) followed by the age of < 40 years old (34.2%) with a mean age of 50.6 ± 15.4 (Table 1).

Clinical Characteristics

Of the total study population, 52 (44.4%) had co-morbidity. Co-morbidity was significantly higher in moderate and severe COVID-19 patients compared to mild COVID-19 patients (24.4%, 53.5% and 62.1% of mild, moderate and severe patients, respectively) (Chi-Square p -value = 0.002). The most common co-morbidities were diabetic Mellitus (17.1%) and hypertension (14.5%) and the most common clinical presentations (signs and symptoms) were fever (57.26%), headache (59.83%), breathing difficulty (51.28%), cough (44.44%), and myalgia (51.28%) (Table 2).

Platelet Parameters of COVID-19 Patients

In the current study, 28 (23.9%) of the COVID-19 patients had thrombocytopenia. The median PLT count was $198 \times 10^9/L$ (IQR; 123) with a mean platelet volume (MPV) of 9.1fl (IQR; 1.5). They also had $63.03 \times 10^9/L$ platelet large cell count (P-LCC) (SD; $26.1 \times 10^9/L$). PLT count was lower in moderate and severe cases. But, it was statistically insignificant (P -value = 0.114). Mean platelet volume also had no significant differences between mild, moderate and severe groups (P -value = 0.891). Moreover, the PCT (p -value = 0.081), P-LCC (P -value = 0.178) and P-LCC% (P -value = 0.237) had no significant differences between mild, moderate and severe groups. However, Platelet distribution width

Table I Socio-Demographic and Behavioral Characteristics of Study Participants Attending at the Tibebe Ghion Specialized Hospital, COVID-19 Treatment Center 2021 (N=117)

Variable	Category	Frequency, n (%)
Age in years	< 40	40 (34.2)
	41–60	43 (36.8)
	>60	34 (29.1)
	Mean $\pm$ SD	50.6 $\pm$ 15.4
Gender	Male	77 (65.8)
	Female	40 (34.2)
Residence	Urban	94 (80.3)
	Rural	23 (19.7)
Educational status	Unable to read and write	10 (8.6)
	Primary school	32 (27.4)
	High school	26 (22.2)
	College and above	49 (41.9)
Occupation	Farmer	17 (14.5)
	Housewife	12 (10.3)
	Merchant	27 (23.1)
	Government employee	42 (35.9)
	Others ^b	19 (16.2)
Marital status	Unmarried ^a	15 (12.8)
	Married	102 (87.2)
Alcohol use	Yes	40 (34.2)
	No	77 (65.8)

Notes: ^asingle, widowed and divorced; ^bprivate, student and unemployed.

(PDW) (P-value = 0.001) and thrombocytopenia (P-value 0.008) had significant differences between mild, moderate and severe groups (Tables 3 and 4).

Factors Associated with Thrombocytopenia

The prevalence of thrombocytopenia was higher in females and older age groups. It was also higher in COVID-19 patients who had co-morbidity and alcohol consumption habits. However, all had no significant association with thrombocytopenia. On the other hand, the clinical stage of the COVID-19 patient had a significant association with thrombocytopenia. The odds of thrombocytopenia were 4.57 (1.30–16.07) and 6.10 (1.54–24.08) times more likely among moderate and severe COVID-19 patients compared to mild COVID-19 patients, respectively (Table 5).

Discussion

Thrombocytopenia was the most common manifestation of the early epidemic of viral infection. It was observed in 20–55% of the SARS epidemic.²¹ Among COVID-19 patients, thrombotic complications become a major

Table 2 Clinical Characteristics of the Study Participants Attending the Tibebe Ghion Specialized Hospital, COVID-19 Treatment Center 2021 (N=117)

Co-Morbidities			Clinical Presentations		
	Category	Frequency, n (%)		Category	Frequency, n (%)
Co-morbidity	Yes	52 (44.4)	Fever	Yes	67 (57.3)
	No	65 (55.6)		No	50 (42.7)
Type of co-morbidity			Headache	Yes	70 (59.8)
Hypertension	Yes	17 (14.5)		No	47 (40.2)
	No	100 (85.5)	Nausea	Yes	32 (27.4)
Digestive system disorder	Yes	10 (8.5)		No	85 (72.6)
	No	107 (91.5)	Breathing difficulty	Yes	60 (51.3)
Diabetic mellitus	Yes	20 (17.1)		No	57 (49.7)
	No	97 (82.9)	Chest pain	Yes	47 (40.2)
Heart disease	Yes	3 (2.6)		No	70 (59.8)
	No	114 (97.4)	Abdominal pain	Yes	43 (36.8)
Liver disease	Yes	1 (0.9)		No	74 (63.2)
	No	116 (99.1)	Cough	Yes	77 (65.8)
Cancer	Yes	3 (2.6)		No	40 (34.2)
	No	114 (97.4)	Sputum production	Yes	34 (29.1)
Tuberculosis	Yes	4 (3.4)		No	83 (70.9)
	No	113 (96.6)	Pharyngalgia	Yes	50 (42.7)
Renal disease	Yes	2 (1.7)		No	67 (57.3)
	No	115 (98.3)	Myalgia	Yes	60 (51.3)
Epilepsy	Yes	3 (2.6)		No	57 (48.7)
	No	114 (97.4)			
Human Immuno virus	Yes	5 (4.3)			
	No	112 (95.7)			
Asthma	Yes	8 (6.8)			
	No	109 (93.2)			
Other respiratory disease *	Yes	10 (8.5)			
	No	107 (91.5)			

Note: *Bronchitis and allergic rhinitis.

concern.^{22,23} These complications may cause thrombocytopenia which intern may associate with disease severity. Thrombocytopenia can prolong hospitalization, increase the need of ventilation, and increase patient mortality.²⁴

In the present study, thrombocytopenia at admission was presented in 23.9% of the COVID-19 patients (95% CI: 17.1–32.5%). This might be due to COVID-19 patients having a higher level of P-selectin expression and activated platelets, elevated circulating platelet-leukocyte aggregates, increased aggregation, and thromboxane generation. This leads to platelet

Table 3 Platelet Parameters of COVID-19 Patients Attending the Tibebe Ghion Specialized Hospital, COVID-19 Treatment Center (N = 117)

Platelet Parameter	Result
PLT $\times 10^9/L$: Median (IQR)	198 (123)
MPV, fl: Median (IQR)	9.1 (1.5)
PDW, fl: Median (IQR)	16.4 (0.75)
PCT, %: Median (IQR)	0.19 (0.1)
P-LCC $\times 10^9/L$: Mean $\pm$ SD	63.03 $\pm$ 26.1
P-LCR, %: Mean $\pm$ SD	31.36 $\pm$ 8.45
Thrombocytopenia n (%)	28 (23.9)

Table 4 Comparison of Baseline Platelet Parameters with the Clinical Stage of COVID-19 Patients Attending the Tibebe Ghion Specialized Hospital, COVID-19 Treatment Center (N = 117)

Variable	Measurement Category	Clinical Stage of the Disease			P-value
		Mild	Moderate	Severe	
PLT $\times 10^9/L$	Median (IQR)	209 (97)	186 (142)	190 (189)	0.114 ^a
MPV, fl	Median (IQR)	9.1 (1.4)	9.1 (1.5)	8.9 (1.75)	0.891 ^a
PDW, fl	Median (IQR)	16.4 (0.65)	16.5 (0.7)	17 (1.55)	0.001^a
PCT, %	Median (IQR)	0.19 (0.09)	0.17 (0.12)	0.17 (0.14)	0.081 ^a
P-LCC $\times 10^9/L$	Mean $\pm$ SD	68.49 $\pm$ 23.7	58.33 $\pm$ 25.64	61.55 $\pm$ 29.45	0.178 ^b
P-LCR, %	Mean $\pm$ SD	30.39 $\pm$ 7.35	30.83 $\pm$ 9.11	33.65 $\pm$ 8.9	0.237 ^b
Thrombocytopenia	Yes; n (%)	4 (8.9)	13 (30.2)	11 (37.9)	0.008^c
	No; n (%)	41 (91.1)	30 (69.8)	18 (62.1)	

Notes: ^aKruskal–Wallis test P-value; ^bANOVA test P-value; ^cPearson Chi-square; the bold text shows significance P-value at 0.05.

consumption.^{25,26} At admission, P-selectin expression was significantly increased in severe COVID-19 patients.²⁷ Rampotas Alexandros and Pavord Sue report that PLTs were aggregated in COVID-19 patients that showed the important role of platelets in thrombotic complications or respiratory failure in COVID-19 patients.²⁸ The direct attack on hematopoietic stem/progenitor cells by the virus, lung injury, and platelet destruction by auto-antibodies may also cause thrombocytopenia.²⁹ Following the viral infection, the cytokine storm destroys bone marrow progenitor cells and leads to a decrease in PLT production. The virus also causes lung damage, resulting in PLT aggregation and micro-thrombi formation in the lungs which causes platelet consumption. Production of autoantibody against PLT glycoproteins also destructs autoantibody-coated PLTs by the reticuloendothelial system leading to thrombocytopenia.³⁰ A similar findings were reported in Turkey (25.1%),³¹ China (17.8%),³² Iran (30%)³³ and USA (20%).²⁶

Early identification of severe patients will improve patient outcomes and reduce mortality. The current study revealed that the assessment baseline thrombocytopenia had significant value in the stratification of COVID-19 clinical stage at admission. It was significantly higher in moderate and severe COVID-19 patients compared to mild cases. It was 4.57 and 6.10 times more likely in moderate and severe COVID-19 patients respectively. The presence of thrombocytopenia may indicate the presence of some forms of coagulopathy with an increased risk of thrombotic events.³⁴ The risk of the

Table 5 Factors Associated with Thrombocytopenia Among COVID-19 Patients (N = 117)

Variable	Category	Thrombocytopenia		AOR (95% CI)	P-value
		Yes; n (%)	No; n (%)		
Sex	Female	10 (25.0)	30 (75.0)	1	0.712
	Male	18 (23.4)	59 (76.6)	0.71 (0.27–1.86)	
Age	<41	8 (20.0)	32 (80.0)	1	0.903
	41–60	10 (23.3)	33 (76.7)	0.93 (0.30–2.92)	
	>60	10 (29.4)	24 (70.6)	1.23 (0.37–4.07)	
Co-morbidity	No	14 (21.5)	51 (78.5)	1	0.522
	Yes	14 (26.9)	38 (73.1)	1.35 (0.54–3.38)	
Alcohol	No	16 (20.8)	61 (79.2)	1	0.822
	Yes	12 (30.0)	28 (70.0)	1.12 (0.43–2.90)	
Clinical stage	Mild	4 (8.9)	41 (91.1)	1	0.018
	Moderate	13 (30.2)	30 (69.8)	4.57 (1.30–16.07)	
	Severe	11 (37.9)	18 (62.1)	6.10 (1.54–24.08)	

Note: The bold text shows statistical significance at P-value < 0.05.

thrombotic event was increased as the disease prognosis is worsening. A higher prevalence of thrombotic events was found in ICU COVID-19 compared to non-ICU COVID-19 patients.^{35–38} Other studies also supported this finding. Jin et al reported that PLT was correlated with COVID-19 disease severity.³⁹ The Sayad et al study was also reported that thrombocytopenia could be important indicator of severe COVID-19 which might associate with mortality.³³ According to the systematic review and Meta-analysis reported by Lippi et al thrombocytopenia increased the risk of disease severity more than five times more likely.⁴⁰ Moreover, a study in the USA by Barrett et al showed that COVID-19 disease severity was 1.4 times more likely among patients with thrombocytopenia compared to those without thrombocytopenia.²⁶

Contrarily, there was another study disagreed with this finding. A study in the USA by Hana et al showed that platelet count at admission had no a significant difference between severe disease versus non-severe disease.⁴¹ The difference might attribute to the difference in the comparison method. In the current study, the comparison of platelet count and disease severities was made by grouping the patients with thrombocytopenia and without thrombocytopenia. On the other hand, the study in the USA used the mean platelet count which is affected by extreme values. This was also demonstrated by the present study. The average PLT count, expressed by median (IQR), had no significant association with disease severity (Kruskal–Wallis P-value = 0.114). This indicated that grouping COVID-19 patients as thrombocytopenia and without thrombocytopenia might have a better clinical significance value than comparing them by the average PLT count. Indeed, there was a study that showed the average PLT count was significantly lower in severe COVID-19 patients than in non-severe cases.⁴²

The current study also showed that PDW was significantly associated with disease severity (P-value = 0.001). Platelet distribution width reflects the variation in the size of PLTs. It increases when PLT destruction increases and when the new large immature platelet production raises for compensation. A study conducted by Bhandary et al also showed that PDW was significantly associated with disease severity and it was higher in severe cases.⁴³ Moreover, Aydinylmaz et al showed that PDW had a statistically significant change between severe (admitted at ICU) and non-severe COVID-19 patients (ward patients).⁴⁴ Tire et al also revealed that PDW had a statistical difference between outpatients, patients who

received standard treatment in the hospital and patients who had to be connected to a mechanical ventilator (ICU patients).⁴⁵

However, other studies revealed that PDW had no significant association with disease severity.^{31,46,47} The difference might be due to the comparison groups. The studies, which were done by Gulu et al and Chaudhary et al, compared the moderate and severe groups while in the current study the comparison was done between mild, moderate and severe groups. On the other hand, Yardimci et al compared the ward and ICU COVID-19 patients.

Strengths and Limitations

Determining the severity of the disease with a simple and inexpensive test might have social benefits and might help the scientific community for future studies. However, the current study had limitations. First, this study was a single-center study. Second, the sample size was relatively low. Therefore, a multicenter study with larger sample size is needed to verify the current findings.

Conclusions and Recommendations

Thrombocytopenia was presented in 23.9% of COVID-19 patients. It was significantly associated with the clinical stage of the disease. However, still, most moderate and severe patients did not have thrombocytopenia (69.8% and 62.1%, respectively). PDW was also associated with disease severity. Therefore, Thrombocytopenia and PDW can be used as risk stratification markers in COVID-19 patients. Furthermore, a multicenter study with larger sample size is needed to verify the current findings.

Abbreviations

CBC, Complete Blood Count; COVID-19, Corona Virus Disease 2019; IQR, Inter Quartile Range; MPV, Mean Platelet Volume; PLT, Platelet; PCT, Plateletcrit; PDW, Platelet Distribution Width; P-LCC, Platelet Large Cell Count; P-LCCR, Platelet Large Cell Count Ratio; RT-qPCR, Real-Time Reverse Transcriptase-Polymerase Chain Reaction; SARS-CoV-2, Severe Acute Respiratory Syndrome Corona Virus-2; SOP, Standard Operating Procedures.

Data Sharing Statement

All data supporting these findings is contained within the manuscript.

Consent for Publication

Not applicable. This manuscript does not contain any individual persons' data.

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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.

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Managing Fatigue in Patients with Paroxysmal Nocturnal Hemoglobinuria: A Patient-Focused Perspective

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Abstract: The most frequently reported symptom in patients with paroxysmal nocturnal hemoglobinuria (PNH), a disease characterized by complement mediated hemolysis and chronic anemia, is “fatigue”. The latter seems the best word to communicate patient’s perception of personal health status and disease impact on daily living, namely quality of life (QoL). Objectivating QoL and grading patient’s fatigue is one of the most difficult medical tasks given the highly heterogeneous communication skills of patients and caregivers and the multitude of meanings that might be attributed to this term. Along with anemia, QoL in PNH is also affected by the emotional burden of a chronic life-long disease with heterogeneous treatment requirement, risk of hemolytic exacerbations (breakthrough hemolysis) and of thrombosis. In the last decade, structured surveys and scores have been adapted from cancer settings to evaluate fatigue and QoL in patients with PNH, and to assess the benefit of complement inhibitors in this setting. Eculizumab was the first drug utilized and was shown to improve QoL scores in the registrative trials. However, the intravenous fortnightly administration, the presence of residual anemia, and the risk of extravascular hemolysis are some of the unmet needs impacting QoL under eculizumab. Several novel drugs have been designed to improve patients’ convenience and alleviate anemia and fatigue. In this review, we focus on available studies that evaluated fatigue and QoL in PNH patients, and the effect of old and new therapeutic strategies.

Keywords: paroxysmal nocturnal hemoglobinuria, novel drugs, eculizumab, quality of life, fatigue

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare condition characterized by chronic hemolysis resulting in anemia of various degree and fatigue, variably affecting quality of life (QoL). Additionally, PNH patients carry the emotional burden of a chronic life-long disease with heterogeneous treatment requirement, risk of hemolytic exacerbations (breakthrough hemolysis) and of thrombosis. A Patient-Reported Outcome (PRO) is any report of the status of a subject’s health condition (QoL and symptoms) that comes directly from the patient, without interpretation of the response by a clinician or anyone else.¹ PROs are used as indicators for assessing health-related QoL (impacts of disease and its treatment and symptoms). Regarding therapy, the beneficial impact on survival and/or on a meaningful aspect of a patient’s life is fundamental, and health features affecting the way a patient feels or functions should be considered.² Fatigue has been identified as a prevalent disturbance in PNH patients. There are many components of fatigue, one of which is anemia, as frequently shown in other diseases, particularly cancer. Objectivating and grading patient’s fatigue is one of the most difficult medical tasks given the highly heterogeneous communication skills of patients and caregivers and the multitude of meanings that might be attributed to this term. Clinicians usually evaluate patients’ QoL by simply asking them “how are you” during routine visits and the ability to elicit PROs varies between professionals. For this reason, there is a need for instruments to measure PROs. To aid the objective evaluation of QoL and the effect of treatment, the use of structured surveys has been implemented in the clinic. In this article,



we will briefly address the unmet needs of PNH treatment and describe how novel therapies might fill the gap in patients' management. Furthermore, we will focus on available reports that evaluate fatigue and QoL in PNH patients.

What is PNH?

PNH is a clonal disorder of the hematopoietic stem cell caused by acquired somatic mutations of PIGA gene. The latter encodes for a glycosyl-phosphatidylinositol (GPI) that anchors several molecules on the cellular membrane,^{3,4} including the complement inhibitory proteins CD55 (decay accelerating factor [DAF]) and CD59 (membrane inhibitor of reactive lysis [MIRL]). GPI deficiency results in complement-mediated intravascular hemolysis^{5–7} and thrombophilia, the main cause of morbidity and mortality in PNH. Chronic anemia mainly results in fatigue, shortness of breath on exertion, and reduction of physical activity, whilst dark urine are the typical symptom of intravascular hemolysis. Along with these, a variety of signs may be present including abdominal pain, bloating, back pain, headaches, esophageal spasms, erectile dysfunction, and fatigue, but also thrombosis of venous and arterial system, renal failure, pulmonary hypertension, so that PNH has been referred to as “the great impersonator”.³ Standard and high-sensitivity flow cytometric procedures for detecting PNH cells are the gold standard for disease diagnosis.⁸ Three different PNH cellular phenotypes have been recognized: type I with normal, PNH II with partial expression, and PNH III with absence of GPI-anchored proteins.⁹ The heterogeneity of PNH clone size (ie, the percentage of PNH+ cells), and the variable presence of complement activating conditions (infections, trauma, pregnancy, surgery) explain the different extent of hemolysis, and anemia, observed in the clinic. The degree of anemia is further affected by the characteristic association of PNH with bone marrow failure syndromes, namely aplastic anemia (AA), and with myelodysplastic syndromes (MDS), particularly hypoplastic ones that show several similarities with AA.^{10–12} Transfusion-dependent anemia along with thrombosis, PNH-related symptoms, and pregnancy (that increases thrombotic risk affecting maternal and fetal outcome), has been the main reason to start therapy with complement inhibitors. The latter revolutionized the natural history of the disease and re-drew the survival curve of PNH patients to that of the general population.

PNH Treatment and Its Limitations

Eculizumab (Soliris®, Alexion Pharmaceuticals, Inc) has been the first complement inhibitor studied in PNH and was approved in 2007. It is a humanized monoclonal antibody that binds the C5 complement fraction, preventing its activation to C5b by the C5 convertase thus inhibiting the formation of the membrane attack complex.¹³ Treatment with eculizumab reduced transfusions, improved anemia and fatigue, and ameliorated PNH symptoms. Furthermore, it reduced thrombosis occurrence and improved survival.^{14–17} Additionally, eculizumab appeared safe during pregnancy, and ameliorated fetal-maternal outcomes, although formal toxicology studies in pregnancy are lacking.¹⁸ The recommended maintenance dose of eculizumab is fixed (900 mg every 2 weeks ± 2 days) intravenously. This implies a high level of medicalization of PNH patients with consequent interference with working and leisure activities. Further unmet needs include patients with residual anemia, the occurrence of hemolytic flares (breakthrough hemolysis, BTH), the presence of an underlying bone marrow failure on which eculizumab has no effect, and the increased risk of infections. All of these clinical issues further burden patients' QoL. Regarding residual anemia,¹⁹ up to 25% of cases will still need blood transfusions. Along with concomitant conditions such as nutrients deficiencies and impaired erythropoiesis due to bone marrow failure, extravascular hemolysis of PNH erythrocytes as a consequence of C3 opsonization should be considered.^{3,19–21} This happens since the complement cascade upstream C5 is not blocked by eculizumab, and C3 continued to be produced and deposits on PNH-erythrocytes. C3 coated erythrocytes are then cleared by the monocyte/macrophage system, mainly in the liver. Clinically, extravascular hemolysis is evidenced by increased reticulocyte counts, indirect hyperbilirubinemia, and decreased haptoglobin in a patient with almost normal LDH values; additionally, the false positivity of direct antiglobulin test with anti-complement anti-sera provides a further demonstration. Moreover, patients of Asian ancestry (ie, Japanese, Han Chinese) may harbor a polymorphism of C5 (Arg885His) that impairs response to eculizumab.²² Homozygosity for the low-affinity allele L of the complement receptor gene CR1 has been associated with poor response to eculizumab.^{23,24} Regarding BTH, it may occur just before the subsequent infusion (pharmacokinetic PK BTH), or during complement activating conditions (pharmacodynamic PD BTH). The first situation may be managed by increasing eculizumab dose or frequency of administration (PK), and the second one requires

a prompt control of the underlying trigger (PD). Concerning infectious risk, patients treated with eculizumab must be vaccinated prior to initiation of therapy to prevent the risk of *Neisseria meningitidis*¹³ A, B, C, W and Y serogroups and in some countries, additional life-long antibiotic prophylaxis is prescribed. Despite this, meningococcal sepsis may still occur, and even fatal episodes have been reported.²⁵ Finally, eculizumab does not eliminate PNH clone but only prevents its complement mediated destruction. As a result, eculizumab needs to be administered lifelong in most cases further giving the patients the idea of a chronic “incurable” disease, with important impact on perceived QoL. Given these drawbacks, there is a need for new agents and approaches to optimally control PNH disease activity, as discussed thereafter.

Novel Therapies

Several novel complement inhibitors are under study. The new approaches aim at increasing the drug half-life or subcutaneous (sc) administration of C5 inhibitors (ie, ravulizumab iv, crovalimab sc), at producing small molecules that may be given orally, and at targeting the complement cascade upstream to C5 (including anti-C1, anti-C3 pegcetacoplan, and the alternative pathway factors B and D inhibitors iptacopan and danicopan).^{26,27} An innovative approach is the use of small interfering RNA (siRNA) molecules capable of inhibiting C5 synthesis, and very preliminary gene therapy.²⁸ Preclinical and clinical trials showed that novel C5 inhibitors (ECU biosimilars, ECU with longer half-life such as ravulizumab IV or sc,²⁹ small molecules,^{30,31} and siRNA³²) are effective in inhibiting complement-mediated hemolysis and imply more manageable administration routes/schedules thus improving patient convenience. In particular, ravulizumab was not inferior to eculizumab and reduced the occurrence of BTH in two clinical trials,^{29,33} and some of the new agents proved effective in patients with C5 polymorphisms (ie, crovalimab).²⁷ Importantly, proximal inhibitors (anti-C1 iv, anti-C3 sc, and oral anti-factor B and D) were effective both in naïve patients and in those with suboptimal response to eculizumab, significantly reducing C3 mediated extravascular hemolysis thus reducing residual anemia and fatigue. These agents appear very promising and are being developed both in combination with eculizumab and as single agents.²⁷

Bone Marrow Transplant (BMT)

Except for anecdotal cases,^{34–36} PNH is a lifelong disease requiring long-term treatment (if indicated), and this represents one of the main factors affecting patient’ perception of severity and illness. The only curative therapy is allogeneic bone marrow transplant (BMT). BMT outcome significantly improved with the introduction of reduced intensity conditioning, and the addition of eculizumab has reduced morbidity and mortality linked to PNH;³⁷ however, it remains a demanding, potentially life-threatening procedure that is limited to selected cases. As a matter of fact, a large European study comparing transplanted versus non-transplanted PNH patients, showed a worse survival after BMT if the indication was life-threatening thrombosis, whilst survival was not inferior if the indication was AA.³⁸ On the whole, BMT is indicated in patients with PNH and severe AA (below 40 years and with available donor) or MDS (high-risk patients or low-risk MDS with life-threatening cytopenias) and has the potential to eradicate both AA/MDS and the PNH clone.

PNH Complications

Not only the occurrence but even the fear of PNH complications, further impact QoL and may lead to a feeling of “life limitation” and “frailty” in these patients. The disease is dominated by increased thrombotic risk, reported in as many as 40% of patients, possibly being the first sign at presentation.³⁹ Thrombosis mainly affects the venous district and may also occur in atypical sites, including the cerebral, mesenteric, and renal vessels. Hepatic veins may also be involved in the so-called Budd–Chiari syndrome.^{40,41} Some studies have suggested that larger PNH clones correlate with increased thrombotic risk,⁴² however, many adjunctive congenital (eg, factor V Leiden or factor II mutations, protein C and S deficiencies, etc.) or acquired risk factors (eg, cigarette smoke, diabetes, obesity, estrogen-progestin compounds, pregnancy, surgery, prolonged immobilization, etc.) may contribute to thrombosis in this setting and deserve consideration. PNH can also be complicated by infectious events, either related to the coexistence of BMF and immunosuppression, or to complement inhibition. As stated before, anti-*Neisseria meningitidis* vaccinations are mandatory before eculizumab, and the addition of anti-*Streptococcus pneumoniae* and anti-*Hemophilus influenzae* vaccines have been required with

novel agents in clinical trials. However, not all vaccinated subjects will get proper immunization and antibody titers are not routinely evaluated. Therefore, patient education remains pivotal for prompt recognition and treatment of infectious episodes. Additionally, infections may trigger PD BTH, further complicating the clinical picture. By a careful discussion of available strategies to prevent/promptly manage thrombotic and infectious risk, clinicians may aid PNH patients to get back to their daily lives.

PNH, QoL and Measures

Besides treating clinical manifestations and preventing major complications, the improvement of patients' QoL is an emerging objective in the current management of PNH. QoL is defined by World Health Organisation (WHO) as

An individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns.⁴³

As stated above, QoL of PNH patients is usually reduced because of both PNH-related symptoms and complications, but also due to treatment-related issues that requires frequent hospital visits heavily interfering with working and leisure activities.^{44,45} As of today, there are no specific scores that have been fully validated to measure QoL in PNH so that questionnaires designed for cancer patients are usually administered in PNH clinical trials. The most frequent instruments are the Functional assessment of chronic illness (FACIT) Fatigue questionnaire (a 40-items score that determines fatigue's impact on QoL)⁴⁶ and the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 Global Health Status/QoL (a questionnaire suitable for measuring cancer patients' physical, psychological and social functions).⁴⁷ FACIT-F scores range from 0 to 52, with lower scores indicating greater fatigue. EORTC-QLQ-C30 domain scales range from 0 to 100, with lower scores indicating worse QoL and higher scores for symptom scales indicating worse symptoms. Data from the International PNH Registry reveals an impaired QoL in PNH patients, with median scores for EORTC QLQ-C30 and FACIT-F score of 58.3 (41.7, 75.0) and 34.0 (27.0, 40.0), respectively.⁴⁵ More recently, other scores have been developed to investigate in a better and more specific way PNH impact on QoL and the perceived benefit of treatment on QoL. The PNH-SQ (Symptom Questionnaire) is a novel questionnaire evaluating the presence and severity of 10 PNH-related symptoms over 24 hours.⁴⁸ Moreover, a PNH/aplastic anemia (AA) specific QoL questionnaire has been created as a specific disease PRO measure, although psychometric evaluations are still undergoing.⁴⁹ Lastly, PNH Patient Preference Questionnaire (PNH-PPQ), a 11-item questionnaire, has been validated to assess patients' preferences in the context of the clinical trials comparing eculizumab with its long half-life analogue ravulizumab.⁵⁰ As a matter of fact, the availability of several novel drugs will allow tailored therapy (ie, the choice of a specific compound for a specific patient) and defining patient's preference also in regards to QoL and improvement of fatigue will be pivotal.

Assessment of QoL in PNH Clinical Trials

Tables 1 and 2 report the main findings regarding PNH therapies and QoL. Concerning eculizumab, in the Phase 3 TRIUMPH study,^{16,51} patients in the eculizumab arm had a mean increase in FACIT-F score of 6.4±1.2 points from baseline to week 26, significantly higher than the placebo arm. Eculizumab group also showed a significant improvement in EORTC QLQ-C30 score in various scales: global health, functioning (5 scales/5), symptom (2 scales/3) and single-item measures (3/6). Results from the phase 3 SHEPHERD study were concordant, with a mean increase in FACIT-F score of 12.1±1.01 from baseline to week 52, and EORTC QLQ-C30 improvement in global health, functioning (5 scales/5), symptoms (3 scales/3) and single-item measures (4/6).^{14,51} More recently, Ueda et al published data from the post-marketing surveillance of eculizumab in Japan:⁵² they showed a significant amelioration after 1 year of eculizumab therapy both in FACIT-F score, with a mean change from baseline of 9.4, and in EORTC QLQ-C30, with a mean change from baseline of 21.4 and 16.3 points for global health status and physical functioning, respectively. Furthermore, they noted that fatigue showed the highest improvement among symptoms (significant amelioration of 1/3 scales for symptoms), along with amelioration of dyspnea, insomnia and appetite loss (3 scales/6 single-item measures).

Table 1 Quality of Life (QoL) and Paroxysmal Nocturnal Hemoglobinuria (PNH) Therapy: Eculizumab and Ravulizumab

Drug	Study Name	Number of Participants	Main Message	Ref
Eculizumab	Triumph	87 (43 eculizumab)	FACIT-F score significantly increased from baseline in eculizumab arm	[16, 51]
	Shepherd	97	Both FACIT-F score and EORTC-QLQ-C30 improved from baseline to week 52	[14, 51]
	Japanese experience	54	Both FACIT-F score and EORTC-QLQ-C30 meaningfully increased	[52]
Ravulizumab	301	246 (125 ravulizumab, 121 eculizumab)	Improvement in FACIT-F score and EORTC-QLQ-C30 similar in the ravulizumab and eculizumab group	[33]
	302	195 (97 ravulizumab, 98 eculizumab)	Change in FACIT-F score from the baseline significantly higher in the ravulizumab group; change in EORTC-QLQ-C30 similar in both groups	[29]
	302 extension	191 (96 ravulizumab, 95 eculizumab to ravulizumab)	Mean FACIT-F score remained stable through study period; mean EORTC-QLQ-C30 was similar in both groups	[53]
	302s	95	Ravulizumab preferred over eculizumab by a significant proportion of patients	[54]

Abbreviations: EORTC, European Organisation for Research and Treatment of Cancer-QLQ-C30 score; FACIT-F, the Functional assessment of chronic illness-Fatigue.

Table 2 Quality of Life (QoL) and Paroxysmal Nocturnal Hemoglobinuria (PNH) Therapy: New Drugs

Drug	Study Name	Number of Participants	Main Message	Ref
Pegcetacoplan	Pegasus	80 (41 pegcetacoplan, 39 eculizumab)	FACIT-F score increased from baseline in pegcetacoplan arm	[55]
Crovalimab	Composer	29	Both FACIT-F score and EORTC-QLQ-C30 improved from baseline	[56]
Danicopan	NCT03053102	10	Significant increase of mean FACIT-Fatigue score from baseline at days 28 and day 84	[57]
	NCT03472885	12	Mean FACIT-Fatigue score increased by 11 points at week 24	[58]
Iptacopan	NCT03439839	10	The FACIT-F score improved significantly in most patients	[59, 60]

Abbreviations: EORTC, European Organisation for Research and Treatment of Cancer-QLQ-C30 score; FACIT-F, the Functional assessment of chronic illness-Fatigue.

The long-lasting anti-C5 ravulizumab achieved an improvement in fatigue and QoL as well. The 301 study,³³ assessing the non-inferiority of ravulizumab to eculizumab in patients naïve from complement inhibitors, demonstrated a similar increase in FACIT-F and EORTC-QLQ-C30 score both in the ravulizumab and eculizumab group. However, a higher percentage of patients in the ravulizumab arm obtained a ≥ 3 -point improvement in FACIT-F score and a ≥ 10 -point improvement in the EORTC QLQ-C30 score (global health status/QoL, physical functioning and fatigue). The subsequent 302 study,²⁹ conducted in patients previously treated with eculizumab, showed a statistically significant change from the baseline in FACIT-F score in ravulizumab versus eculizumab group, while EORTC QLQ-C30 scores remained stable in both groups. In the 302 extension study,⁵³ patients continued on ravulizumab or were switched from eculizumab to ravulizumab by week 26: mean FACIT-F and EORTC QLQ-C30 scores remained stable from week 26 to week 52, suggesting that the switch from eculizumab to ravulizumab did not affect fatigue or QoL. Finally, the 302s study⁵⁴ included patients from the registrative trial with the aim of assessing the preference for ravulizumab or eculizumab using the PNH-PPQ survey: 93% of the patients preferred ravulizumab over eculizumab, 6% had no preferences and 1% preferred eculizumab. The main reasons for ravulizumab preference were the frequency of infusions (98%; this was selected as the most relevant factor determining treatment choice),

the ability to plan activities (98%), the improvement in overall QoL (88%), the convenience of receiving treatment (85%) and the effectiveness of medication until the next infusion (78%). Regarding the latter, PK BTH seems to significantly impact QoL in a patient perspective analysis,⁵⁰ particularly with eculizumab that is not adjusted on patient's body weight or individual response. Conversely, ravulizumab decreased PNH-related symptoms for a longer duration, including those occurring pre-administration and consistent with the longer half-life and effective plasma concentrations of the drug.⁵⁴

In the last few years, different therapies for PNH have been studied, all of them showing an improvement in terms of fatigue or QoL. Pegcetacoplan, a C3 inhibitor, obtained an increase of FACIT-F score of 9.2 points versus a decrease of 2.7 points with eculizumab, with 73% of patients in the pegcetacoplan arm achieving an increase of at least 3 points (versus 0% in the eculizumab arm).⁵⁵ Data from the COMPOSER study showed that both FACIT-F score and EORTC QLQ-C30 scores improved at week 10 with crovalimab (of 8.8 and 21.1 points, respectively) a subcutaneous C5 inhibitor also active in patients with the Japanese polymorphism.⁵⁶ Danicopan, an oral factor D inhibitor, achieved an increase of FACIT-F score both when used in patients naïve from complement inhibitors (34 points at baseline, increased by 9 and 13 points at days 28 and 85, $p < 0.05$) and when added to eculizumab (mean FACIT-F increased by 11 points at week 24).^{57,58} Finally, latest data from trials evaluating iptacopan, an oral factor B inhibitor, showed a significant amelioration of FACIT-F score.^{59,60}

Conclusions

PNH-related fatigue and symptoms are a puzzle of physical and psychological constraints to patients' QoL. These include inability to perform working and leisure activities, but also a detrimental effect on daily living. To better assess these issues, clinicians may profit of several scores developed for chronic anemia due to cancer and other conditions, but also of the advent of novel PNH specific PRO measures that are being evaluated in clinical trials. The latter will allow to objectivate and measure QoL in PNH patients while also putting them and their expectations at the center when designing clinical trials. As a matter of fact, eculizumab revolutionized PNH management in the last 15 years, and also ameliorated QoL. However, recent studies with novel drugs clearly showed that the boundaries of QoL in PNH patients may be pushed far beyond those reached with eculizumab. Given the variety of novel compounds, including intravenous, subcutaneous, and oral drugs, with different half-lives, availability of auto-injection, and the potential to differently improve residual anemia in PNH, patients' perspective and efficacy on QoL will become more and more crucial in treatment selection. Despite the advancement in disease treatment, the lifelong nature of PNH and the occurrence of BTH and thrombotic and infectious complications still burden the clinical course and affect individual health perception. Only education of clinicians and patients, along with maximization of preventive measures will improve the management of these unmet clinical needs.

Data Sharing Statement

All data are available within the manuscript and further may be available upon reasonable request to the corresponding author.

Consent for Publication

All Authors approved present submission.

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.

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Clinical Epidemiology, Treatment Outcome and Mortality Rate of Newly Diagnosed Immune Thrombocytopenia in Adult Multicentre Study in Malaysia

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Background: Immune thrombocytopenia (ITP) is well characterized in Western, European and other Asia-Pacific countries. Nevertheless, the clinical epidemiology, treatment pattern and disease outcome of ITP in Malaysia are still limited and not well known.

Objective: This study aimed to describe the clinical epidemiology, treatment outcome and mortality of ITP patients in haematology tertiary multicentre in Malaysia.

Methods: Clinical and laboratory data of newly diagnosed adults with ITP by a platelet count $<100 \times 10^9/L$ from January 2010 to December 2020 were identified and analyzed.

Results: Out of 500 incident ITP, 71.8% were females with a striking age preponderance of both genders among those aged 18–29 years. The median age was 36 years. The median platelet count was $17.5 \times 10^9/L$, 23.0% had a secondary ITP, 34.6% had a Charlson's score ≥ 1 , 53.0% had bleeding symptoms including 2.2% intracranial bleedings (ICB). *Helicobacter pylori* screening was performed in $<5\%$ of cases. Persistency and chronicity rates were 13.6% and 41.8%, respectively. Most (80.6%) were treated at diagnosis onset and 31.2% needed second-line treatment. Throughout the course of ITP, 11.0% of patients died; 3.0% and 8.0% with bleeding and non-bleeding related ITP.

Conclusion: This study confirms the epidemiology of ITP is comparable with worldwide studies. Our incidence is high in the female, Malay ethnicity, primary ITP and events of cutaneous bleeding at ITP onset with 18–29 years predominance age group for both genders. The frequency of persistent and chronic ITP is inconsistent with published literature. Corticosteroids and immunotherapies are the most prescribed first-line and second-line pharmacological treatments. Thrombopoietin receptor agonist medications (TPO-RAs) usage is restricted and splenectomy is uncommon. Our mortality rate is similar but ITP related bleeding death is fourth-fold lower than previous studies. Mortality risks of our ITP patients include age ≥ 60 years, male, severe bleeding at presentation, CCI ≥ 1 and secondary ITP.

Keywords: epidemiology, immune thrombocytopenia, mortality, treatment outcome

Introduction

Immune thrombocytopenia (ITP) formerly described as idiopathic or immune thrombocytopenic purpura¹ is an acquired autoimmune bleeding disorder secondary to premature platelet destruction, decelerated platelet production or a combination of both leading to thrombocytopenia and life-threatening bleeding in some patients.^{2–5} In the last several years, the basic understanding of the pathophysiology of ITP has significantly improved but the epidemiology and clinical course of ITP have not been well investigated in the general population, particularly in the Asian population. The

current literature includes a limited number of published studies, each of which describes a relatively small number of ITP patients.⁶ ITP is an old disease characterized by bleeding disorder comprises heterogeneous clinical characteristics and response to therapy. Isolated thrombocytopenia (defined as platelet count $<100 \times 10^9/L$) without an evident predisposing etiology is considered primary ITP as opposed to secondary ITP that is associated with coexisting systemic disease.^{7,8} ITP remains the exclusion of other causes of thrombocytopenia as no gold standard test to confirm ITP diagnosis. In adult ITP patients, bleeding is the most significant cause of morbidity, mortality and decision to start treatment.⁹ Treatment is indicated if the platelet counts less than $20 \times 10^9/L$ to $30 \times 10^9/L$, even in the absence of bleeding manifestation. However, the risk factors that influence bleeding such as older age (eg >65 years), type of ITP, previous bleeding history, exposure to anticoagulants or antiplatelet drugs, the presence of comorbidities such as renal impairment and the risk of trauma from daily activities should be assessed before a decision to start treatment.^{10–13}

First-line therapy for ITP is based on oral corticosteroids, plus administration of intravenous immunoglobulin (IVIg) in case of severe bleeding.^{11,14} Majority of patients will respond to initial corticosteroid treatment but relapse after the dose is reduced. Only about one-third of treated patients can expect a long-term response.¹⁵ Although chronic refractory patients are a minority, they appear to be at the highest risk of severe bleeding and mortality.^{16–18} ITP becomes persistent (lasting >3 months) or chronic (lasting >12 months) in about 70% of adult cases.¹⁹ Various corticosteroid-sparing agents are used in these cases such as splenectomy, rituximab or thrombopoietin receptor agonists (TPO-RAs) such as eltrombopag or romiplostim.^{11,14,20} Splenectomy is now frequently delayed until late in the disease course as it is an effective treatment for steroid-refractory or steroid-dependent ITP.^{21,22}

Although ITP disease has been studied for more than a century, its robust clinical epidemiological data is not well known in Malaysia. This retrospective study was conducted to analyze the clinical epidemiology, treatment outcome and mortality of newly diagnosed adult ITP patients in the tertiary hospitals in Malaysia. To the best of our knowledge, this is the first article involving a multicentre study on the clinical epidemiology, treatment outcome and mortality of adult ITP in Malaysia at present.

Methods

Study Design

All consecutive adult patients with newly diagnosed ITP from January 2010 to December 2020 were identified through the discharge codes (International Classification of Diseases, 7th–10th). Codes D69.3 (immune thrombocytopenia) from the 10th revision of the International Classification of Diseases (ICD-10) was used in this retrospective, observational multicentre study. All data were retrieved from the database of the International Centre for Casemix and Clinical Coding for Hospital Canselor Tuanku Mukhriz, Universiti Kebangsaan Malaysia Medical Centre (HCTM, UKMMC), Kuala Lumpur, Malaysia and from the database of new cases in Haematology Department, Ampang Hospital, Selangor, Malaysia. Diagnosis of ITP was validated using medical records and Integrated Laboratory Information System (ILMS). There was no duplicated data between the two registries of patients included in this study.

Study Population

An adult was defined as a patient by an age of 18 years and above. Platelet count $<100 \times 10^9/L$ without a secondary cause was defined as ITP in our study as outlined by Rodeghiero et al.¹⁹ We obtained information on patients' demographic and clinical data which includes age, gender, ethnicity, hospital visits, date of ITP diagnosis, platelet count and bleeding types at presentation, laboratory values, types and initiation date of pharmacological therapy and duration of ITP-treatment from the registered data and medical records.

Definitions

Phases of ITP disease were defined according to the International Working Group (IWG); 1. Newly diagnosed ITP: from the time of diagnosis up to 3 months; 2. Persistent: between 3 and 12 months from diagnosis; 3. Chronic phase: disease duration of more than 12 months.^{23,24} We defined treatment outcome to ITP treatments following American Society of Haematology (ASH) terminology: Complete response (CR): platelet count $\geq 100 \times 10^9/L$ and absence of bleeding;

Response (R): platelet count $\geq 30 \times 10^9/L$ and at least 2-fold increase the baseline count and absence of bleeding; No response (NR): platelet count $< 30 \times 10^9/L$ or less than 2-fold increase of baseline platelet count or bleeding; Loss of CR or R: platelet count below $100 \times 10^9/L$ or bleeding (from CR) or below $30 \times 10^9/L$ or less than 2-fold increase of baseline platelet count or bleeding (from R).¹⁹

Mortality

The outcomes of the patients including the date of death were recorded. For patients who passed away during the observation period, the cause of death was investigated by manually reviewing their medical records to identify diagnoses listed at the time of death. These were grouped into ITP related to bleeding and ITP with no specific bleeding.

Statistical Analysis

Patients' clinical characteristics were analyzed using simple descriptive statistics, mean $\pm$ standard deviation or median and range for quantitative variables and percentages, n [%] for qualitative or categorical variables. All potential risk associations were assessed by Pearson chi-square test for categorical data. Data were considered statistically significant at p -values < 0.05 . Mortality relative risk was computed by odds ratio (OR) and 95% confidence interval (CI) for observed number of deaths. Statistical Package for the Social Sciences (IBM-SPSS) version 27 was used to perform all data analysis.

Ethics Approval

This study was conducted in accordance with the Declaration of Helsinki. As this is a retrospective study, informed consent is not required. The study was approved by the Universiti Kebangsaan Malaysia Medical Research and Ethics Committee (UKM MREC FF-2021-080) and National Medical Research Register (NMRR-20-2969-57701), regional ethical board in Malaysia. Permission to obtain data was approved by the respective unit and was kept anonymized and confidentiality was maintained using coding.

Results

Patient Selection

In a database of 740604 of total admission over the study period, we identified 832 patients with a diagnosis encoded D69.3 ICD-10 at any age. Following exclusion criteria, 330 patients were excluded including 12 with a platelet count at ITP diagnosis of $\geq 100 \times 10^9/L$ but $< 150 \times 10^9/L$. Consequently, only 500 patients were included in the study.

Clinical Epidemiology at ITP Diagnosis

In total, 500 adult patients with newly diagnosed ITP were included in the cohort. Demographic characteristics of ITP patients are expressed in Table 1. The median age at ITP diagnosis of the entire population was 36 years, range 18–84 years. The incidence was more common in females, 359 (71.8%) with a median age of 35 years (18–84 years). The male-to-female ratio was 1:2.5. Male patients were slightly older with a median age of 41 years (range 18–82 years). The most frequent age at diagnosis was those aged 18–29 years in both genders (Figure 1).

Ethnic background is presented in Table 1. The percentage of patients was higher in Malays and smaller in the other groups. Other ethnicities include Indonesian, Siamese, Myanmar, Australian and African. Two hundred and five patients (41%) cases had one or more comorbidities including hypertension, diabetes mellitus, autoimmune disease, renal and heart disease. We observed 173 patients had Charlson comorbidity index (CCI) of ≥ 1 (14.2% had a CCI of ≥ 3).

Out of all cohort patients, only 19 patients were on antiplatelet drugs and 13 patients were exposed to the anticoagulant, but none had exposure to non-steroidal anti-inflammatory drugs (NSAIDs). A total of 385 patients with primary ITP and 115 with secondary ITP were diagnosed on both participating sites. The secondary causes were related to other conditions including autoimmune disease ($n = 79$, 15.8%), viral infections ($n = 22$, 4.4%), malignancy ($n = 6$, 1.2%) and drug-induced ($n = 8$, 1.6%). Eight patients were identified with Evans syndromes.

Table I Demographic Characteristics of ITP Patients

Variables	Study Subjects, n (%)
Total number of patients	500 (100)
Age at diagnosis, year	
18–29	163 (32.6)
30–39	109 (21.8)
40–49	69 (13.8)
50–59	69 (13.8)
60–69	56 (11.2)
70–79	27 (5.4)
≥ 80	7 (1.4)
Sex	
Male	141 (28.2)
Female	359 (71.8)
Ethnic background	
Malay	274 (54.8)
Chinese	122 (24.4)
Indian	68 (13.6)
Others	36 (7.2)
Charlson comorbidity index (CCI)	
0	327 (65.4)
1	48 (9.6)
2	54 (10.8)
≥ 3	1 (14.2)
Concomitant drugs	
Antiplatelet	19 (3.8)
Anticoagulant	13 (2.6)
NSAIDS	0 (0.0)
Type of ITP	
Primary	385 (77.0)
Secondary	115 (23.0)
Platelet count at presentation ($\times 10^9/L$), median	17.5 [0–99]
Bleeding symptoms at ITP diagnosis	
Yes	265 (53.0)
No	235 (47.0)

Abbreviations: ITP, immune thrombocytopenia; NSAIDS, non-steroidal anti-inflammatory drugs.

Clinical symptoms of bleeding at diagnosis were assessed by location; no bleeding, skin, oral cavity, nose, gynaecological, gastrointestinal tract (GIT), genitourinary, intracranial, pulmonary, and ocular (Figure 2). Bleeding manifestations (any site) were observed more frequently in females (194 patients, 73.2%) than in males (71 patients, 26.8%) ($p = 0.458$) but no difference in age between males and females (peak at age 18–28 years). Multiple sites (≥ 1 site) of bleeding have been observed in 134 patients of the bleeding subgroup with cutaneous bleeding as the most common presentation.

The median platelet count at diagnosis was $17.5 \times 10^9/L$ (range $0-99 \times 10^9/L$) and 257 (51.4%) patients showed value $<20 \times 10^9/L$ at presentation. The distribution of the platelet count at initial diagnosis is shown in Figure 3. If data are analyzed in patients with a presenting platelet of $<20 \times 10^9/L$, bleeding occurred in 190 (71.7%) of cases ($p < 0.001$). ICB occurred in 11 of cases (2.2%) ($p = 0.002$), by which 2 patients with the platelet of $\geq 20 \times 10^9/L$. The mean age of patients with intracranial hemorrhage was 40 years. The median platelet at diagnosis was $7.0 \times 10^9/L$ (range $1-23 \times 10^9/L$). Among them, only one patient died due to massive ICB after 5 days of admission. No association was found between risk

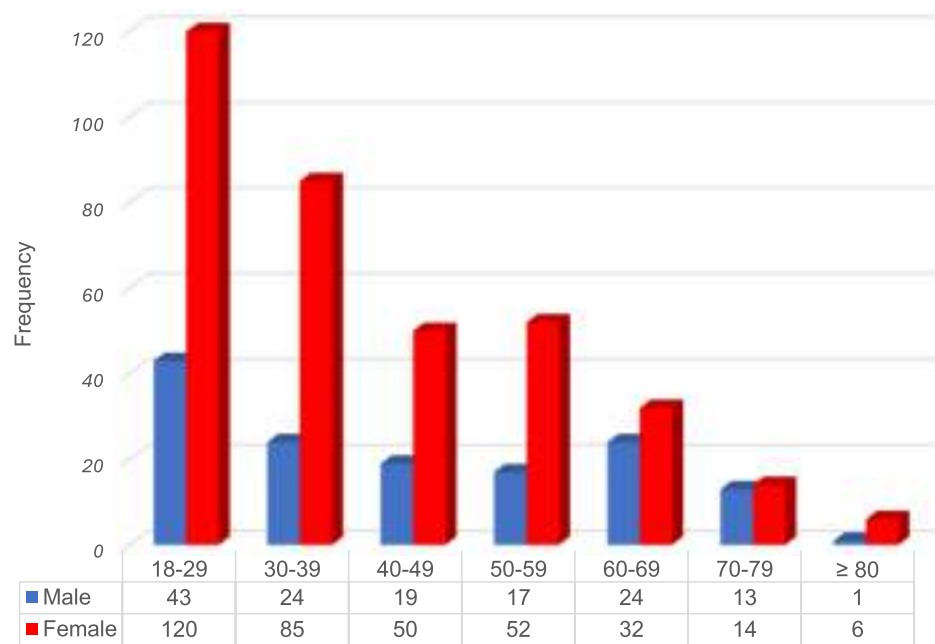


Figure 1 Distribution of age and gender of ITP patients.

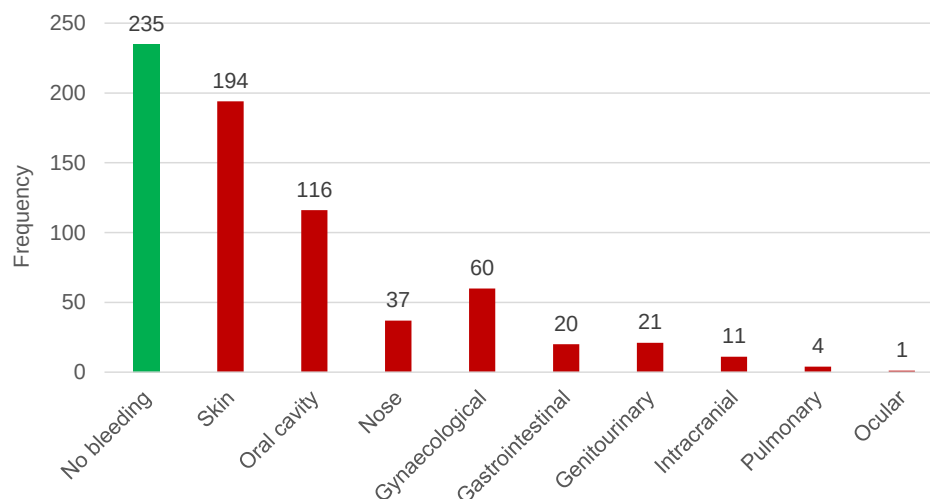


Figure 2 Bleeding symptoms at presentation by anatomical site.

of ICB and any of the following; age, gender, ethnicity, comorbidity, type of ITP or drug exposure except for platelet counts ($p = 0.041$).

Bone marrow examinations were performed in 134 (26.8%) cases, more often in patients who needed corticosteroids in contrast to other treatments. An antiphospholipid antibody, rheumatoid factor and *Helicobacter pylori* (*H.pylori*) testing were performed in less than 50% of patients. Antinuclear antibody (ANA) was the frequently positive laboratory test, while testing for *H. pylori* was not routinely done in ITP patients (Table 2).

Treatment Exposure at Diagnosis

A total of 403 (80.6%) patients received the first ITP treatment during the study period. Among this subgroup, 246 (61.0%) were presented with bleeding and 157 (39.0%) without bleeding. All patients have recorded platelets count at the

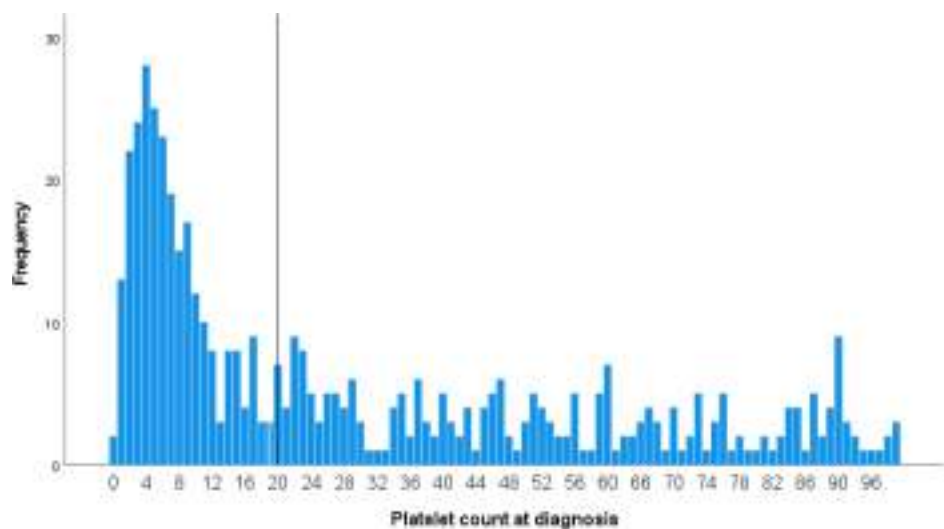


Figure 3 Platelet count at the time of ITP diagnosis. The vertical line indicates a platelet count of $20 \times 10^9/L$.

clinic before the start of treatment. Among the patients who did not need ITP treatment, the mean platelet count was $60.3 \times 10^9/L$. The median platelet count at treatment start was $11.0 \times 10^9/L$ (range $0\text{--}99 \times 10^9/L$). Among them, 298 (59.6%) and 234 (46.8%) had platelet counts checked at 3 months and 12 months, respectively, after treatment started. In the subgroup of patients requiring treatment, 77 (19.1%) had severe bleedings and were mostly in the age group 18–29 years. Seventeen (4.2%) patients were aged 60 and above.

Corticosteroids were the most used first-line treatment. Corticosteroids alone were given in 398 patients (98.8%) in which all of them received high-dose prednisolone 1mg/kg at the initial treatment. Intravenous (IV) methylprednisolone was pulsed in 258 (51.6%) patients at starting of therapy while concurrent intravenous hydrocortisone and dexamethasone were given in 0.7% and 0.5% patients, respectively. Out of 104 (20.8%) who received platelet transfusion, 83 (79.8%) was due to platelet $<20 \times 10^9/L$, 84 (80.8%) presented with any bleeding and 47 (45.2%) with severe bleeding. Among them, 4 patients with platelet $>20 \times 10^9/L$ were given platelet transfusion prior to surgical procedures. Second-line therapy is illustrated in Table 3.

Treatment Outcomes and Mortality

The mean follow-up was 69 months after the first diagnosis. ITP course at 3 and 12 months is observed in the whole cohort by platelet counts. Out of the remaining 97 patients who did not require initial treatment, 15 (15.5%) had normal platelet count $>100 \times 10^9/L$ at 3 months and 23 (23.7%) at 12 months without any active treatment. Among those who did not require initial treatment, only 2 (2.1%) patients had platelet count $<30 \times 10^9/L$ at 3 months and none at 12 months monitoring. The initial response to first-line treatment is shown in Table 3. Disease outcome was assessed at the time of

Table 2 Laboratory Test Results

Test	Positive (%)	Negative (%)	Not Done (%)
Antiphospholipid antibodies	24 (4.8)	39 (7.8)	437 (87.4)
Antinuclear antibodies	129 (25.8)	267 (53.4)	104 (20.8)
Rheumatoid factor	18 (3.6)	47 (9.4)	435 (87.0)
Hepatitis B	14 (2.8)	433 (86.6)	53 (10.6)
Hepatitis C	5 (1.0)	441 (88.2)	54 (10.8)
HIV	11 (2.2)	411 (82.2)	78 (15.6)
<i>Helicobacter pylori</i>	6 (1.2)	8 (1.6)	486 (97.2)

Abbreviation: HIV, human immunodeficiency virus.

Table 3 Treatment Exposure at Diagnosis

Treatment	All ITP, n = 500
General data	
No therapy, number (%)	97 (19.4)
Platelet transfusion at diagnosis, number (%)	104 (20.8)
First-line therapy, number (%)	
IV Methylprednisolone (pulses) + corticosteroids	258 (51.6)
IV Hydrocortisone + corticosteroids	3 (0.6)
IV Dexamethasone + corticosteroids	2 (0.4)
IVlg + corticosteroids	136 (27.2)
Corticosteroids alone	398 (79.6)
Second-line treatments (%)	156 (31.2)
Azathioprine	134 (26.8)
Eltrombopag	27 (5.4)
Danazol	45 (9.0)
MMF	25 (5.0)
Vincristine	3 (2.6)
Cyclosporin	8 (1.6)
Cyclophosphamide	2 (0.4)
Rituximab	35 (7.0)
Splenectomy	9 (1.8)
Initial response rate to first line treatments, with reference to;	All patients, n = 403
At 3 months, number (%)	
Complete response	147 (36.5)
Partial response	91 (22.6)
No response	60 (14.9)
No count (loss of follow-up)	105 (26.1)
At 12 months, number (%)	
Complete response	105 (26.1)
Partial response	75 (18.6)
No response	54 (13.4)
No count (loss of follow-up)	169 (41.9)

Abbreviations: IV, intravenous; IVlg, intravenous immunoglobulin; MMF, mycophenolate mofetil.

analysis according to their platelet count response at least of 3 to 12 months consequence follow-up. Acute ITP was observed in 185 (37.0%) patients, 68 (13.6%) became persistent ITP and 209 (41.8%) progress to chronic ITP (cITP). Another 38 (7.6%) cases were lost to follow-up. Among those who progress to persistent and cITP, 23 (33.8%) and 85 (40.7%) required second-line therapy, respectively.

At the time of analysis, 445 patients were alive (89.0%) whereby 55 patients had died (11%). Table 4 shows the mortality risk observed in our cohort. The death was two-fold in male patients with a mortality risk of 2.0 (95% CI, 1.2–3.2). When patients were reclassified as young age (<60 years) and old age (≥60 years), the old age had a higher mortality risk 2.8 (95% CI, 1.5–5.1). ITP patients presenting with severe bleeding symptoms suffered a 2.6-fold (95% CI, 1.4–4.9) increased mortality risk. CCI ≥1 and secondary ITP accelerated the mortality risk by 3.9 (95% CI, 2.2–7.0) and 2.1 (95% CI, 1.2–3.8), respectively.

Causes of death were classified into ITP related and non-ITP related deaths. A total of 15 (3.0%) patients with newly diagnosed ITP died due to bleeding ITP-related deaths during the entire period of follow-up. Among this subgroup, 7 were ICB, 7 with upper gastrointestinal bleeding (UGIB) and one case with epistaxis and massive bleeding. Causes of death considered unrelated to ITP or bleeding ITP consisted of malignancy (6), cardiovascular disease (7), severe infection (15), multiorgan failure (3), multiple underlying comorbid (3), both sepsis and underlying malignancy (1), trauma (1), old age (3) and one (1) undetermined cause of death.

Table 4 Mortality Risk of ITP Patients

Variables	Dead	Alive	Relative Risk (95% CI)	P value
Age, years				
<60 (n = 410)	36 (8.8)	374 (91.2)	0.4 (0.3–0.7)	0.001
≥60 (n = 90)	19 (21.1)	71 (78.9)	2.8 (1.5–5.1)	
Gender				
Male (n = 141)	24 (17.0)	117 (83.0)	2.0 (1.2–3.2)	0.007
Female (n = 359)	31 (8.6)	328 (91.4)	0.5 (0.3–0.8)	
Initial platelet				
<20 × 10 ⁹ /L (n = 257)	26 (10.1)	231 (89.9)	0.8 (0.5–1.4)	0.516
≥20 × 10 ⁹ /L (n = 243)	29 (11.9)	214 (88.1)	1.2 (0.7–2.1)	
Bleeding at diagnosis				
Yes (n = 265)	30 (11.3)	235 (88.7)	1.1 (0.6–1.9)	0.808
No (n = 235)	25 (10.6)	210 (89.4)	0.9 (0.6–1.6)	
Severe bleeding at diagnosis				
Yes (n = 82)	17 (20.7)	65 (79.3)	2.6 (1.4–4.9)	0.002
No (n = 418)	38 (9.1)	380 (90.9)	0.4 (0.3–0.7)	
Charlson comorbid index				
0 (n = 327)	20 (6.1)	307 (93.9)	0.3 (0.2–0.5)	0.000
≥1 (n = 173)	35 (20.2)	138 (79.8)	3.9 (2.2–7.0)	
Type of ITP				
Primary (n = 385)	35 (9.1)	350 (90.9)	0.5 (0.3–0.9)	0.013
Secondary (n = 115)	20 (17.4)	95 (82.6)	2.1 (1.2–3.8)	
Anticoagulant				
Yes (n = 13)	3 (23.1)	10 (76.9)	2.5 (0.7–9.4)	0.159
No (n = 487)	52 (10.7)	435 (89.3)	0.5 (0.2–1.3)	
Antiplatelet				
Yes (n = 19)	2 (10.5)	17 (89.5)	1.0 (0.2–4.2)	0.946
No (n = 481)	53 (11.0)	428 (89.0)	1.0 (0.3–4.0)	

Abbreviations: ITP, immune thrombocytopenia; CI, confidence intervals.

Discussion

Clinical epidemiology, laboratory tests and mortality of newly diagnosed ITP in adults have not been systematically described in previous studies including in Asia and Malaysia. Published research suggests that there are differences in the clinical manifestations of ITP with variable clinical presentations, clinical consequences and treatment responses in different ages.^{11,25} Understanding the similarities and differences could affect the clinical practice and may provide a starting point for future study of the underlying etiologies and pathophysiological causes in each age group. The establishment of a regional ITP registry plays an important role to gather all the data, as a valuable resource for studies of rare diseases at the population level, clinical trials, improve treatment, disease burden and outcomes for all ITP patients.²⁶ In addition, the local population study also will be able to guide future planning of social, health policies and services.²⁷ Although there are few tertiary centres for referral for haematology cases in Malaysia, the national ITP registry has not yet been established. Currently, Japan is the only country in the Asia-Pacific region that has a national ITP registry.^{19,28}

The incidence of ITP is reported in 1.5 to 5 per 100000 persons in the general population for both adults and children.^{6,29–33} The incidence of adult ITP patients is illustrated in Table 5. A multimodal incidence with 1 peak seen in childhood whereby the second and third peaks observed in young adults and the elderly.³⁴ Our study confirms the higher incidence of ITP in females in younger adults as reported by previous population-based literature but not in the elderly (>65 years). The prevalence of ITP in men and women in the elderly is almost even in previous studies.^{4,6,18} We identify only one predominance peak of age at 18–29 years old for both genders. We did not see any multimodal peak as

Table 5 Incidence of Adult ITP Patients in General Population

Year	Study	Country	Design	Incidence	Year of Studied
1999	Frederiksen et al ³⁸	Denmark	Retrospective by ICD code	2.68	1973–1995
2009	Marieke Schoonen et al ⁶	England	Retrospective by administrative data	3.90	1990–2005
2010	Terrell et al ³⁰	Europe	Critical review of published reports	3.30	1966–2009
2010	Kurata et al ²⁸	Japan	Retrospective by registered data	2.16	2004–2007
2014	Michel et al ³⁹	France	Retrospective by cohort study	4.00	2009–2012
2017	Lee et al ⁴⁰	Korea	Retrospective by registered data	1.50	2010–2014
2018	Hung et al ⁴¹	Taiwan	Retrospective by registered data	2.00	2006–2013

described in European⁴ and Japanese studies.²⁸ It has previously been reported that most cases of adult ITP occur between 20 and 40 years old in female patients and that it is less common after 50 years old.^{35–37} These epidemiological data were similar in our study, instead not only in female but also in male patients. The incidence of ITP was found to be higher in Malay (54.9%) ethnicity. This is most likely contributed by the Malay being the highest ethnicity group in Malaysia.

This study demonstrated that infective screening (hepatitis B, hepatitis C and HIV) is frequently done (>80%) in our practice compared to autoimmune antibodies screening (<10% except ANA test). The antiphospholipid antibody, ANA and rheumatoid factor were not performed in all patients, restricting the analysis to exclude secondary causes and may underestimate the incidence. Furthermore, ANA provides information about the baseline risk of thrombosis which is associated with systemic lupus erythematosus (SLE)⁴² or antiphospholipid syndrome (APS).⁴³ ITP patients concomitant with APS have an increased risk of thrombotic complications which may be higher if given TPO-RAs medications. For ITP patients at risk of thrombotic complications, there is limited evidence for the use of anticoagulation but stopping anticoagulation can result in more harmful outcomes than continuing anticoagulation.⁴⁴ Positive *H. pylori* testing is common with increasing age and the prevalence of pathogenic strains can depend upon the country of birth. The overall prevalence rate of *H. pylori* infection was 35.9% in Malaysia and differs in the different regions within Malaysia. The Malay populations have the lowest prevalence of *H. Pylori* infection (11–29%) compared to Indians and Chinese, 49–52.3% and 26.7–57.5%, respectively.^{45,46} Despite the fact that the prevalence of *H. pylori* infection is high in Malaysia, the screening test by *H. pylori* serology and confirmation of active infection with a C14 urea breath test is not routinely done in our study (<5.0%). Moreover, eradication of *H. pylori* may rise a platelet count up to 50% of ITP patients with *H. pylori*-positive based on studies that were mostly conducted in Japan.^{47,48} Our frequency of secondary ITP (23%) is almost equal to the estimated incidence (~20%) by the expert opinion.^{41,49–51} In contrast, the prevalence of secondary ITP was substantially higher (64.8%) as compared to primary ITP (35.2%) in Pakistan.⁵² Our data also support that the female patients have a higher association with autoimmune disorders (n = 67, 18.6%) for secondary ITP, which may be an explanation for the greater number of women with ITP.

The incidence of bleeding events (53.0%) is in following previous French and the United Kingdom (UK) cohorts.^{12,53,54} Figure 2 presents the incidence of bleeding according to the site of bleeding at presentation. Estimated incidence, the weighted proportion of ICB and severe non-ICB bleeding have been reported in 1.4% (95% CI, 0.9–2.1%) and 9.6% (95% CI, 4.1–17.1%), respectively, in adults with ITP.⁵⁵ The event of bleeding and rarity of serious bleeding at ITP onset in our cohort study is comparable with previous studies. Nonetheless, the incidence of ICB is higher but fewer GIT bleeding cases (2.2% and 4.0% respectively) compared to <1% for ICB and ~6.0% for GIT in the past.^{54,56,57} There was no association of ICB with age, gender, ethnicity, comorbidity, type of ITP or drugs exposure except for platelet counts ($p = 0.041$). The incidence of persistent and chronic disease in our study is smaller (<50%), inconsistent with France and Swiss populations.^{58–60} Interestingly, the persistent disease (13.6%) is much lower but higher rate of cITP (41.8%) in our population compared to Taiwanese population (24% persistent and 12.3% cITP). Our study demonstrated that 23 (18.0%) and 8 (6.3%) of partial response and no response ITP patients, respectively, at 3 months monitoring has switched into remission status at 12 months follow-up ($p < 0.001$). Out of 23 partial response who became remission,

only 6 (26.1%) patients needed second-line treatment while 2 patients did not require further therapy among no response cases.

The rate of patients treated at the onset of ITP diagnosis (80.6%) was in accordance with the recent study by Grimaldi-Bensouda et al (87.4%).⁶⁰ Corticosteroids remain the most frequently used first-line treatment followed by IVIg. A scoring system such as Khellaf's bleeding score >8 has been recommended by the French group to aid clinicians in adding IVIg to corticosteroids particularly in patients with life-threatening bleeding when a rapid increase in platelet counts is required.^{61,62} This practice can also be implemented among Malaysian physicians. The use of anti-D Ig in the Asia-Pacific region is uncommon due to its ability to cause hemolysis and lack of availability in many countries in Asia.^{63,64} None of the patients was given anti-D Ig in our study. Second-line therapy (31.2%) is less frequently used in our population as compared to French nationwide population-based study (47.1%).^{20,51} Recent ASH ITP guidelines recommend TPO-RAs rather than rituximab;⁶⁵ however, our rate of TPO-RAs usage is not as high as reported in ITP World Impact Survey (iWISh study).⁶⁶ TPO-RAs such as eltrombopag or romiplostim are an option in patients after failure of steroids and splenectomy, prior to the use of rituximab and also can reduce the use of concomitant ITP medication.^{67–70} Nevertheless, long-term treatment should be used with caution as the most frequent adverse effects were headache, thrombocytosis and the risk of fibrosis in the bone marrow.^{69,71} Splenectomy is hardly practiced nowadays and was performed in only 9 (1.8%) of our refractory patients, as compared to 6–34% in the other countries.⁶ Splenectomy is not advisable in secondary ITP associated with an autoimmune or connective tissue disease since a subsequent flare of the connective tissue disease may necessitate immunosuppression. Asplenic patients have a higher risk of infection and the use of immunosuppressants is associated with poor survival in these patients.⁷²

The mortality rate (11.0%) in our cohort is relatively consistent with the UK study (12.1%) but slightly lower compared to Netherland's study (15.8%). Mortality risks of our ITP patients include age ≥ 60 years, male, severe bleeding at presentation, CCI ≥ 1 and secondary ITP. Surprisingly, platelet counts at ITP onset and medications (anticoagulant and antiplatelet) were not related to mortality risk in our study population. Of the 55 ITP patients for whom a cause of death was recorded, 3% died due to bleeding, which is a very small proportion than reported previously ($\sim 13.0\%$).^{53,73} This is probably due to established guidelines from the ITP-IWG and ASH on ITP management, with the inclusion of eltrombopag, romiplostim and rituximab in the treatment of cITP. Newer agents such as sutimlimab, fostamatinib and decitabine were shown promising results in recent a ITP study.⁷⁴

The strengths of our study include the ability to include all cases of adult ITP during the study period. Thus, there is no selection bias of a certain group of ITP patients. The records from the computerized database were able to provide a relatively large number of cases. The study findings are comparable with other studies worldwide because we used IWG definitions and standardized terms and definitions. Nevertheless, this study has some limitations. As this study was done retrospectively in the tertiary hospital of haematology centres, the data were restricted which may contribute to analysis discrepancies. The local national ITP registry is not available in Malaysia, thus the completeness of the data registry cannot be exactly assessed. Consequently, the results cannot be extended to other regions or countries, particularly regarding laboratory results and treatment exposure.

Conclusions

In conclusion, this study confirms the epidemiology of newly diagnosed adults ITP is comparable with nationwide studies. Our study population demonstrated the high incidence in the female, Malay ethnicity, primary ITP and events of cutaneous bleeding with a predominance 18–29 years age group for both genders. Corticosteroids and immunotherapies are the most prescribed first-line and second-line pharmacological treatments. Splenectomy and TPO-RAs prescriptions are infrequent. Our mortality rate is even, but ITP-related bleeding death is fourth-fold lower than reported before. Mortality risks of our ITP patients include age ≥ 60 years, male, severe bleeding at presentation, CCI ≥ 1 and secondary ITP.

Future Directions

This study has paved the way for the development of a local national ITP registry or a population-based surveillance system. This registry will enable us to obtain data which is useful for standardization of local clinical practice, explore

new research and novelty findings in ITP patients in Malaysia including study for possible platelet disorders in refractory and cITP group. It is also our hope that with this data/registry, we are able to pave easier access to TPO-RAs that will benefit all individuals diagnosed with ITP.

Data Sharing Statement

Study data are available on request from the corresponding author.

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Disclosure

The authors report no conflict of interests.

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ABO and Rh Antigen Distribution Among Pregnant Women in South Western Uganda

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Introduction: ABO and Rh are the major blood group systems in Transfusion Medicine, the ABO system based on two red cell antigens (A, B) while the Rh has about 50 antigens of which five are highly clinically significant (D, C, c, E, e). These vary among races and ethnic groups. Blood type phenotype incompatibility between mother and fetus may result in antigen mismatch, triggering alloimmunization, and thus causing hemolytic transfusion reaction (HTR), which results in hemolytic disease of fetus and newborn (HDFN). This study aimed to determine the frequencies of ABO and rhesus blood group antigen in the pregnant women in South Western Uganda.

Methods: A cross-sectional study was carried out on 1369 pregnant women who were recruited and provided consent to participate during their regular antenatal visits between August 2020 and July 2021. Four milliliters (4mL) of EDTA-anti-coagulated blood samples were collected and ABO and Rh-blood grouping including Rh antigen screening was done using the agglutination technology comprised of glass beads and reagent contained in a column of the Ortho Biovue ID Micro Typing System (Ortho Clinical Diagnostics, New Jersey, USA). The Rh antigen phenotypes and frequencies were then determined.

Results: There was percentage distribution of 99.8%, c 99.3%, D 94.3%, C 19.2% and E 15.9%, with Rh cDe/cDe (65.1%) being the most common phenotype followed by cDe/CDe (15%), cDe/cDE (10.8%) and cDE/cDE 0.1% least common. The ABO grouping frequency was obtained as O 49.4%, A 29.5%, B 17.0% and AB 4.1%, with D positivity at 94.3%.

Discussion: Population genetic variations result in varied expressions of red cell antigens that may have clinical complications. Knowledge of the presence of these Rh antigen distributions and phenotype frequencies during pregnancy help in rational management of the pregnancy, alloimmunization and better approach to safe blood transfusion.

Keywords: ABO, Rh antigens, Rh phenotypes, pregnant women, South Western Uganda

Introduction

Blood groups are useful antigenic and genetic markers in the study of immune-haematological complications in a population. The Rh system, having many antigens, is easily classifiable into various phenotypes and genotypes in different populations and can be associated with particular complications.¹ The distribution patterns of ABO and Rh blood group systems are complex around the world and their antigenic variations often occur in populations within one small particular area. A significant regional heterogeneity has been reported in the ABO and Rh blood group gene frequencies.² However, one population may exhibit a high degree of similarity with a distant population that can be attributed to the common history of these populations.³

The frequency distribution of ABO group antigens varies across ethnic populations; for example, Asians have been found to have higher frequency occurrence of blood group B⁴ than White Europeans and among Africans, but the distribution of ABO blood groups is generally: B, 20%; A, 27%; AB, 4%; and O, 49%.⁵

The Rh blood group antigen has more than 50 antigens, including D, C, E, c, e, f, Cw, Cx, which have been linked to haemolytic diseases of the fetus and the newborn.⁶ Clinically, D antigen has high immunogenicity thus classified as D⁺ (Rh-positive) or D⁻ (Rh-negative). Amongst the White population, d⁺ frequency occurrence is 85%, while amongst the Black population it is 92%.^{7,8} Most D⁻ individuals will automatically produce anti-D when they are exposed to D⁺. The anti-D will then cause haemolysis of the recipient's red cells, which is the alloimmune haemolytic disease.⁹ The other Rh antigens like C, c, E, and e are of less immunogenic activity. They only become important in patient care upon the development of the corresponding antibody. D and E antibodies are mainly IgG in nature, although there are some of them that are IgM in nature, and mostly react at 37°C with anti-human globulin.²

Blood group antigen data in a population is important for effective management of related conditions. A 2.2% maternal alloimmunization prevalence has been reported in Uganda, especially in the south west. This study was done with the aim of determining the prevalence and trends of ABO and Rh antigen among the pregnant women attending antenatal clinics in South Western Uganda. Blood group antigen data are important in guiding population genetic studies that are not easily captured by current screening methods during antenatal visits but important in some complications that may arise.^{10,11}

Materials and Methods

This was a cross-sectional study where 1396 pregnant women attending antenatal clinic, not critically ill, were consented by use of informed consent system and recruited during their regular antenatal visits in the months of August 2020 to July 2021. 4mL of EDTA-anti-coagulated blood samples were collected, and ABO and Rh antigen testing was done using the agglutination technology comprised of glass beads and reagent contained in a column of gel to capture agglutinates in a semi-solid medium of the Ortho Biovue ID Micro Typing System (Ortho Clinical Diagnostics, New Jersey, USA). The gels column is about 75% packed gel and 25% liquid in micro-tubes embedded in a plastic card to allow ease of handling, testing, reading, and disposal.

Known positive and negative control samples were included in accordance with the relevant guidelines of quality assurance. ID cards were identified with the individual's initials and study identification number. The aluminum foil was removed, 10µL red cell suspension of 5% concentration added to the respective micro-tubes and the ID cards centrifuged for 15 minutes. The ID cards were read visually for agglutination or hemolysis.

Results

A total of 1396 pregnant women were recruited and they had a median age of 25 years and a median parity of 2, with median gravidity of 3. Most of them (65%) were in their second trimester (Table 1).

The distribution of ABO antigens in the participants showed most pregnant women having blood group O (49.4%), followed by A (29.5%), in the order of O, A, B, AB. The D antigen was observed in 94.3% of the participants (Table 2).

The Rh antigens c and e were common in most participants (99.3% and 99.8%, respectively), while E was least prevalent (15.9%). The Rh profile cDe/cDe (65.1%) was most common, followed by cDe/cDE (10.8%) while cDE/cDE (0.1%) was the least common (Tables 3 and 4). There was no significant difference in the distribution of antigens among the respective blood groups. The most common antigens were highly prevalent in those groups with high appearance (Table 5).

Table 1 Demographic Characteristics of the Participants (N=1369)

Variable	Category	Frequency	%
Age (n=1365)	(Min=14yrs; Max=45yrs); (Med= 25yrs; IQR=10yrs)		
Parity (n=1350)	(Min=0; Max=11); (Med= 2; IQR= 3)		
Gravidity (n=1354)	(Min=0; Max=12); (Med= 3; IQR= 3)		
Trimester (n=1327)	First	88	6.6
	Second	862	65.0
	Third	377	28.4

Table 2 The ABO, Rh Antigen Distribution in the Study Participants (N=1369)

Variable	Category	Frequency	%
ABO blood group	A	404	29.5
	B	233	17.0
	AB	56	4.1
	O	676	49.4
Rh group	Pos	1281	94.3
	Neg	78	5.7

Table 3 The Rh Antigen Distribution (N=1369)

Variable	Frequency	%
Rh Antigen - D	1299	94.9
Rh antigen - C	263	19.2
Rh antigen - E	218	15.9
Rh antigen - c	1360	99.3
Rh antigen - e	1366	99.8

Table 4 The Rh Antigen Phenotype Combination Distribution (N=1369)

Variable	Category	Frequency	%
Rh antigen	ccDee (cDe/cDe)	891	65.1
	CcDee (cDe/CDe)	205	15.0
	Ccdee (Cde/cde)	17	1.2
	CcDEe (cDe/CDE)	39	2.8
	ccDEe (cDe/cDE)	148	10.8
	CCDee (CDe/CDe)	7	0.5
	ccdee (cde/cde)	60	4.4
	ccDEE (cDE/cDE)	2	0.1

Table 5 Distribution of Rh Antigens by ABO Blood Groups

Blood Group	Rh Antigens				
	D (%)	C (%)	E (%)	c (%)	e (%)
A	387 (29.8)	81 (30.8)	61 (28.0)	401 (29.5)	404 (29.6)
B	214 (16.5)	45 (17.1)	33 (15.1)	230 (16.9)	232 (17.0)
AB	54 (4.1)	13 (4.9)	9 (4.1)	55 (4.0)	56 (4.1)
O	644 (49.6)	124 (47.1)	115 (52.8)	674 (49.6)	674 (49.3)

Discussion

Variability in genetics of different populations results in varied expression of red cell antigens. The distribution of the ABO blood group in this study was in the order of O > A > B > AB, which is similar to that obtained in other studies. Mbalibulha et al,¹² Apecu et al¹³ and Natukunda et al¹⁴ also reported similar findings amongst pregnant women and the general population in this area. This is the same order of blood group distribution in Ogoni ethnic group in Nigeria and North India, as reported by Christian et al¹⁶ and Mungwana,¹⁵ respectively.

Prevalence of D-positive in this study was 94.3%, which is similar to previous reports by Mangwana (94.5%)¹⁵ and Mbalibulha et al (96%)¹² in India and Africa, respectively. There are variations in prevalence of D in populations worldwide that range from 83–89% in European and American populations and up to 100% in Far East populations. This variation in the distribution of population groups has significant clinical impact as regards the potential for hemolytic disease of newborn and of hemolytic transfusion reaction as a result of alloimmunization from these antigens.

The highest prevalence among the Rh antigens was shown by *e* antigen (99.8%) followed by *c*, *D*, *C*, (99.3%, 94.3%, 19.2%, respectively) and *E* (19.66%) being the lowest. These findings are very similar to studies in Nigeria by Mukhter¹ and Erhabo et al⁸ but different when compared to those studies by Gupta et al,¹⁶ Dholakiya et al,¹⁷ and Gundrajukuppam et al¹⁸ done in India and other Far East populations. This difference is mainly attributed to the difference in ethnic and multi-racial group composition of these populations around the world.

The Rh phenotype distribution observed in this study among the pregnant women shows the order of commonest phenotypes to be cDe/cDe, CDe/cDe and then cDe/cDE. Similar findings have been observed in various studies in Africans.^{19,20} However, similar results are observed in a few white populations and about 2.9% of the Indian population.^{1,21,22} These findings are not similar to those obtained by Sarkar,²³ Makroo et al²⁴ Prinja et al²⁵ and Basu et al,⁴ who observed the order of prevalence to be CDe/CDe, CDe/cDe, CDe/cDe, and cDe/cDE. This is possibly due to the ethnic differences in the populations in these areas.

It's evident that the Rh antigen frequency in this population is unique to this population and would be helpful in serological investigations and transfusion support to pregnant women. Knowledge of varied frequency and phenotypic expression of major clinically relevant antigens in these blood group systems are helpful in more rational approach for pregnancy management, monitoring the initiation of alloimmunization by the antigens and its follow-up. This will help in the decision of who receives RhIG prophylaxis, thus minimizing costs. Methodological studying of these Rh antigens and blood group data in relation to alloimmunization in this population continues to be worthwhile.

Ethical Approval

All the participants were taken through consenting procedures to provide a written informed consent and the study was conducted following the participant protection procedures in conformity with the Helsinki Declaration. Ethical clearance was obtained from the ethical review board of the school of medicine, College of Health Science Makerere University (REC Ref No 2019-114) and the Uganda National Council for Science and Technology (HS508ES).

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Disclosure

The authors declare having no competing conflicts of interest relevant to the work presented in this article.

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Comparison of HIT Tests in Patients with COVID-19 and Thrombocytopenia

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Abstract: Thrombosis and coagulopathy have been found to be the most prevalent complications in patients with COVID-19. Thromboprophylaxis to prevent thromboembolic events is recommended for hospitalized COVID-19 patients. Heparin-induced thrombocytopenia (HIT) is a known complication of heparin use. This study aimed to determine the incidence of HIT among admitted patients with confirmed COVID-19 by PCR. In this study, two different HIT assays, rapid immunoassay (STic Expert HIT, Stago) and H-PF4 specific enzyme-linked immunosorbent assay (Asserachrom[®] HPIA – IgG), were performed. Of 200 patients with confirmed COVID-19, we identified 49 patients who met the possibility of HIT (low platelet count and high D-Dimer level). Only five (10.2%) had a positive HIT rapid test. However, none of the tested samples tested positive by ELISA. Thrombosis was reported in two of five (40%) patients. Further extensive studies are required to determine the prevalence and clinical significance of a positive HIT test among patients with COVID-19.

Keywords: COVID-19, thrombosis, coagulopathy

Introduction

COVID-19 has a systemic effect on the respiratory and cardiovascular systems. The correlation between thrombosis and COVID-19 is clinically important. Venous thromboembolism (VTE) has been reported in approximately 20–30% of patients, especially in critical cases.¹ Heparin-induced thrombocytopenia (HIT) is a known complication of heparin therapy, which changes the characteristics of heparin from anticoagulant to procoagulant.² The incidence of HIT in COVID-19 patients has been reported to be 0.8%.³ A meta-analysis of seven studies revealed that the combined incidence of HIT was higher in patients with severe COVID-19 who received heparin (2.2%) than those without severe symptoms (0.1%).³ HIT is caused by produced antibodies directed against a complex of platelet factor 4 (PF4) and polyanions such as heparin. Pathogenic immunoglobulin G (IgG) binds to PF4/heparin and activates cellular human immunoglobulin receptor IIa (FcRIIa) in platelets and monocytes, causing hypercoagulability and potentially fatal thrombosis.⁴

Materials and Methods

This study was conducted on 200 patients with COVID-19 who were admitted to our institute, between 1 March and 17 June 2021. Any patient who has confirmed COVID-19 via PCR and meets any of the following criteria should be admitted (clinical or radiological evidence of pneumonia; age >65 years; low oxygen saturation SpO₂ <94% on room air; Acute respiratory distress syndrome (ARDS), chronic pulmonary disease, chronic kidney disease history of comorbidities; diabetes Mellitus or/and hypertension; history of cardiovascular disease; obesity (BMI ≥40); use of immunosuppressant medications; history of organ transplant or another immunosuppression disease; history of active malignancy; and other Co-illness). Plasma samples were collected (in 3.8% sodium citrate) from patients (age range: 17–86 years old; median: 59 years old) with

confirmed COVID-19. All samples were centrifuged for 15 minutes at 2000–2500 RCF within two hours of sample collection, divided into aliquots, and stored at -80°C until testing.

Patient Sample Size and Clinical Presentation

Out of 200 patients with confirmed COVID-19, 49 (24.5%) met the inclusion criteria (platelet count less than $140 \times 10^9/\text{L}$; D-dimer more than 1 ng/mL). These patients had thrombocytopenia (median platelet count $83 \times 10^9/\text{L}$; range: 8 to $138 \times 10^9/\text{L}$) and had high D-dimer levels (median: 2.87 ng/mL; range: 1.12 to >20). All patients had received an anticoagulant (enoxaparin or unfractionated heparin) on the same day of hospital admission or two days later, according to the hospital guidelines for the management of COVID-19 patients.

Laboratory Assay

Plasma samples from these patients were tested for HIT by two different tests: rapid immunoassay and H-PF4 specific ELISA.

Rapid Immunoassay (STic Expert HIT, Stago)

STic Expert HIT is a qualitative detection of IgG antibodies against the PF4/polyanion complex in human plasma. It is a lateral-flow immunoassay that is based on the capillary action that induces a flow of the test sample along a test strip. A conjugate, which is a gold nanoparticle coated with an anti-ligand, is included in the test strip. The plasma was pipetted into a sample pad, and then drops of buffer that contained biotinylated human PF4/polyanion (PA) complex were added.

Human anti-PF4/PA antibodies in the plasma bind to the PF4/PA complexes. When the fluid passes through the test line on the strip, complexes develop. A biotinylated PF4/PA system, biotin coated gold nanoparticles and human antibodies are fixed in place by an immobilized goat antibody. This goat antibody was chosen for its ability to immobilize human IgG antibodies in a specific manner (which serve as the capture antibody printed onto the membrane). A strongly colored line can be interpreted visually to indicate a positive reaction (test line). A second line is included on the test strip (the control line). The presence of the control line indicates that the test was carried out correctly.⁵

H-PF4 Specific ELISA (Asserachrom[®] HPIA-IgG)

HPIA-IgG is a highly sensitive and specific ELISA for the detection of H-PF4-specific IgG antibodies. It is a quantitative assay based on the ability of the H-PF4 antibody to activate platelets in the presence of heparin, whereas immunoassays confirm the presence of the antibody without considering its ability to cause platelet activation. Heparin–PF4 complexes were coated on the inside of the ELISA. Anti-human IgG antibodies (from each plasma sample), combined with peroxidase, bound to any antigenic determinants of immobilized antibodies that were available. The action of the attached enzyme, peroxidase, on the Tetramethylbenzidine substrate reveals. A strong acid was used to terminate this reaction. The plate reader was set to read absorbance values at 450 nm, and the intensity of the color produced was proportional to the number of antibodies present in the sample. As a result, optical density units (cut-off $\text{OD} \leq 0.39$) values are used to detect the actual concentration computed for each sample against a standard via interpolation in the standard curve.

Results

Clinical Results

The 4Ts is an HIT pre-score method designed to improve and standardize clinical diagnosis. It includes four common aspects of HIT: (1) the severity of thrombocytopenia; (2) the timing of thrombocytopenia in relation to heparin exposure; (3) thrombosis or other HIT complications; and (4) the likelihood of additional thrombocytopenia causes. The algorithm generates an integer score ranging from 0 to 8, with 0–3, 4–5, and 6–8 indicating low, intermediate, and high HIT pretest probability, respectively.⁶ For all patients who met the required criteria for possible HIT, the 4T clinical probability score was calculated. The median HIT 4T score was 5 (range: 2–8).

Table I Laboratory and Clinical Results of Five Suspected Cases of HIT with COVID-19

Patient	Platelets/ 10 ⁹ L	D-Dimer	HIT 4T Score	VTE Score	STic Expert HIT	H-PF4 ELISA OD (≤0.39)	Asserachrom® HPIA -IgG	Thrombosis	Type of Anticoagulant	Dose
1	139	1.17	5	14	Weak positive	0.09	Negative	PE	Heparin	5000, 0.2 mL q8hr
2	101	2.9	5	9	Weak positive	0.178	Negative	NO	Enoxaparin	0.4 mL of 40 mg q12hr
3	119	1.52	2	13	Weak positive	0.103	Negative	NO	Enoxaparin	0.4 mL of 40 mg q24hr
4	84	>20	8	9	Positive	0.142	Negative	STROKE	Enoxaparin	0.4 mL of 40 mg q24hr
5	98	4.06	5	11	Positive	0.112	Negative	NO	Heparin	2500 units q8hr

Abbreviations: HIT, heparin-induced thrombocytopenia; VTE, venous thromboembolism; OD, optical density; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G.

The risk of developing VTE was assessed for all cases using the value of a simple risk assessment model proposed by the Padua Prediction Score.⁷ The Padua Prediction Score was created to predict the risk of venous thromboembolism (VTE) in medical patients who were hospitalized. Very low risk (score 0), low risk (1–2), moderate risk (3–4), or high risk (score 5) are the five risk levels assigned to patients.⁷ The median VTE score was 9 (range, 5–16). Thrombosis was reported in two (40%) out of five patients.

Laboratory Results

Rapid Immunoassays (STic Expert HIT, Stago)

The results showed that only five (10.2%) of the 49 patients were positive for the rapid test. Strong positive reactions were recorded in two (4.08%) patients, weakly positive in three (6.12%) patients, while all other patients (44; 89%) had a negative result. Remarkably, one patient who had a positive STic expert HIT suffered a stroke. It developed on the same day as enoxaparin treatment (0.4 mL of 40 mg q24h). Interestingly, this patient had elevated D-dimer levels (>20 ng/mL) and thrombocytopenia ($84 \times 10^9/L$). Pulmonary embolism (PE) was reported in another patient (20%) who had a weakly positive STic Expert HIT assay. PE started five days after heparin administration (5000 iu sc, q8h). This patient had a mildly elevated D-dimer level (1.17 ng/mL) and platelet count of $139 \times 10^9/L$ (Table 1).

H-PF4 Specific ELISA (Asserachrom[®] HPIA-IgG)

No positive results were detected in all 49 suspected cases of HIT.

Discussion

In this study, we report a higher incidence of HIT in patients with COVID-19, as detected by a screening method, while a more specific test (ELISA) was negative in all cases. In addition, we report the development of VTE in two patients. The first patient who developed a stroke one day after starting heparin treatment was probably not related to HIT positivity but rather to COVID-19 infection severity. However, the second case was probably due to HIT, although the ELISA test result was negative.

Conclusions

HIT can occur in COVID-19 patients receiving heparin and should be recognized as a possible cause of thrombocytopenia in these individuals. Overall, our results provide further support to show that rapid HIT immunoassays are more sensitive and less specific compared to PF4-specific enzyme-linked immunosorbent assays. Further investigations with a larger sample size are warranted to confirm this finding and to determine the incidence of HIT among COVID-19 patients.

Abbreviations

HIT, heparin-induced thrombocytopenia; VTE, venous thromboembolism; PF4, platelet factor 4; IgG, immunoglobulin G; FcRIIA, F cellular human immunoglobulin receptor IIa; ARDS, acute respiratory distress syndrome; OD, optical density; PA, polyanion.

Data Sharing Statement

Data are available upon request to the corresponding author.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of King Faisal Hospital and Research Center (KFSH&RC), Riyadh, Saudi Arabia. Informed consent was obtained from the study participants prior to the commencement of the study. This study was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors report no conflict of interest in this work.

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Thromboelastometry Predicts Thromboembolic Events, Hospital Length of Stay, and Mortality in Patients with COVID-19 Infection and Mild Hypoxemia: A Prospective Observational Study

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Thromboelastometry Predicts Thromboembolic Events, Hospital Length of Stay, and Mortality in Patients with COVID-19 Infection and Mild Hypoxemia: A Prospective Observational Study

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Background: The aim of this study was to assess changes in hemostasis and associated outcome of hospitalized patients with COVID-19 infection and mild hypoxemia.

Methods: Adult patients with COVID-19 infection and hypoxemia admitted to ICU were included in this prospective observational study. The primary outcome was defined as an unfavorable course of the disease if a patient: (1) developed a thromboembolic event while receiving anticoagulation prophylaxis, (2) had prolonged ICU stay, or (3) died. Demographic data, laboratory parameters and thromboelastometry (ROTEM) test results were collected.

Results: Twenty-five patients were recruited into the study. There were 16 patients with an unfavorable course of the disease. Compared to the 9 patients in the favorable course group, patients with an unfavorable course had a lower platelet count, median difference of 154 (95% CI, 26 to 223 $\times 10^9/L$), $P = 0.012$, and lower clot firmness parameters in EXTEM assay: amplitude at 20 minutes (A20), median difference of 7 (95% CI, 2 to 11) $P = 0.006$, maximum clot firmness (MCF), median difference of 6 (95% CI, 3 to 10) $P = 0.006$ and area under the curve (AUC) with a median difference of 671 (95% CI, 244 to 1029) $P = 0.005$. They also demonstrated suppression of fibrinolysis: higher lysis index 60, median difference of -3 (95% CI, -6 to 0), $P = 0.023$. Results of functional fibrinogen (FIBTEM) assay were similar between the groups.

Conclusion: The platelet count and the results of EXTEM assay, but not FIBTEM assay, were associated with the difference in clinical outcome among patients with COVID-19 infection and hypoxemia. The role of platelets in the outcome of COVID-19 infection calls for further investigation. Future studies on adjusting anticoagulant therapy based on the results of viscoelastic testing may be beneficial.

Keywords: intensive care unit, thromboelastometry, coronavirus disease 2019, hypoxemia, hemostasis, thrombosis, recovery, mortality

Background

More than one million people have died in the US from the coronavirus infection (COVID-19) since the beginning of the pandemic.¹ In addition to severe pulmonary and renal injury, abnormality of hemostasis is recognized as a contributing factor to the severity of the disease.² Thromboembolic complications were diagnosed in 31% of patients with COVID-19 infection admitted to an intensive care unit, despite anticoagulation therapy.³ Assessment of hemostasis with traditional plasma coagulation tests (eg, prothrombin time, activated thromboplastin time) is often not helpful because they evaluate only the initial phase of clot formation and frequently do not demonstrate significant abnormality.⁴

Viscoelastic tests (eg, thromboelastography (TEG), rotational thromboelastometry (ROTEM), or ClotPro) were developed to assess in real time all stages (dynamic) of clot formation and fibrinolysis using whole blood samples. In patients with bacterial sepsis abnormal coagulation detected by viscoelastic testing has shown to be of negative prognostic value.⁵ Moreover, among critically ill patients with COVID-19 infection, viscoelastic tests have presented signs of hypercoagulation (elevated clot firmness parameters, ie, extrinsic rotational thromboelastometry (EXTEM) MCF > 70 mm) and suppression of fibrinolysis (high lysis indexes, ie, EXTEM LI60 >95%) and also associated with negative prognosis.^{6,7} The association between the results of viscoelastic testing and the clinical outcomes may be different in critically ill patients compared to less critically ill hospitalized patients.^{8,9} It is currently unknown if the results of viscoelastic testing in patients with COVID-19 infection and mild hypoxemia (patients who did not require mechanical ventilation or received treatment with vasopressors) are associated with severity of the infection and outcome of the disease.

The main objective of the current investigation was to evaluate if assessments of hemostasis by traditional and research thromboelastometry tests among patients with COVID-19 infection and mild hypoxemia performed at the time of admission to the intensive care unit were associated with higher risk of thromboembolic events, prolonged hospitalization or death. We hypothesized that patients with an unfavorable course of COVID-19 disease would demonstrate higher clot firmness parameters using ROTEM analysis compared to the patients with a favorable course of the disease.

Methods

This single center prospective cohort study complied with the Declaration of Helsinki and was approved by the Lifespan Institutional Review Board (IRB# 1591584, October 27, 2020). Written informed consent was obtained from all participants and the study period extended from January 27, 2021 to April 2, 2021. The study is reported following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting observational studies.¹⁰

Inclusion and Exclusion Criteria

All consecutive patients 18 years or older who were admitted to the step-down unit or intensive care unit of our hospital due to respiratory insufficiency were considered for inclusion on the first morning following admission. Inclusion criteria were a positive PCR COVID-19 test result and respiratory insufficiency requiring supplemental oxygen. Exclusion criteria included patient's refusal to participate or the inability of the patient to consent due to their physical condition. All patients received medications and respiratory treatment (supplemental oxygen and respiratory support) for COVID-19 related hypoxemic respiratory failure and pre-existing medical conditions according to standards of care. No patients received mechanical ventilation prior to enrollment. All patients received prophylactic anticoagulation therapy (heparin or low-molecular-weight heparin).

Data Collection

Data collection was performed by using a pre-designed collection form and included the following variables: (1) patient demographics (eg, age, gender, body mass index), (2) clinical characteristics (eg, comorbidities and vaccination status), (3) laboratory results (eg, white blood cell count, hematocrit, platelet count, potassium, albumin, glucose, D-dimer, C-reactive protein), and (4) treatment information (eg, antiviral medication, glucocorticoid treatment, antibiotics and anticoagulants). The blood sampling for the routine laboratory panels was performed by the patient's clinical providers. All patients were followed until discharge from the hospital or death.

Study Grouping

The primary study endpoint was an unfavorable course of the disease defined by the presence of one or more of the following outcomes: (1) development of a thromboembolic event (deep venous thrombosis or pulmonary embolism) while receiving prophylactic anticoagulation (2) prolonged (greater than 30 days) intensive care unit (ICU) stay, and (3) death. It is well documented that patients with respiratory insufficiency due to COVID-19 are at higher risk for

thromboembolic events, mortality, and prolonged ICU stays.^{11–13} Patients who did not develop these conditions were assigned to the favorable course of the disease group.

Thromboelastometry Procedure

Rotational thromboelastometry was performed by trained personnel (DS) using a ROTEM delta device (TEM Innovations GmbH, Munich, Germany). Quality control tests were performed in accordance with manufacturer's recommendations. After obtaining consent, the blood samples for the ROTEM analysis were collected within two to three hours after the administration of the next scheduled dose of the prescribed anticoagulant medication. Venous blood samples (3.6 mL) were collected in blue top tubes containing sodium citrate anticoagulant. The blood specimens were processed at 37°C within 30 minutes of collection.

The following ROTEM tests were performed: (1) EXTEM assay consisting of recombinant tissue factor and Polybrene (heparin inhibitor) added to re-calcified whole blood to activate the extrinsic pathway and initiate coagulation, (2) In the FIBTEM assay, the contribution of platelets to clot formation is eliminated allowing to assess fibrinogen contribution (functional fibrinogen) to hemostasis. It is performed by an addition of recombinant tissue factor, polybrene and platelet inhibitor cytochalasin D added to the whole blood sample to activate the extrinsic pathway while suppressing platelets.

Thromboelastometry Parameters

The following ROTEM parameters were recorded for all of the assays: (1) Clotting time (CT) in seconds from the beginning of the measurement until the beginning of clotting illustrated by a clot firmness amplitude of 2 mm; (2) Clot formation time (CFT) measured in seconds is the time needed for the clot firmness amplitude to increase from 2 to 20 mm; (3) Alpha angle (ALP) which is tangent to the clotting curve at the 2 mm clot firmness amplitude point; (4) A10 and A20 represent clot amplitude in millimeters reached at 10 and 20 minutes after CT, respectively; (5) Maximum clot firmness (MCF) measured in millimeters is the maximum amplitude of clot firmness reached during testing run time; (6) Lysis index LI30 and LI60 indicates the percentage of the remaining clot firmness in percentage of MCF at 30 and 60 minutes after CT. (7) Maximum lysis (ML) clot firmness reduction in percentage of MCF during the run time of 90 minutes.

ROTEM parameters reflecting the velocity of clot formation are recognized in the European Union; however, they are still considered research parameters in the United States. The research parameters we analyzed included: (1) Maximum clot velocity (maxV) measured in mm/second; (2) time to maximum velocity (maxV-t) is measured in seconds, (3) Area under the velocity curve (AUC) is defined as the area under the first derivative curve from start of the derivative curve until MCF is reached. It correlates to endogenous thrombin potential.^{14,15} The velocity parameters MaxV, maxV-t and AUC are the first derivative of the ROTEM curve.

Statistical Analysis

No statistical power calculation was performed due to the investigative nature of the study. Data were tested for normality using Kolmogorov–Smirnov test. Categorical variables were evaluated by using a Chi-square test of independence or Fisher exact test were appropriate. Non-normally distributed interval and ordinal data are reported as median (interquartile range) and compared among groups by using Mann–Whitney *U*-test. The estimate median difference was calculated using the Hodges-Lehmann method. All statistical tests were two-tailed and a *p* value <0.05 was considered statistically significant. The analyses were performed using Stata 11.1 (Stata Corporation, College Station, TX) and SAS software version 9.4 (SAS Institute Inc., Cary, North Carolina).

Results

Thirty-one patients were approached and 25 patients agreed to participate in the study. Four patients refused to participate, one patient was unable to consent and one patient was missed by the recruitment team. Sixteen patients were assigned to the unfavorable course group as they developed deep venous thrombosis (*n* = 5), pulmonary embolism (*n* = 3), prolonged hospitalization (*n* = 5) or died during their hospitalization (*n* = 8). Nine patients were assigned to the

favorable course group as they recovered from respiratory failure within three weeks of admission and did not develop any thromboembolic events. One patient quickly recovered from COVID-19 related respiratory failure but died following re-admission due to a perforated bowel in the setting of newly discovered obstructive colon cancer. The patient refused surgery and died 3 days after their initial discharge from the hospital.

Patients' demographics and hospital stay are presented in Table 1. There were no differences between the two study groups in patients' age, body mass index, or gender distribution. There was no difference in the time period from admission to ROTEM sampling between groups with a median time of 3 days (range 1–15). Pre-existing medical conditions were also similar in both groups, with hypertension, cardiovascular disease, and diabetes as the most frequent co-morbidities. Patients in the favorable course group experienced a shorter hospital stay than those patients in the unfavorable course group, median difference of -10 (95% CI, -22 to -1), $P = 0.014$.

Patients received antiviral, antibiotics, glucocorticoids, and prophylactic anticoagulation therapy treatment as directed (Table 2). At the time of our study, other treatment modalities such as anti-interleukin-6 receptor monoclonal antibody, tocilizumab was not routinely used for patient care.

The laboratory parameters are presented in Table 3. There was no difference between the groups regarding white blood cell count, hematocrit, potassium, albumin, glomerular filtration rate, blood glucose, D-dimer and C-reactive protein. The

Table 1 Patient Characteristics and Hospital Stay

	Total COVID Patients (n=25)	Unfavorable Course (n=16)	Favorable Course (n=9)	P value
Age, (years)	66 (62 to 74)	70 (63 to 78)	64 (53 to 71)	0.164
Sex, n (%)				
Male	14 (56)	9 (56)	5 (56)	1
Female	11 (44)	7 (44)	4 (44)	
Body mass index, (kg/m ²)	30 (25 to 39)	31 (25 to 36)	29 (24 to 40)	0.840
Body mass index > 40, (kg/m ²)	4 (17)	2 (12)	2 (22)	0.602
Comorbidities, n (%)				
Cardiovascular disease	14 (56)	9 (56)	5 (56)	0.973
Chronic kidney disease	5 (20)	4 (25)	1 (11)	0.621
Diabetes	15 (60)	8 (50)	7 (78)	0.229
Hypertension	20 (80)	12 (75)	8 (89)	0.621
Malignancy	9 (36)	7 (44)	2 (22)	0.401
Smoker*	8 (32)	7 (44)	1 (11)	0.182
COPD/Asthma	10 (28)	6 (25)	4 (33)	0.673
Oxygen-dependent	3 (12)	1 (6)	2 (22)	0.238
OSA	6 (24)	4 (25)	2 (22)	1
COVID vaccine, n (%)				
1 dose	2 (8)	1 (6)	1 (11)	1
2 dose	2 (8)	2 (12)	0	
Length of hospitalization (days)	14 (8 to 26)	20 (11 to 31)	10 (4 to 12)	0.013

Notes: Data is presented as median (interquartile range) or n %. *Previous or current smoker.

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; OSA, obstructive sleep apnea.

Table 2 Patient Treatment and Prophylactic Anticoagulation Therapy During Hospital Course

	Total COVID Patients (n=25)	Unfavorable Course (n=16)	Favorable Course (n=9)	P value
Medication/Treatment				
Antiviral medication				
Remdesivir	21 (84)	14 (88)	7 (78)	0.524
Glucocorticoid				
Dexamethasone	23 (92)	16 (100)	7 (78)	0.120
Antibiotics	20 (80)	13 (81)	7 (78)	0.835
Anticoagulants				
Lovenox	14 (56)	8 (50)	6 (67)	0.420
Heparin				
SQ	8 (32)	4 (25)	4 (44)	0.317
IV	2 (8)	1 (6)	1 (11)	0.667
Quality of Insurance				
Commercial Payer	17 (68)	10 (63)	7 (78)	0.432
Government Payer	8 (32)	6 (37)	2 (22)	0.432

Note: Data is presented as n, %.

Abbreviations: SQ, subcutaneous; IV, intravenous.

Table 3 Laboratory Parameters

	Reference Range	Total COVID Patients (n= 25)	Unfavorable Course (n=16)	Favorable Course (n=9)	P value
White Blood Cell Count, (x10 ⁹ /L)	3.5–11.0	8.6 (7.5 to 12.6)	8.9 (7.7 to 12.3)	8.5 (6.8 to 13)	0.651
Hematocrit, (%)	32–45	36 (28 to 40)	36 (31 to 40)	33 (27 to 40)	0.734
Platelet counts, (x10 ⁹ /L)	150–400	231 (161 to 348)	191 (132 to 248)	348 (273 to 364)	0.011
Potassium, (mEq/L)	3.6–5.1	3.9 (3.7 to 4.2)	4 (3.5 to 4.3)	3.8 (3.7 to 4.1)	0.777
Albumin, (g/dL)	3.5–5.0	3.2 (2.9 to 3.6)	3.2 (2.7 to 3.5)	3.1 (2.9 to 3.6)	0.929
Glucose, (mg/dL)	67–99	153 (120 to 210)	156 (111 to 208)	153 (139 to 210)	0.865
D-dimer, (ng/mL)	0–300	442 (338 to 1253)	462 (300 to 1046)	356 (338 to 1301)	0.910
C-reactive protein, (mg/L)	0–10	79 (38 to 138)	107 (49 to 144)	54 (35 to 100)	0.246

Note: Data is presented as median (interquartile range) or n, %.

median platelet count was within normal range in both groups; however, patients in the favorable course had higher platelet counts compared to patients in the unfavorable course, median difference of 154 (95% CI, 26 to 223 x10⁹/L, $P = 0.012$).

In the EXTEM assay, there were signs of elevated coagulation activity in both groups (EXTEM AUC >7000), though patients in the favorable course had significantly higher clot firmness parameters compared to patients in the unfavorable course group: A10 median difference of 8 (95% CI, 2 to 13), $P = 0.012$, A20 median difference of 7 (95% CI, 2 to 11), $P = 0.006$ and MCF median difference of 6 (95% CI, 3 to 10), $P = 0.006$ (Table 4). They also had higher fibrinolytic activity, which was demonstrated by a lower LI60, median difference of −3 (95% CI, −6 to 0), $P = 0.023$ and higher ML, median difference of 4 (95% CI, 1 to 7), $P = 0.018$. Values of CT, CFT and alfa angle were approaching, but did not

Table 4 Results of the Thromboelastometry Extrinsic Activation (EXTEM) Assay

Characteristics	Unfavorable Course (n=16)	Favorable Course (n=9)	Hodges Lehmann Estimator of Shift (95% CI)	P value
CT, (sec)	75 (72 to 87)	68 (64 to 73)	-9 (-22 to 0)	0.057
CFT, (sec)	59 (53 to 78)	53 (47 to 56)	-9 (-27 to 0)	0.051
MCF, (mm)	66 (63 to 69)	73 (71 to 74)	6 (3 to 10)	0.006
Alpha angle	78 (76 to 79)	79 (79 to 80)	1 (0 to 5)	0.057
A10	61 (56 to 66)	68 (67 to 69)	8 (2 to 13)	0.012
A20	66 (62 to 69)	73 (70 to 74)	7 (2 to 11)	0.006
LI30	100 (99 to 100)	99 (99 to 100)	0 (-1 to 0)	0.100
LI60	95 (92 to 97)	90 (90 to 94)	-3 (-6 to 0)	0.023
ML	10 (7 to 12)	15 (11 to 16)	4 (1 to 7)	0.018
Max V	22 (18 to 25)	23 (22 to 27)	2 (-1 to 8)	0.221
MaxV-t	100 (91 to 132)	112 (98 to 115)	2 (-27 to 21)	0.799
AUC	6590 (6312 to 6847)	7282 (7032 to 7384)	671 (244 to 1029)	0.005

Notes: Data presented as medians (IQR). Comparison between groups was performed Mann-Whitney U-Test and Hodges-Lehmann Estimator.

Abbreviations: CT, clotting time; CFT, clotting formation time; MCF, maximum clotting firmness; A10, amplitude 10 minutes after clotting time; A20, amplitude 20 minutes after clotting time; LI30, lysis index 30 minutes after clotting time; LI60, lysis index 60 minutes after clotting time; ML, maximum lysis; Max V, maximum clot velocity; MaxV-t, time to maximum velocity; AUC, area under the curve reflecting the overall platelet aggregation.

reach, significant difference ($P = 0.057$, 0.051 and 0.057 , respectively). Among the research parameters, clot velocity maxV and maxV-t was not different, but AUC demonstrated a significant increase in the favorable course group, with a median difference of 671 (95% CI, 244 to 1029), $P = 0.005$.

In the FIBTEM assay, the parameters A10, A20, and MCF were above the normal limits in both groups but did not demonstrate significant difference between patients who had favorable compared to unfavorable outcomes. Similarly, there was no significant difference in fibrinolytic parameters and AUC in FIBTEM between the groups (Table 5).

Table 5 Results of the Thromboelastometry Extrinsic Activation (FIBTEM) Assay

Characteristics	Unfavorable Course (n=16)	Favorable Course (n=9)	Hodges Lehmann Estimator of Shift (95% CI)	P value
A10	33 (29 to 37)	35 (28 to 45)	2 (-6 to 12)	0.630
A20	35 (30 to 39)	38 (30 to 49)	2 (-6 to 13)	0.650
MCF, (mm)	36 (32 to 40)	38 (31 to 49)	2 (-6 to 13)	0.630
LI30	100 (100 to 100)	100 (100 to 100)	0 (0 to 0)	0.211
LI60	99 (98 to 100)	96 (95 to 100)	-2 (-5 to 0)	0.057
ML	2 (1 to 3)	7 (0 to 10)	4 (-1 to 8)	0.196
AUC	3611 (3163 to 4057)	3817 (3031 to 4883)	216 (-626 to 1240)	0.671

Notes: Data presented as medians (IQR). Comparison between groups was performed Mann-Whitney U-Test and Hodges-Lehmann Estimator.

Abbreviations: A10, amplitude 10 minutes after clotting time; A20, amplitude 20 minutes after clotting time; MCF, maximum clotting firmness; LI30, lysis index 30 minutes after clotting time; LI60, lysis index 60 minutes after clotting time; ML, maximum lysis; AUC, area under the curve reflecting the overall platelet aggregation.

Discussion

This prospective cohort study identified early changes in hemostasis among patients with COVID-19 infection and mild hypoxemia, which were associated with clinical outcomes. The changes in hemostasis were demonstrated among patients who, at the time of ROTEM testing, did not have signs of thrombosis, require mechanical ventilation for respiratory failure, exhibit signs of hemodynamic instability or were treated with vasopressors. Contrary to our expectations, higher pro-coagulation activity detected by thromboelastometry at the time of admission to the ICU was associated with shorter hospitalization, lower incidence of thromboembolic event or death among patients with COVID-19 related hypoxemia. A higher platelet count, higher EXTEM clot firmness parameters (A10, A20 and MCF), a higher EXTEM AUC together with fibrinolytic activity indexes (lower LI60 and higher ML at 90 minutes, but not LI30) were associated with a favorable course of the disease.

Our findings demonstrated that fibrinolytic shutdown is associated with negative prognosis not only in critically ill COVID-19 patients, as reported previously,^{6,7,16} but in less severely ill patients with mild hypoxemia, who did not require treatment with vasopressors and/or mechanical ventilation. The association between lower clot firmness parameters, lower (but still within normal limits) platelet count and worse clinical outcome in COVID-19 patients is presented for the first time.

Alterations observed in the hemostasis of patients with COVID-19 infections is not well understood; however, it is likely related to the hyperinflammatory response due to the COVID-19-infection with significant and prolonged increase in the production of cytokines such as IL-6, TNF- α .¹⁷ This hyperinflammatory response is different from the cytokine storm observed in patients with bacterial sepsis or other types of severe respiratory infection and is correlated with the disease severity and patient prognosis.¹⁷ The hyper-production of proinflammatory cytokines is associated with elevated levels of fibrinolysis inhibitors such as plasmin activator inhibitor 1 (PAI-1) and thrombin activated fibrinolysis inhibitor.^{18,19} This creates an imbalance between fibrinolysis activators and inhibitors which results in fibrinolytic shutdown. Other potential factors that may contribute to the development of fibrinolysis suppression such as consumption of the pro-enzyme plasminogen or a lack of fibrinolysis activators (tissue plasminogen activator, tPA) have not been demonstrated in patients with COVID-19.^{6,7} Furthermore, elevated factor XIII activity is unlikely the cause of fibrinolysis suppression in patients with COVID-19, since COVID-19 infection is associated with acquired factor XIII deficiency.²⁰

The hypercoagulable status observed in patients with COVID-19 disease is different from patients with other severe respiratory infections.^{21,22} In addition to elevated levels of D-Dimer, elevated clot firmness parameters such as maximum clot firmness (MCF), clot amplitude at 20 minutes (A20) and area under the curve (AUC) were previously reported.^{21,22} The increase in proinflammatory cytokines, an elevated release of neutrophil extracellular traps and the expression of tissue factor by monocytes may contribute to the increase in clot firmness parameters.²³ The changes in the dynamics of clot development demonstrated by viscoelastic testing, such as an increase in clot firmness parameters and suppression of fibrinolysis, reflect the prothrombotic profile of hemostasis in patients with COVID-19 infection. It is interesting to note that contrary to our expectations, those changes in the results of viscoelastic testing had an opposite prognostic value among our COVID-19 patients. While fibrinolytic shutdown was associated with an unfavorable outcome, more significantly elevated clot firmness parameters were associated with a favorable outcome.

Since the difference in clot firmness parameters was observed in the EXTEM assay and not in the FIBTEM assay (where contribution of platelets to clot formation is eliminated), we postulate that the difference in hemostasis and clinical outcomes observed between the study groups may be associated with the difference in platelet contribution to clot formation. Moreover, even though the patients in both study groups had normal platelet counts, the patients in the favorable course group had significantly higher platelet count. In two previous reports that investigated platelet function between healthy controls and COVID-19+ patients reported no significant differences between groups.^{24,25} At the same time, it has been shown that in patients with COVID-19 infection, an increase in platelet function as assessed by ROTEM platelet ARATEM (stimulation with arachidonic acid) and ADPTM (stimulation with adenosine diphosphate) during a 14-day study period was associated with a favorable outcome.²⁶ The hyperproduction of proinflammatory mediators seen in patients with COVID-19 infection, such as increased von Willebrand Factor, may affect platelet activity and

contribute to the observed difference in platelet count, ROTEM results and clinical outcomes observed in our study.²⁷ The role of platelets in the pathogenesis of COVID-19 disease and alteration of platelet function as a possible therapeutic goal will need further investigation.

Here, it is important to consider that COVID-19-associated coagulopathy is a very dynamic process and can change significantly during the course of the disease,^{28–30} which may require adjustment of anticoagulation therapy. Indeed, a recent randomized controlled trial did not demonstrate a benefit of therapeutic anticoagulation over prophylactic anticoagulation among critically ill COVID-19 patients.³¹ The implementation of a personalized anticoagulation regimen adapted to the individual coagulation status might be advantageous.³² Thromboelastometry testing may be a viable strategy to optimize coagulation status and the subsequent prognosis for patients with COVID-19 related hypoxemia.

Limitations

Our study does have several limitations. Due to the small sample size, our statistically significant results should be confirmed with future randomized clinical trials with sample size calculation and power analysis. Second, the absence of dynamic observation of changes in hemostasis. Serial measurements of the hemostasis components would provide a more accurate description of the dynamic changes occurring in hemostasis in this population. However, a larger study population employing continuous or repeated measures over time is needed to assess whether the prognostic value of platelet count and EXTEM clot firmness are modified by bacterial superinfection and organ failure. Last, the COVID-19 infection has affected racial minorities in the United States more severely than any other racial group. The majority of patients in our study were Caucasian and thus our findings cannot be generalized to other racial groups.^{33–35}

Conclusions

Suppression of fibrinolysis, lower (though still within normal limits) platelet count and lower clot firmness parameters (in EXTEM, but not FIBTEM assay) were associated with unfavorable course of the disease in patients with COVID-19 infection and mild hypoxemia. The role in the outcome of COVID-19 infection calls for further investigation. An investigation into adjusting anticoagulant therapy based on the results of viscoelastic testing may be beneficial.

Abbreviations

ALP, alpha angle; A10, amplitude 10 minutes after clotting time; CCT, conventional coagulation tests; CFT, clot formation time; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; CT, clotting time; DVT, deep vein thrombosis; ICU, intensive care unit; INR, international normalized ratio; LI60, lysis index 60 minutes after clotting time; Max V, maximum velocity; MaxV-t, time to maximum velocity; MCF, maximum clot firmness; ML, maximum lysis; ROTEM, rotational thromboelastometry; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; TEG, thromboelastogram.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval

This study was approved by the Lifespan Institutional Review Board (IRB# 1591584, October 27, 2020). Our study conforms to provisions of the Declaration of Helsinki. Written consent was obtained from all participants.

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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.

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Disclosure

Dr. Klaus Görlinger has been the Medical Director of TEM Innovations GmbH, Munich, Germany since 2012 and reports no other potential conflicts of interest in relation to this work. All other authors (DS, MCK, AL, RS, DP, and GDO) report no conflicts of interest in relation to this work.

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Peripheral Cytopenia and Its Associated Factors in Type 2 Diabetes Mellitus Patients, Northwest Ethiopia

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Background: Hematological abnormalities are linked with diabetes mellitus (DM) and play a major role in diabetes-related micro- and macro-vascular complications. Therefore, this study aimed to investigate the magnitude of peripheral cytopenia and associated factors in type 2 diabetes (T2DM) patients.

Methods: A cross-sectional study was conducted from March to May 2021 at the University of Gondar Comprehensive Specialized Hospital. A total of 357 T2DM participants were selected using a simple random sampling technique. A total of 3 mL of venous blood samples were collected using the vacutainer method for the complete blood count (CBC). A univariate and multivariate regression analysis were used to investigate the association between dependent and independent variables. P-value <0.05 was considered statistically significant.

Results: The magnitude of cytopenia, bicytopenia, and pancytopenia were 21% (95% CI: 17.1, 25.53), 1.1% (95% CI: 0.44, 2.85), and 0.56% (95% CI: 0.01, 1.12), respectively. Furthermore, the magnitudes of anemia, leucopenia, and thrombocytopenia were 8.7% (95% CI: 6.18, 12.06), 10.9% (95% CI: 8.09, 14.59), and 5.3% (95% CI: 3.43, 8.16), respectively. Being male (AOR: 3.23; 95% CI: 1.43, 7.56), lack of exercise (AOR: 2.70; 95% CI: 1.137, 6.43), and never married (AOR: 3.90; 95% CI: 1.248, 12.18) were all associated with anemia.

Conclusion: This study showed that T2DM causes disturbances in the hematological parameters and leads to a mild level of cytopenia. It is, therefore, suggested that hematological abnormalities, especially cytopenia, should be monitored and controlled on a regular basis in T2DM patients for better prognosis and quality of life.

Keywords: peripheral cytopenia, type 2 diabetes mellitus, Gondar, Ethiopia

Background

Diabetes mellitus has become one of the leading global diseases of the twenty-first century, with over 422 million diabetics worldwide.¹ It is a life-threatening disease that causes nephropathy, retinopathy, and neuropathy, as well as a variety of metabolic diseases.² DM develops when the body's cells are unable to utilize glucose for energy. It can be classified as Type 1 diabetes (T1DM), T2DM, and gestational diabetes. T2DM is defined as a combination of low peripheral insulin resistance and insulin production from pancreatic cells.³

Peripheral pancytopenia is defined as a reduction in all three major constituents of the blood to levels below their lower normal range, manifesting as anemia, leucopenia, and thrombocytopenia all occurring at the same time. Cytopenia, on the other hand, is a decline in one of the produced elements.⁴ DM impacts the erythropoiesis system by inducing a hyperglycemic situation that causes protein glycation. Hence, cytopenia can result in this case. Of the different types of cytopenia, anemia is the most common type of disorder in T2DM. Anemia can be caused by a decrease in erythropoietin production, which is linked to microvascular complications and renal impairment, systemic inflammation that affects iron metabolism, and the release of multiple inflammatory cytokines and free radicals that increase hepcidin (which leads to ferroportin degradation and

iron deficiency anemia), and the ferroportin degradation leads to a blockage of duodenal iron transfer.^{5–9} Besides, red blood cell (RBC) count has been found to be associated with microvascular and macrovascular complications, which negatively affect the circulatory system. All of these changes will have an impact on blood cell count, particularly RBC count.¹⁰

White blood cells (WBC) and platelet counts are affected by T2DM and its medications.¹¹ Immune-based pathways are the most common cause of thrombocytopenia, which is linked to nonspecific immune complexes binding to platelets, as seen in autoimmune disorders.¹² Besides, the microvascular and macrovascular complications are associated with the dysfunction of platelets.¹³ The WBC is considered an inflammation marker in different chronic diseases.¹⁴ An increased WBC count has been linked to macro and microvascular complications, activation of glycation end products, oxidative stress, and angiotensin II as a result of hyperglycemia, as well as the production of factors like tumor necrosis factor and interleukin 1 that are involved in the pathogenesis of chronic diabetes complications.^{15,16}

In Ethiopia, DM is estimated to affect half a million people.⁵ Even though previous studies in Ethiopia had primarily focused on the prevalence of anemia and its associated factors, the goal of this study is to determine the prevalence of and associated factors of cytopenia in T2DM, which had not been addressed in previous studies.

Materials and Methods

Study Setting, Population, and Period

A cross-sectional study was conducted from March to May 2021 on a total of 357 T2DM patients attending at the University of Gondar Comprehensive Specialized Hospital chronic illness clinic. All adult T2DM study participants that are on follow-up in the chronic illness clinic of the hospital were considered as a source of the population. All adult T2DM study participants who were on follow-up at the hospital chronic illness clinic during the study period and volunteered to participate in the study were included in the study population. The study participants were recruited by using a simple random sampling technique.

Inclusion and Exclusion Criteria

All adult study participants who were diagnosed with T2DM and who were willing to participate were conveniently included in the study. Participants with severe bleeding, patients undergoing current surgery, cancer, hyperthyroidism, and severely ill patients unable to consent were excluded from this study.

Study Variables

The magnitude of hematological abnormalities was considered as an outcome variable and socio-demographic characteristics (gender, age, residence, educational level, occupation, marital status, religion) and clinical and behavioral characteristics (duration of DM, duration of antidiabetic drug use, malaria infection, history of chronic illness, estrogen-containing oral contraceptives like choice and style (for female patients), hypertension, body mass index (BMI), physical exercise habit, habitual cigarette smoking, history of alcohol consumption, and using traditional medicine were considered as the independent variables.

Operational Definitions

Cytopenia: was defined as a reduction in either RBCs, WBCs, or platelets.¹⁷

Bi-cytopenia was defined as two of the three lineage cell counts falling below the levels.¹⁸

BMI: was calculated as weight in kg divided by height in m² and the result is categorized as the following: severely underweight, underweight, normal, overweight, and obese were considered when the BMI is < 16.5, < 18.5, 18.5–24.9, 25–29.9, and ≥ 30, respectively.¹⁹

Alcohol consumption: was defined as the act of ingesting (typically orally) a drink containing ethanol or ethyl alcohol.²⁰

Data Collection Procedure

Sociodemographic Data Collection

After obtaining an informed written consent, a structured and pretested questionnaire was used to collect sociodemographic and behavioral data from eligible study participants. The questionnaire was used to conduct face-to-face interviews with the participants. The questionnaire has two parts. The first part of the questionnaire consists of sociodemographic characteristics of study participants, and the second part consists of questions regarding the behavioral information of study participants. The sociodemographic data were gathered through the use of questionnaires and face-to-face interviews conducted by trained nurses working in the chronic illness clinic of the hospital.

Clinical and Behavioral Data Collection

Clinical data including duration of DM, duration of antidiabetic drug intake, blood pressure, and other related data were collected by trained nurses from the patient's medical chart using data collection sheets. The weight and height of the study participants were measured and were used for the calculation of BMI. The BMI is calculated after careful measurement of the weight and height of the study participants.

Blood Sample Collection

A total of 3 milliliters of venous blood samples were collected using the vacutainer method. The blood was dispensed into a tube containing tri-potassium Ethylene Diamine Tetraacetic Acid (EDTA). The blood sample was used for the CBC examination. The blood sample was collected aseptically by strictly following standard operating procedures.

Complete Blood Cell Count Determination

The Beckman Coulter UniCel DxH 800 fully automated hematology analyzer was used to determine the results. The coulter principle is used in the DxH 800 CBC analysis, which is based on a suspension of blood cells being passed through a small orifice simultaneously with an electric current. Individual blood cells passing through the orifice introduce an impedance change in the orifice determined by the size of the cell. The system counts the individual cells and provides a cell size distribution. The coulter volume, conductivity, and scatter established WBC differential technology using three measurements: individual cell volume, high-frequency conductivity, and laser-light scatter.

Data Quality Control Measures

The sociodemographic, behavioral, and clinical data quality was maintained by preparing the questionnaire in English and translating it to the local language (Amharic), then converting it back to English to examine the uniformity of the data collection tools. The pretest of the questionnaire was performed among 5% of the total participants. All study participants were notified about the objective and importance of the study before the data collection. The gathered data was inspected daily for consistency and accuracy. Further, the laboratory data quality control measures were kept by maintaining the sample's quality by removing hemolysis, clotting, blood to anticoagulant proportion, and delaying the laboratory analysis. Besides, to remove the hemolysis, the blood was dispensed to the wall of the EDTA test tube. Furthermore, any reagents that are used for CBC, peripheral morphology, and blood film were checked for expiration dates and prepared in compliance with the manufacturer's guidelines. The commercial Beckman Coulter UniCel® DxH 800 hematology cell controls (low, normal, and high) were run on a daily basis before the samples were examined. The quality of the smear and Giemsa stain were checked using known malaria positive and negative slides.

Data Processing and Analyzing

The information was coded, entered into Epi Info version 7.2.4.0, and then transferred to SPSS version 25 for analysis. Descriptive statistics were used to summarize the characteristics of the study participants. The normality of the data was checked by the Shapiro–Wilk test. Univariate and multivariate logistic regression analyses were used to determine factors associated with cytopenia in T2DM. The odds ratio, with its 95% confidence interval, was used to determine the strength of the association between the independent variable and the outcome variable. Variables with a p-value of less

than 0.25 on the univariate analysis were entered into a multivariate logistic regression analysis. A p-value of less than 0.05 was considered statistically significant.

Ethical Considerations

The current study was carried out in accordance with the Helsinki Declaration. Besides, an ethical clearance was delivered by the Ethical Review Committee of the School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, the University of Gondar (Ref. No. SBLS/2746/2021). A permission letter was obtained from the university of Gondar comprehensive specialized hospital chief executive clinical director. An informed written consent was obtained from each patient, and the findings were kept confidentially. The confidentiality of the data was protected by only using codes for specimens and results, and no personal identification was used. In the event of abnormal results, their medical doctors were notified so that they could receive appropriate treatment.

Results

Sociodemographic Characteristics

A total of 357 T2DM patients were enrolled in the current study. Of those, 186 (52.1%) and 288 (80.7%) were females and urban residents, respectively. The mean age of the study participants was 54 ± 12 years old and ranged from 23 to 87 years old (Table 1).

Table 1 Socio-Demographic Characteristics of Participants in the 2020 University of Gondar Comprehensive and Specialized Hospital Chronic Illness Clinic

Sociodemographic Characteristics		Frequency	Percentage
Gender	Male	171	47.9%
	Female	186	52.1%
Age	18–39	34	9.5%
	40–64	257	72.0%
	>65	66	18.5%
Place of residence	Urban	288	80.7%
	Rural	69	19.3%
Educational status	Unable to read and write	97	27.2%
	Elementary	110	30.8%
	High school	90	25.2%
Occupation	College and above	60	16.8%
	House wife	89	24.9%
	Office work	66	18.5%
	Merchant	61	17.1%
	Farmer	53	14.8%
	In retirement	45	12.6%
	Private	22	6.16%
Marital status	Driver	9	2.5%
	Other	12	3.36%
	Single	20	5.6%
	Married	276	77.3%
	Divorced	23	6.4%
Religion	Widowed	38	10.6%
	Orthodox	316	88.5%
	Muslim	31	8.7%
	Protestant	10	2.8%

Clinical and Behavioral Characteristics

About 43.7% (156/357) of the study participants had a history of chronic disease. Besides, 99.7% (356/357) of the study participants were taking antidiabetic drugs. Furthermore, the number of patients with hypertension was 216 (60.5%). About 42.0% (150/357), 20.7% (74/357), and 44.5% (159/357) have a physical exercise habit, are alcohol drinkers, and have abnormal BMI, respectively (Table 2).

Magnitude of Hematological Abnormalities

The magnitudes of anemia, leucopenia, and thrombocytopenia were 8.7% (95% CI: 6.18, 12.06), 10.9% (95% CI: 8.09, 14.59), and 5.3% (95% CI: 3.43, 8.16), respectively. Instead, leukocytosis, thrombocytosis, neutrophilia, and lymphocytosis were 4.8% (95% CI: 2.99, 7.49), 1.1% (95% CI: 0.44, 2.85), 4.8% (95% CI: 2.99, 7.49), and 5.3% (95% CI: 3.43, 8.16), respectively (Table 3). The magnitude of cytopenia, bicytopenia, and pancytopenia were 21% (95% CI: 17.1, 25.53), 1.1% (95% CI: 0.44, 2.85), and 0.56% (95% CI: 0.01, 1.12), respectively (Table 3 and Figure 1).

Factors Associated with Cytopenia and Anemia

Bivariate and multivariate analysis were performed to identify the association of the independent variable with the occurrence of cytopenia. In bivariate analysis, gender, age in years, residence, educational level, occupation, physical exercise, and alcohol consumption habits were associated with cytopenia, but in multivariate analysis, none of the variables was associated with cytopenia. However, the multivariate analysis of anemia demonstrated that being male (AOR: 3.23, 95% CI: 1.43, 7.56), reducing physical exercise (AOR: 2.70, 95% CI: 1.137, 6.43), and never married (AOR: 3.90, 95% CI: 1.248, 12.18) were significantly associated with anemia (Tables 4 and 5).

Table 2 Clinical and Behavioral Characteristics of Participants in 2020 the University of Gondar Comprehensive and Specialized Hospital Chronic Illness Clinic

Clinical and Behavioral Characteristics		Frequency	Percentage
Duration of diabetes in years	<6	235	65.8%
	≥6	122	34.2%
Antidiabetic drug use status	Yes	356	99.7%
	No	1	0.3%
Duration of antidiabetic drug use in years	<6	236	66.1%
	≥6	121	33.9%
Traditional medicine use	Yes	27	7.6%
	No	330	92.4%
Consumption of oral contraceptive	Yes	7	3.8%
	No	179	96.2%
Physical exercise status	Yes	150	42.0%
	No	207	58.0%
Cigarette smoking status	Yes	5	1.4%
	No	352	98.6%
Alcohol drinking status	Yes	74	20.7%
	No	283	79.3%
History of chronic illness	Yes	156	43.7%
	No	201	56.3%
Body mass index	Abnormal	159	44.5%
	Normal	198	55.5%
Blood pressure	Hypertensive	216	60.5%
	Normotensive	141	39.5%

Table 3 The Magnitude of Hematological Abnormality Among Participants in the 2020 University of Gondar Comprehensive and Specialized Hospital Chronic Illness Clinic

Variable	Category	Frequency	Percent (%)
RBC	Normal	322	90.2
	Anemia	31	8.7
	High count	4	1.1
WBC	Normal	301	84.3
	Leukopenia	39	10.9
	Leucocytosis	17	4.8
Platelet	Normal	334	93.6
	Thrombocytopenia	19	5.3
	Thrombocytosis	4	1.1
Neutrophil	Normal	223	62.5
	Neutropenia	117	32.8
	Neutrophilia	17	4.8
Lymphocyte	Normal	294	82.4
	Lymphopenia	44	12.3
	Lymphocytosis	19	5.3

Abbreviations: RBC, red blood cell; WBC, white blood cells.

Discussion

Diabetes is associated with peripheral cytopenia, which plays a crucial role in diabetes-related micro and macrovascular complications. As a result, the goal of this study was to assess the degree of cytopenia in T2DM. Anemia was identified in 8.7% (95% CI: 7.44, 9.92) of the T2DM patients in the current study. The current study confirmed that anemia is a mild public health problem among T2DM in the study area, according to the WHO classification of anemia's public health relevance in populations.²¹ The reason for the mild anemia prevalence in T2DM patients is that patients with DM have systemic inflammation, inhibition of Epo release, damage to the renal interstitium, severe symptomatic autonomic neuropathy causing efferent sympathetic denervation of the kidney, drugs, altered iron metabolism, and hyperglycemia are some of the factors that lead to the earlier onset of anemia in DM patients.²² Additionally, anemia in DM patients is linked to older age, worsening renal function, peripheral vascular disease, lower weight or BMI, lower diastolic blood

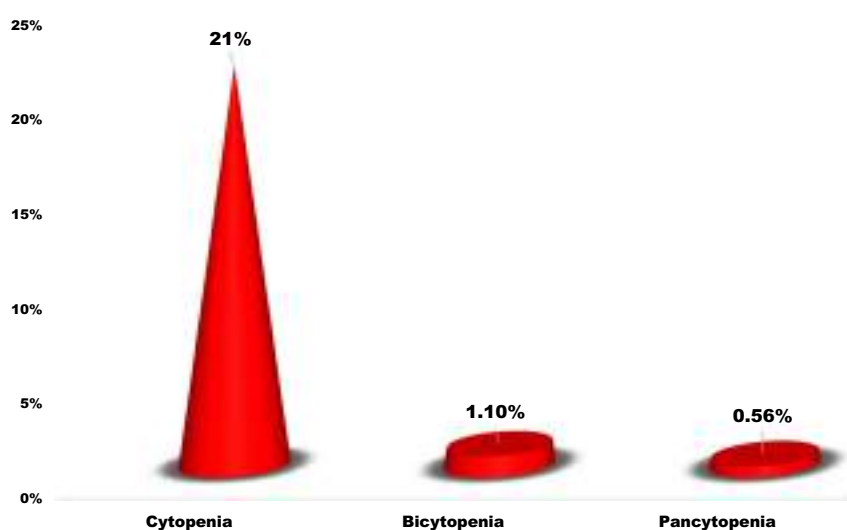
**Figure 1** The magnitude of cytopenia among participants in the 2020 University of Gondar comprehensive and specialized hospital chronic illness clinic.

Table 4 Factors Associated with Cytopenia Among Participants in the 2020 University of Gondar Comprehensive and Specialized Hospital Chronic Illness Clinic

Variable	Category	Cytopenia		COR (95% CI)	P-value	AOR (95% CI)
		Yes	No			
Gender	Male	49	122	1.93 (1.17, 3.20)	0.011	0.76 (0.40, 1.461)
	Female	32	154	1		1
Age in years	18–39	4	30	1	0.199	2.679 (0.77, 9.33)
	40–64	60	197	0.66 (0.36, 1.19)		1.122 (0.557, 2.26)
	>65	17	49	0.49 (0.26, 0.92)		1
Residence	Urban	61	227	0.88(0.50–1.54)	0.113	0.613 (0.175, 2.144)
	Rural	20	49	1		1
Educational level	Unable to read and write	24	73	1.31(0.60, 2.88)	0.20	0.847 (0.279, 2.571)
	Elementary	30	80	1.50(0.70, 3.20)		0.591 (0.231, 1.513)
	High school	15	75	0.80(0.35, 1.86)		1.138 (0.453, 2.861)
	College and above	12	48	1		1
Occupation	House wife	10	79	1	0.011	1
	Office work	13	53	1.94(0.79, 4.74)		2.33 (0.889, 6.125)
	Merchant	16	45	2.81 (1.18, 6.71)		0.927 (0.361, 2.382)
	Farmer	19	34	0.34 (0.16, 0.73)		0.927 (0.409, 2.102)
	Other	23	65			0.803 (0.169, 3.802)
Marital status	Never married	6	14	1	0.416	-
	Married	75	262	0.67 (0.248, 1.798)		-
Monthly income	<500	6	12	1.59 (0.568, 4.47)	0.98	-
	500–1500	16	60	0.57 (0.30, 1.06)		-
	1501–2500	11	51	0.69 (0.33, 1.42)		-
	>2500	48	153	1		-
Religion	Orthodox	74	242	1.49 (0.632, 3.489)	0.291	-
	Other	7	34	1		-
	Hypertensive	18	59	1		-
BP	Normotensive	28	113	0.76 (0.45, 1.28)	0.303	-
	Hypertensive	53	163			-
Hypertension	Yes	19	78	0.77 (0.44, 1.39)	0.478	-
	No	62	198	1		-
Heart Disease	Yes	7	26	0.91 (0.40, 2.18)	0.831	-
	No	74	250	1		-
Chronic disease	Yes	32	124	0.80 (0.48, 1.33)	0.445	-
	No	49	152	1		-
Physical exercise	Yes	28	122	0.67 (0.40, 1.12)	0.127	0.638 (0.359, 1.137)
	No	53	154	1		1
Alcohol consumption habit	Yes	22	52	1.60 (0.90, 2.85)	0.119	1.181 (0.618, 2.257)
	No	59	224	1		1
Traditional medicine	Yes	7	20	1.20 (0.493, 2.97)	0.638	-
	No	74	256	1		-

Abbreviations: AOR, adjusted odds ratio; COR, crude odds ratio; BP, blood pressure.

pressure, polypharmacy, and longer duration of T2DM, as well as not utilizing an angiotensin converting enzyme inhibitor.^{23,24}

The finding of this study was similar to a previous study in Gondar by Kebede et al where the magnitude of anemia was 8.06%.²⁵ However, it is lower than studies conducted in Debre Berhan (20.1%)²⁶ and Debre Tabor (29.81%)²⁷ that reported a higher magnitude of anemia than the current study. In general, a systematic review and meta-analysis conducted by Adane et al showed that the overall magnitude of anemia was 31.12% (95% CI; 9.66, 52.58) in DM patients.²⁸ The discrepancy might be as a result of variations in the type of DM. The present study included T2DM

Table 5 Factors Associated with Anemia Among Study Participants

Variable	Category	Anemia		COR (95% CI)	P-value	AOR (95% CI)
		Yes	No			
Gender	Male	22	149	2.91 (1.29, 6.49)	0.009	3.29 (1.43, 7.56)*
	Female	9	177	1		
Age	18–64	24	267	1	0.54	-
	>65	7	59	0.49 (0.26, 0.92)		
Residence	Urban	24	264	0.801 (0.33, 1.95)	0.632	-
	Rural	7	62	1		
Educational level	Unable to read and write	8	89	0.99 (0.31, 3.18)	0.978	-
	Elementary and High school	18	183	1.08 (0.38, 3.05)		
	College and above	5	55	1		
Marital status	Never married	5	15	3.99 (1.34, 11.84)	0.013	3.90 (1.248, 12.18)*
	Married	26	311	1		
Monthly income	<1500	9	85	0.15 (0.49, 2.68)	0.934	-
	1501–2500	5	57	0.95 (0.34, 2.69)		
	>2500	17	184	1		
BP	Normotensive	9	132	1	0.216	-
	Hypertensive	22	194	1.66 (0.74, 3.73)		
BMI	Normal	19	179	1	0.858	-
	Abnormal	12	147	0.77 (0.36, 1.64)		
Hypertension	Yes	23	237	1.08 (0.47, 2.50)	0.858	-
	No	8	89	1		
History of chronic disease	Yes			0.45 (0.20, 1.03)	0.559	-
	No			1		
Physical exercise	Yes	8	142	1	0.061	1
	No	23	184	0.67 (0.40, 1.12)		
Alcohol consumption habit	Yes	24	259	0.89 (0.37, 2.15)	0.790	-
	No	7	67	1		

Note: *Shows statically significant.

Abbreviations: AOR, adjusted odds ratio; COR, crude odds ratio; BP, blood pressure; BMI, body mass index.

patients only. This is in line with the fact that T2DM patients are more vulnerable to various forms of both short-term and long-term complications due to the commonness of this type of DM, its insidious onset, and late recognition, especially in developing countries.²⁹ Anemia has a major negative impact on DM patients' quality of life and is linked to disease progression and the development of comorbidities. It's linked to a quick deterioration in renal function and a greater requirement for renal replacement therapy, which is frequently unavailable or costly in most underdeveloped nations, including Ethiopia.³⁰ Anemia in DM patients is harmful to their health since it increases the risk of cardiovascular disease and hypoxia-induced end-organ damage such as diabetic retinopathy, nephropathy, and foot ulcers.³¹

The result of the logistic regression showed that males are 3 times more anemic as compared to females. The findings of the study are similar to those of studies conducted in India³² and Ethiopia.³³ This might be explained by the fact that low testosterone levels and hypo-gonadotropic hypogonadism are common in male DM patients. Since testosterone stimulates erythropoiesis, low testosterone levels may contribute to anemia in male patients.³⁴ Moreover, T2DM patients with reduced or lack of physical exercise are 2.70 times more at risk of anemia than their counterparts. Notably, in T2DM subjects, reduced exercise capacity appears to be a predictor of all-cause mortality.³⁵ During exercise, optimal oxygen delivery and utilization imply a delicate interplay of multiple physiological functions, such as pulmonary ventilation, gas exchange, cardiac output, muscle blood distribution and diffusion, skeletal muscle aerobic and force-generating capacity, as well as fatigue perception.³⁶ According to the findings of this study, the never married participants are also 3.9 times more anemic than their counterparts.

Leukopenia and leukocytosis were detected in 10.9% (95% CI: 8.09, 14.59) and 4.8% (95% CI: 2.99, 7.49) of T2DM patients, respectively. Our study is in consonance with a report by Arkew et al in which 3.7% (95% CI: 2.46, 4.95) of DM patients developed leukocytosis.³⁷ Total peripheral WBC count, a nonspecific marker of inflammation, has been suggested to be associated with diabetes risk. The influence of hyperglycemia and the pathophysiology of T2DM may explain the mechanism driving the increase in total and differential WBC counts in T2DM patients. In T2DM patients, chronic inflammation, as shown by a higher WBC count, is linked to microvascular problems. In DM, impaired WBC function is also reported.³⁸

Thrombocytopenia was found in 5.3% (95% CI: 3.43, 8.16) of T2DM patients. While 1.1% of the participants had thrombocytosis. By mediating the first phase of hemostasis, blood platelets play a critical role in the clotting process. Platelets in diabetic individuals have dysregulated signaling pathways, resulting in an increased tendency to activate and aggregate in response to a stimulus. Increased clotting, poor clot breakdown, and endothelial dysfunction are all linked to platelet abnormalities.^{39,40} Platelets in patients with diabetes are reported to exhibit hyper-reactivity to sub-threshold stimuli and undergo rapid consumption, resulting in accelerated thrombopoiesis of more reactive platelets.⁴¹ The enhanced platelet reactivity seen in diabetic individuals has been linked to a number of processes. These mechanisms include hyperglycemia, insulin deficiency and resistance, associated metabolic conditions (obesity, dyslipidemia, and increased systemic inflammation), and other cellular abnormalities.⁴² Acute hyperglycemia results in increased platelet activation, as documented by elevated levels of surface adhesion molecules such as P-selectin and soluble markers of platelet activation.^{43,44}

The magnitude of cytopenia, bicytopenia, and pancytopenia were 21% (95% CI: 17.1, 25.53), 1.1% (95% CI: 0.44, 2.85), and 0.56% (95% CI: 0.01, 1.12), respectively. Hyperglycemia in T2DM and/or its treatment may cause a reduction in RBC, WBC, and platelets. However, we did not get enough available literature to evaluate our findings. In addition to this, none of the analyzed variables were associated with cytopenia. Therefore, it paves a path for researchers to investigate the magnitude of cytopenia and its contributing factors in T2DM patients in large cohorts of participants.

Conclusion and Recommendation

This study showed that T2DM causes disturbances in the hematological parameters and leads to a mild level of cytopenia. It is, therefore, suggested that hematological abnormalities, especially cytopenia, should be monitored and controlled on a regular basis in T2DM patients for better prognosis and quality of life.

Abbreviations

AOR, Adjusted Odds Ratio; BMI, Body Mass Index; BP, Blood Pressure; CBC, Complete Blood Count; COR, Crude Odds Ratio; DM, Diabetes Mellitus; EDTA, Ethylene Diamine Tetra acetic Acid; RBC, red blood cell; T2DM, Type II Diabetes Mellitus; WBC, White blood cells.

Data Sharing Statement

All the data supporting these findings is contained within the manuscript.

Acknowledgment

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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 there is no conflicts of interest regarding the publication of this manuscript.

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Lipid and Haematologic Profiling of Regular Blood Donors Revealed Health Benefits

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Background: Blood banks have been suffering a shortage of blood worldwide due to limited donations. By and large, it is widely believed that blood donation has multiple health benefits. However, there are limited studies that support it. As a result, assessing the biochemical profiles of the regular blood donors is indispensable to evaluating an individual's risk for chronic inflammation.

Objective: We strived to compare lipid and haematological profiles of the regular and first-time blood donors in the National Blood Bank Service of Ethiopia.

Materials and Methods: A comparative cross-sectional study, involving 104 blood samples (52 each of regular and first-time donors), was designed to analyze lipid and haematological profiles and anthropometric parameters were measured. Data were analyzed using SPSS version 25, Chi-square (χ^2) was used to compare the relationship between categorical variables and an independent Student's *t*-test was used to compare the mean of the two groups. A *p*-value <0.05 was considered statistically significant.

Results: Regular blood donors had lower mean TC (144.3 ± 28 mg/dL), TG (159.3 ± 88.2 mg/dL), LDL-c (75.9 ± 25.9 mg/dL) than the first-time blood donors with values of TC (158.1 ± 38.94 mg/dL), TG (163.9 ± 82.7 mg/dL), LDL-c (93.1 ± 31.5 mg/dL), respectively. The ratio of LDL-c/HDL-c and TC/HDL-c was found to be lower in regular blood donors when compared to the first-time donors ($P < 0.05$). Even though the level of HDL-c was higher (39.8 ± 8.8 mg/dL) in regular blood donors compared to first-time blood donors (36.8 ± 7.7 mg/dL), it was not statistically significant. The mean of some haematologic parameters like a platelet, RDW, lymphocyte, and MCH was significantly lower in regular blood donors than in first-time blood donors. BMI and WHR in regular donors were less than the first-time donors, albeit statistically insignificant.

Conclusion: Interestingly, blood donation has a significant health benefit by lowering TC, TG and LDL-c, which have the potential risk of developing chronic inflammation.

Keywords: blood donation, lipid profile, hematologic parameters, National Blood Bank Service of Ethiopia, chronic inflammation

Introduction

Blood is an important element of human life, and there is no compensation for its Nubila.¹ Blood donation is a life-saving process for people who have lost large volumes of blood from serious accidents, obstetric and gynaecological haemorrhages and during various surgical procedures. The blood taken from donors could be either whole blood or blood products.²

WHO gives a global rate of 117.5 million blood donations annually, of which 60% are voluntary donors and the rest are for family or paid donors.³ Although blood is very essential to save every life, acute shortage of blood and blood products encountered even in big cities with high populations fail to supply 50% of their demand.²

Blood transfusions in sub-Saharan Africa have emphasized areas of concern, yet there have been very few studies of its history. Record found that transfusions were first reported in Africa in the early 1920s and organized transfusion practices were established before the Second World War and then blood transfusion grew rapidly after 1945. However, there is a great shortage of blood and blood products in sub-Saharan Africa.⁴ In Ethiopia, there has been inadequate and inequitable access to blood. According to a report from the Ethiopian Ministry of Health, Ethiopia, collected 223,000 units of blood in 2019/20, meeting only 22% of its requirements as per the standard of the World Health Organization.⁵

Blood donation makes blood less viscous and lowers LDL-c and TC due to loss of fibrinogen, albumin and lipid loss during donation; Therefore, evaluating lipid profile is very important to assess risk factors for chronic illnesses like diabetes mellitus, hypertension, heart disease, and so on.⁶ Oxidized LDL-c directly delivers various lipid oxides and hydroperoxides to target cells. These compounds variably act as cytotoxins, monocyte, chemoattractant, and stimulators of cholesterol ester accumulation by macrophages.⁷ The accumulation of LDL-c in the vessel wall contributes to the formation of fatty strikes.^{8,9} According to a study, blood donation was to reduce the enzyme responsible for the oxidation of LDL-c, which is linked with the development of atherosclerosis.¹⁰

The study conducted in Argentina reported that HDL-c of regular blood donors were higher than irregular or non-donors.¹¹ HDL-c associated enzyme protects LDL-c from oxidizing because oxidized LDL-c facilitates the formation of platelet activation factor and production of free radicals that are potential to produce pro-inflammatory cytokine.¹² HDL-c also inhibits platelet-activating factor (PAF) synthesis in the endothelial cell by inhibiting PAF acetyltransferase.^{6,13}

A comparative study done on various hematologic parameters among voluntary first-time and regular blood donors in India, Indicated that the level of Hb, MCV, MCH, RBC and serum ferritin were significantly decreased in regular blood donors.¹⁴

Another study conducted on CBC in Sudan Hospitals Blood Bank showed that 70% with the normal range, 26% increased, and 4% decreased in RBCs count. On WBCs count, 82% and 18% were in the normal range and decreased, respectively. In addition, when 90% of the PLTs count is in the normal range, 8% and 2% were increased and decreased in PLTs count, respectively.¹⁵

Some studies have reported that blood donation decreases the risk of cardiovascular and other chronic diseases.^{6,16} However, limited studies were conducted worldwide and to our knowledge, there were no studies in Ethiopia that compared the lipid profile and hematologic parameters among regular and first-time blood donors.

Therefore, this study was primarily focused on comparing the lipid profile and hematologic parameters among regular blood donors and first-time blood donors at the National Blood Bank Service of Ethiopia.

Methods and Materials

Study Design, Area, and Period

An institutional-based comparative cross-sectional study was conducted among 104 regular and first-time blood donors at the National Blood Bank Service of Ethiopia, from January to December 2020.

Population and Eligibility Criteria

The source population for this study was all blood donors who have voluntarily donated their blood at the National Blood Bank Service of Ethiopia, whereas all blood donors who have visited the National Blood Bank Service of Ethiopia during the study period were considered as the study population. All regular and first-time blood donors between the ages of 18–65 years were included in this study; however, donors who were smokers, alcohol drinkers, on lipid-lowering medication, weight less than 45 Kg, pregnant and lactating women, hypertensive, diabetic, cardiovascular and anaemic patients were excluded from the study.

Sample Size Determination and Sampling Technique

The sample size was determined based on the specific mean $\pm$ standard deviation for each sub-groups since there were no reported studies in Ethiopia. The mean $\pm$ SD of HDL for regular blood donors was 0.86 ± 0.24 (mmol/L) and for non-donors was 1.0 ± 0.26 (mmol/L), which was taken from the study done in Nigeria entitle “lipid profile among blood donors”.⁶ The sample size is determined by using the G power software Statistical Power Analyses for Windows (3.1.2.9 version) with the following assumptions: Significance level = 95%, power = 80%, type of test = two independent mean *T*-test, $Z_{\alpha/2}$ = the critical value at 95% ($\alpha = 0.05$) and 1:1 ratio. The computed sample size based on the above assumption was 104, which comprised 52 samples from each category. Convenient sampling methods were used, for both regular and first-time blood donor groups until the required sample size was attained.

Sampling Methods and Producers

A structured questionnaire translated to the local language, Amharic, was used for the socio-demographic data collection. Anthropometric measurements including weight, height, waist-circumference and hip-circumference were measured with the subjects wearing light clothing. Body mass index (Kg/m^2) was calculated by dividing the weight in Kg by height in m^2 and waist-to-hip ratio (WHR) was calculated.

Blood Collection and Processing

About 6 mL of blood sample was drawn by qualified health care professionals and divided into two (3 mL for lipid profile and 3 mL for CBC). While the haematological analysis was performed right away at the time of sample collection, lipid profiles were performed one time after we reached our sample size. Three-milliliter blood was added in SST and allowed to stand for 30 minutes at room temperature for complete clotting and clot retraction. Then, Serum was separated by centrifugation at 3500 rpm for 15 minutes by Megafuge r 1.0 Heraeus centrifuge (Germany, 2017). The serum was used to determine the levels of TC, HDL-c and TG by a Cobas 6000 Chemistry Analyzer (Germany, 2020). LDL-c cholesterol was calculated using the Fried-Wald formula ($\text{LDL-c} = \text{TC} - (\text{TG}/5 + \text{HDL})$).¹⁷ Finally, serum lipid profile ratios (TC/HDL-C, TG/HDL-C and LDL-C/HDL-C) were calculated. Another 3 mL for CBC was added to the EDTA tube to determine hematologic parameters by Beckman haematology Analyzer (Germany, 2018).

Study Variables

Serum lipid profile (TC, LDL-c, HDL-c and TG) and CBC were dependent variables, whereas socio-demographic factors and anthropometric indicators were our independent variables.

Operational Definition

Lipid Profile: is a panel of blood tests that serves as an initial broad medical screening tool for abnormalities in lipids, such as cholesterol, HDL-c, LDL-c, and triglycerides.

Regular blood donors: individuals who had donated blood every three months, at least for the last consecutive two years.

First-time blood donors: an individual who has donated blood for the first time with no history of blood donation previously.

Data Quality Control and Management

Data collection tools were prepared to meet the highest quality and monitored at the time of sampling. Professional laboratory technologists handled all the experimental procedures and all instruments were operated according to the manufacturer's instructions. High-quality standards were used as a control and calibration was used to calibrate the instruments and no analyses were done if the control is out of normal range.

Data Processing and Analysis

Data obtained from the questionnaire and laboratory analysis were checked for completeness and refined. It was coded and entered into the Epi-Data statistical software version 3.1 and exported to SPSS software version 25 package and different variables were tested and analyzed. Simple descriptive statistics were used to present the socio-demographic characteristics of the study subjects. While the Chi-square (χ^2) test was used to compare categorical variables, the continuous variable was present as mean $\pm$ SD and compared using the Student's t-tests for groups. A p-value of less than 0.05 at a 95% confidence level was considered to be statistically significant.

Results

Socio-Demographic Characteristics of the Blood Donors

Table 1 illustrates the socio-demographic characteristics of the study populations. The study included 104 study participants: 52 of each were regular and first-time blood donors. Among the regular blood donors, 84.6% and 15.4% were donated four and three times a year, respectively.

Table 1 Socio-Demographic Characteristics of the Study Participants at the National Blood Bank Service, Ethiopia, from June to July 2020 (n = 104)

S. No.	Variables	Category	RBD (%)	FTB (%)	P-value
1	Gender	Male	84.6	71.2	0.098
		Female	15.4	28.8	
2	Age group	18–38	65.3	73.1	0.095
		39–58	34.6	26.9	
3	Marital status	Married	50.0	55.8	0.84
		Unmarried	48.1	42.3	
		Divorced	1.9	1.9	
4	Religion	Orthodox	57.5	51.9	0.11
		Muslim	11.5	25.0	
		Protestant	30.8	19.2	
		Others	0.0	3.8	
5	Residence	Urban	100.0	96.2	0.15
		Rural	0.0	3.8	
6	Level of education	Illiterate	1.9	3.8	0.75
		Primary school	7.7	1.9	
		Secondary school	25.0	25.0	
		Undergraduate	21.2	17.3	
		Graduate	44.2	51.9	

Among regular blood donors, 65.3% were in 18–38, while 34.6% were in 39–58 years: from first-time donors, 73.1% and 26.9% were 18–38 and 39–58 years, respectively ($P > 0.05$). Eighty-four percent (84.6%) of regular blood donors and 71.15% of first-time blood donors were found to be males ($P > 0.05$).

Among regular blood donors, 57.69% were Orthodox, 30.77% Protestant, and 11.5% Muslim. Likewise, of first-time blood donors, 51.9% were Orthodox, 25% Muslim and 19.23% protestant ($P > 0.05$).

More than 98.5% and 50% were urban dwellers and married, respectively, regular blood donors. Among regular blood donors, 1.9% have no formal education and 44.2% graduated from college or university level. Of first-time blood donors, 3.8% have no formal education and 51.9% graduated from college or university level ($p > 0.05$). Our socio-demographic data reflected an ideal matching between the study participants.

Anthropometric Parameters of Study Participants

This study showed that the mean $\pm$ SD of BMI of regular blood donors was $23.33 \pm 2.77 \text{ kg/m}^2$, while that of first-time blood donors was $23.86 \pm 2.84 \text{ kg/m}^2$. Regarding the waist-to-hip ratio, the mean $\pm$ SD was found 0.85 ± 0.03 and 0.86 ± 0.04 for regular blood and first-time blood donors, respectively ($P > 0.05$; Table 2). The result indicated that the physical status of donors does not affect the level of lipid profile and haematological parameters.

Lipid Profile of the Study Participants

The lipid profile data showed that the regular blood donors had lower mean TC ($144.3 \pm 28 \text{ mg/dL}$), TG ($159.3 \pm 88.2 \text{ mg/dL}$), LDL-c ($75.9 \pm 25.9 \text{ mg/dL}$) and higher HDL-c ($39.8 \pm 8.8 \text{ mg/dL}$) than the first-time blood donors with

Table 2 Anthropometric Parameters of Study Participants at the National Blood Bank Service, Ethiopia, from June to July 2020 (n = 104)

S.No	Variables	Mean $\pm$ SD		P-value
		RBD	FBD	
1	Height (m)	1.73 $\pm$ 0.07	1.70 $\pm$ 0.08	0.11
2	Weight (Kg)	70.4 $\pm$ 10.1	69.5 $\pm$ 11.2	0.68
3	BMI (Kg/m ²)	23.3 $\pm$ 2.78	23.9 $\pm$ 2.84	0.34
4	WC (cm)	84.4 $\pm$ 4.82	83.9 $\pm$ 5.74	0.67
5	HC (cm)	98.0 $\pm$ 4.0	97.8 $\pm$ 3.9	0.85
6	WHR	0.86 $\pm$ 0.03	0.87 $\pm$ 0.04	0.74

values of 158.1 ± 38.94 mg/dL, 163.9 ± 82.7 mg/dL, 93.1 ± 31.5 mg/dL and 36.8 ± 7.7 mg/dL, respectively (Figure 1). LDL-c and TC in regular blood donors were significantly lower than in the first-time blood donors. Succinctly, the ratio of LDL-c/HDL-c and TC/HDL-c was found to be lower in regular blood donors when compared to the first-time donors ($P < 0.05$). Except for a few participants, the mean value of lipid profile among the groups falls within the normal biological range indicating there was no prior history of abnormal lipid profiles that affect the outcome of the present result.

Hematologic Parameters

Table 3 illustrates the haematologic parameters of the 104 study participants classified as regular and first-time donors. The result showed that values of WBC, Neu%, and Lymph% (all in mean $\pm$ SD) were found in regular blood donors at $5.59 \pm 1.5 \times 10^3/\mu\text{L}$; 58.08 ± 10.5 ; 29.9 ± 9.1 compared to the first-time donors $5.85 \pm 1.7 \times 10^3/\mu\text{L}$; 52.2 ± 9.7 ; 35.7 ± 8.6 . The neutrophils of regular blood donors were significantly higher than the first-time blood donors. However, the lymphocyte of regular blood donors was significantly lower than first-time blood donors. Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and RDW-SD were found to be significantly lower in regular blood donors compared to first-time donors. However, the majority of RBC indices were found to be similar.

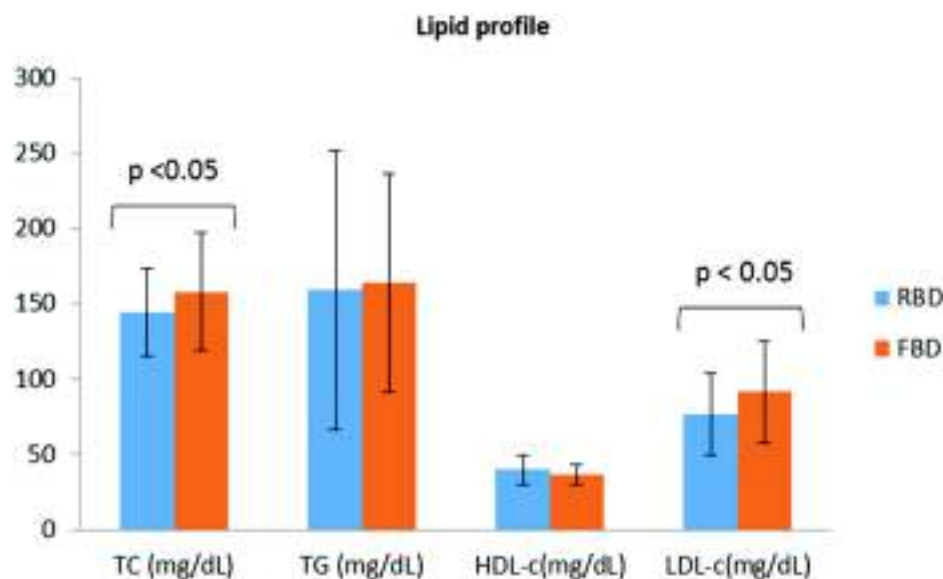
**Figure 1** Levels of lipid profiles in regular and first-time blood donors at National Blood Bank Service, Ethiopia, from June to July 2020 (n = 104).

Table 3 Levels of Haematological Parameters in Regular and First-Time Blood Donors at National Blood Bank Service, Ethiopia, from June to July 2020 (n = 104)

S.No	Variables	Mean $\pm$ SD		P-value
		RBD	FBD	
1	WBC* $10^3/\mu\text{L}$	5.6 ± 1.5	5.8 ± 1.7	0.43
2	Neu (%)	58.2 ± 10.5	52.2 ± 9.7	0.004*
3	Lymph (%)	29.9 ± 9.1	35.7 ± 8.6	0.001*
4	Plt* $10^3/\mu\text{L}$	208.3 ± 24.8	229.5 ± 59.5	0.04*
5	Mpv (fL)	9.2 ± 1.1	9.3 ± 1.1	0.89
6	RBC ($10^3/\mu\text{L}$)	4.76 ± 0.6	4.71 ± 0.5	0.65
7	HCT (%)	42.0 ± 4.3	42.3 ± 4.3	0.40
8	HGB (g/dL)	14.7 ± 1.7	15.0 ± 1.7	0.75
9	MCH (pg)	30.8 ± 2.1	31.7 ± 1.7	0.016*
10	MCHC (g/dL)	35.0 ± 1.0	35.3 ± 1.1	0.017*
11	RDW (%)	13.7 ± 1.1	14.2 ± 2.2	0.133
12	RDW-SD	42.2 ± 2.8	43.8 ± 3.7	0.01*
13	Neu/Lymph	2.4 ± 1.0	1.7 ± 0.8	0.003*
14	Platelets/Lymph	115.5 ± 24.3	113.3 ± 31.6	0.48

Note: The *indicates $p < 0.05$.

Concerning platelet indices, the values (mean $\pm$ SD) of regular blood donors versus first-time blood donors are as follows: PLT (208.3 ± 42.8 and $229.5 \pm 59.5 \times 10^3/\mu\text{L}$) and mean platelet volume (MPV) (9.25 ± 1.08 and $9.28 \pm 1.09\text{fL}$), respectively (Table 3). The platelets of regular blood donors were significantly lower when compared to first-time blood donors. The ratio of Neutrophil to lymphocyte (NLR) and platelet-to-lymphocyte ratio (PLR) were found to be lower for the first time compared to regular blood donors.

Generally, the mean values of all hematologic parameters were in the normal range for both regular and first-time blood donors.

Discussion

This study assessed the lipid and haematologic profiles of the regular blood donors (those who donate every three months in a year) and first-time donors (no recorded history of blood donation) voluntarily coming to the National Blood Bank Services of Ethiopia. The present study showed that the socio-demographic status of the blood donors, viz., age, religion, marital status and education level were well matched. This indicated that sociodemographic characteristics did not affect the results as a confounding, which is in congruence with previous reports.^{6,18} However, the number of male donors was found to be higher in regular blood donors (RBD) and first-time blood donors (FTD), compared to females, albeit statistically insignificant. Rizvi's report showed a similar trend.¹⁹ This might be due to the very low-haemoglobin levels or/and the susceptibility of females to various anaemia.

In addition, BMI and WHR were found to be similar between the regular and first-time blood donors. While our report on BMI value agrees with two researchers in Iran and Palestine,^{6,18} other groups claimed that BMI was lower²⁰

and higher²¹ in regular blood donors when compared to the first-time donors. The latter is due to the high interest of obese people who understood the health benefits of blood donation.²²

Two decades back, regular blood donors have been identified as having a reduced risk of cardiovascular disease when compared with non-donors.¹⁰ The present study compared the lipid profile of regular blood donors with that of first-time donors. Our study vividly proved that TC, LDL-c, and the ratios of TC/HDL-c and LDL-c/HDL-c were found to be significantly lower in regular blood donors (RBD) than the first-time donors (FTD). TG and HDL-c were lower and higher, respectively, in RBDs compared to FTDs, although statistically insignificant.

Most of the previous studies here under were found to be in agreement with our outcomes: lower TC, LDL-c, LDL-c/HDL-c and TC/HDL-c;^{6,18} lower LDL-c;²³ lower TG and LDL-c;²⁴ lower TC and LDL-c;²⁵ healthier HDL-c and TC/HDL-c;⁸ higher HDL-c^{10,11,13} levels in RBDs compared to FBDs. However, others reported that there was no change in the values of TC, TG and LDL-c¹³ for both regular donors and higher HDL-c for non-regular donors.²³

From our result, lower LDL-c and higher HDL-c have health benefits for the regular blood donor. HDL-c is the type of lipoprotein, which mainly contains Apolipoprotein A-I as a major protein component. The atheroprotective activity of HDL-c is often explained by the unique ability of these lipoproteins to remove cholesterol from peripheral tissues, including the arterial wall, and transport it to the liver.²⁶ Therefore, the higher the concentration of HDL-c in regular blood donors reduced the level of cholesterol by scavenging from peripheral tissues and transporting it to the liver for synthesis of other important biomolecules. In addition, the lower LDL-c in regular blood donors is also advantageous over first-time donors. LDL-c is responsible for spasm of the blood vessels that are easily blocked by plaques²⁷ and oxidized LDL-c delivers lipid oxides and hydroperoxides to target cells variable acts as cytotoxins, monocytes, chemoattractants and stimulators of cholesterol ester accumulation by macrophages, which leads to fatty strikes.⁹ In our study, blood donation lowers LDL-c which increases its oxidative (not peroxidation) potential, in which the chance of formation of oxides and hydroperoxides, plaques, and fatty strikes (all the major contributors of atherosclerosis and other complications) are significantly reduced. As a result, we can deduce that regular blood donors have benefited more than the first time and non-donors.

Higher TC/HDL-c and LDL-c/HDL-c ratios are predictors of high cardiovascular risk and coronary artery disease.^{28,29} The present study indicated that these ratios were found to be significantly lower in regular blood donors compared to first-time donors, which is in agreement with the previous study done in Cameroon.³⁰ This confirmed that regular blood donors or blood donation, in general, have reduced the chance of developing cardiovascular risk and coronary heart disease.¹¹

This study was also designed to compare the haematological parameters (RBC, WBC, PC, and packed cell volume) of regular blood donors with first-time blood donors. The analysis of our data showed that the levels of WBC, lymphocyte, platelet, MPV, HCT, HGB, MCH, RDW, and RDW-SD were lower in RBD compared to the FTD, albeit statistical significance was merely observed in lymphocyte, platelet, MCH, and RDW-SD ($P < 0.05$). However, the levels of RBC ($p > 0.05$) and Neutrophil ($p < 0.01$) were higher in RBD compared to FTD. None of the hematologic parameters of both groups deviated from the normal biological ranges, which is highly pronounced in regular blood donors. Elnour's group data is incongruous with ours.¹⁵ This proved how safe is the procedure and adequate interval between consecutive blood donations. The latter allows erythropoiesis to compensate for any minor decline in any of these haematological profiles.³¹

The HCT, also known as packed cell volume or erythrocyte volume fraction, is the volume percentage of RBCs in blood and functions as a major determinant of blood viscosity, blood pressure, venous return, cardiac output, and platelet adhesiveness.³² Although statistically insignificant, our result showed a lower HCT level in regular blood donors which is in agreement with previous reports.¹⁸ As the frequency of donation increases (for example, first to fourth times), the haematocrit value significantly decreases.³³

In our study, the mean value of platelet counts was significantly lower in regular blood donors than in first-time donors ($p < 0.05$). It coincided with the following research.^{33–35}

RDW is a quantitative measure of the red blood cell volume (RBCV) heterogeneity. Increased RDW causes impairment of RBCs deformability and negatively affects blood flow through microcirculation due to the complete standstill movement of RBCs.³⁶ Our analysis showed a significant decline ($p < 0.05$) in the level of RDW in regular

blood donors compared to the first-time donors. This asserted regular blood donation may be attributed to lowering the risk of chronic inflammation.

NLR and PLR can be an important measure of systemic inflammation as it is cost-effective, readily available, and could be calculated easily.³⁷ The previous study showed that NLR of chronic diseases like diabetes and hypertension were higher than a cut-off value.³⁸ In our study, both NLR and PLR were lower in FTD compared to RBD.

We believe our current study answered the important question of how donating blood is indispensable both while saving millions of lives, and the health status of frequent blood donors is improved. Despite that, our study has a limitation: Since the sample size is small and our request to Ethiopian Blood Bank Services to increase it was challenged by the Covid-19 pandemic severe situation, we could not be able to stratify regular blood donors into subgroups.

Conclusion

Our study proved that the TC, TG, LDL-c and the ratios TC/HDL-c and LDL-c/HDL-c were found to be lower in regular blood donors. Interestingly, HDL-c was also found to increase in regular blood donors compared to first-time blood donors. Body-mass index (BMI) and waist-hip ratio (WHR) as well as some haematological profiles like a platelet, haematocrit, white blood cell, lymphocyte and RDW, were also found to be lower in regular blood donors. Combining all the above-mentioned parameters, regular blood donors have gotten less chance of developing the risk of cardiovascular complications and other chronic inflammation. As a result, we can deduce that blood donation has multiple health benefits to our best knowledge, this is the first-ever research in Ethiopia.

Abbreviations

BMI, body mass index; CBC, complete blood count; CVD, cardiovascular disease; FTD, first-time donors; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; NBBS, National Blood Bank Service; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; RBC, red blood cell; RBD, regular blood donors; TC, total cholesterol; TG, triglyceride; VLDL-c, very-low density lipoprotein cholesterol; WBC, white blood cell; WHR, waist to hip ratio.

Data Sharing Statement

Anyone interested in the full data in excel format can have the data by writing to solomon.tebeje@aau.edu.et.

Ethics Approval, Consent to Participate and Publication

Before the study, an ethical clearance letter (with a reference number SOM/DRERC/BCHM/088/2012) was obtained from the Research and Ethics Review Committee of the Department of Medical Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University. A collaboration letter for data collection was also obtained from the National Blood Bank Service. The research proposal was presented and thoroughly discussed at the National Blood Bank Service of Ethiopia as well.

The objective of the study was clarified and explained to each participant before enrolling any of the eligible study participants. Written informed consent for samples, data collection, and publication was obtained from the study participants ([Supporting Information](#)). Confidentiality, anonymity, neutrality, accountability and academic honesty were maintained throughout the study.

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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.

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Disclosure

All authors declare that they have no competing interests.

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Anemia and Associated Factors Among Under Five Year Old Children Who Attended Bule Hora General Hospital in West Guji zone, Southern Ethiopia

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Anemia and Associated Factors Among Under Five Year Old Children Who Attended Bule Hora General Hospital in West Guji zone, Southern Ethiopia

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Background: Anemia is a major global public health problem in the early stages of childhood, particularly in developing countries. In Africa, anemia causes higher morbidity and mortality among children less than five years old, especially in Ethiopia.

Objective: This study aimed to assess the prevalence of anemia and its associated factors among children under five years old attending Bule Hora General Hospital, West Guji Zone, southern Ethiopia.

Methods: A hospital-based quantitative cross-sectional study was conducted at Bule Hora General Hospital, Southern Ethiopia from October to November 2020. A convenience sampling technique was used to include 375 under five children enrolled in the study. The pretested structure questionnaire was used to collect socioeconomic and demographic characteristics of study individuals after taking appropriate written informed consent. Then, a venous blood sample was collected from each child and analyzed for hemoglobin determination using a Cell-DYN 1800 machine. Binary logistic regression models were used to identify associated factors of anemia. A P value ≤ 0.05 was considered statistically significant.

Results: The overall prevalence of anemia among under five children was 13.2% (50) [95% CI=5.2–21.2%] of them anemic children, 11.1% (1) had mild anemia, and 33.3% (3) had moderate anemia and 55.6% (5) had severe anemia. In this study, anemia was significantly associated with recent intestinal protozoan infection [AOR=2.13, 95% CI=1.35–9.270], malaria infection [AOR=5.42, 95% CI=0.307–11.034] and soil-transmitted helminths infection [AOR=6.09, 95% CI=2.047–27.54].

Conclusion: Anemia among under five children in this study was found to be a mild public health problem. It could be managed by preventing malaria infection, intestinal protozoa and soil-transmitted helminthic infection.

Keywords: anemia, under five, Bule Hora, general hospital, West Guji

Introduction

Anemia is a condition that causes a decline in erythrocyte concentration in circulation or hemoglobin in the blood and a concomitant impairment of oxygen transportation.¹ The World

The Health Organization (WHO) defined hemoglobin (Hgb) <12 g/dL in adult nonpregnant women, Hgb <11 g/dL in pregnant females, Hgb <13 g/dL in adult men, Hgb <11 g/dL in children aged 6–59 months, Hgb <11.5 g/dL in children aged 5–11 years, Hgb <12 g/dL for children aged 12–14 years and Hgb <13 g/dL in newborns.^{2,3}

Globally, 1.3 billion individuals suffer from anemia, making it one of the most important public health issues on the international agenda.⁴ Globally, on average, approximately 9.6 million children are severely anemic.⁵ It affects people in both developing and developed countries.⁶ By 2017, 293.1 million (47.4%) children under five years of age were anemic worldwide, and 67.6% of these children lived in Africa.¹ In Ethiopia, 57% of children are anemic, and 6–59 months is anemic according to the Ethiopian Demographic and Health Survey (EDHS) report.⁷

Iron deficiency is the major cause of anemia in developing countries and results in insufficient red blood cell production. In some individuals, infections such as peptic ulcers may cause blood loss and anemia. In developing countries, iron deficiency impacts all vulnerable groups. Additionally, geographically specific infections such as malaria and helminthic contribute to excessive red blood cell destruction and cause excessive red blood cell loss. Other infectious diseases may also be at play.⁸

Anemia impairs mental, physical, and social development and causes negative behavioral and cognitive effects, resulting in poor school performance and work capacity in later years.⁹ In early childhood, poor feeding habits, especially during the weaning period, exacerbate the problem. Anemia frequently develops as breast milk is replaced by foods that are poor in iron and other nutrients, including vitamin B12 and folic acid. Low oxygenation of brain tissues, a consequence of anemia, may lead to impaired cognitive function, growth and psychomotor development, especially in children. Infants, children under 5 years old and pregnant women have greater susceptibility to anemia because of their increased iron requirements due to rapid body growth and expansion of red blood cells.⁹ Moreover, anemia leads to immune system compromise, resulting in a decreased ability to fight infections and increased mortality in African children, where resources to determine the basic etiology remain poor.^{10,11}

However, there are national and regional data on the prevalence of anemia and its risk factors among children under five years of age in specific settings in Ethiopia.⁷ To our knowledge, no previous study has been undertaken in the study area of Bule Hora. Therefore, this study aimed to assess the prevalence of anemia and its associated factors among children from 6–59 months of age in Bule Hora Hospital, southern Ethiopia.

Methodology

Study Area, Period and Design

The health facility-based quantitative cross-sectional study was conducted from October 26 to November 20, 2020, at Bule Hora General Hospital, Guji Zone: Oromia Region, southern Ethiopia. The hospital found in Bule Hora town 467 km from Addis Ababa capital city of Ethiopia. According to the 2007 national census, the total population of Bule Hora town is 27,820. Geographically, the town is located between latitude 5°35'N and longitude 38°15'E and an altitude of 1716 meters above sea level. Regarding Bule Hora General Hospital, the hospital provides different services, including pediatrics, emergency, delivery, outpatient, patient, laboratory, pharmacy, medical and surgical services. Currently, the hospital has given the service for 5 million people in the area.

Study Population and Selection

All children aged between 6 months and 59 months who attended Bule Hora General Hospital during the data collection period and fulfilled the selection criteria were included in the study. Children who were recently treated or who took iron supplements were excluded from the study.

Sample Size and Sampling Technique

The sample size was determined using a single population proportion formula considering the prevalence of anemia from a previous study 66.8%.¹²

$$n = \frac{Z^2 p(1-p)}{d^2} = \frac{(1.96)^2 * 0.668(1-0.668)}{(0.05)^2} = 341$$

where d=Margin of error between the sample and the population (d=5%), n=Sample size, Z $\alpha/2$ =95% confident interval (1.96), P=66.8% Prevalence. Then, a 15% nonresponse rate was added considering the response rate of a previous study; thus, the final sample size was 392. A convenience sampling technique was used for all under five children who fulfilled the inclusion criteria during the study period.

Data Collection Instruments and Procedure

The data were collected using a structured questionnaire adapted from previous literature.¹³ The questionnaire was prepared in English in written form, orally translated into the Afan Oromo and Amharic languages, and then returned to

English to ensure its consistency. Five percent of the total sample respondents were interviewed during the pretest in another health institution. After this, the questionnaire was edited accordingly, and then the final version of the questionnaire was adapted to interview children's parents/caregivers. The data collectors explained the objective of the study to the children's parents/caregivers. Highlighting on the benefit of being tested for Hgb and what would be done if the child is anemic. The data collectors were given details to the patient/caregiver that no name of participant written on the questionnaire and confidentiality were protected, and verbal consent was obtained. Data were collected through pretested and structured questionnaires by face-to-face interviews with the children's patients/caregivers (Annex 1). The questionnaire was used to collect sociodemographic data and associated factors.

Blood Sample Collection

By strictly following the standard operating procedure (SOPs), a 3 mL venous blood sample was collected. Experienced laboratory technicians collected the samples in tubes containing ethylenediaminetetraacetic acid (EDTA). The complete blood count (CBC) reports from the hematology analyzer (Cell-DYN 1800) in the hospitals, including hemoglobin (Hgb) analysis, were performed as per the manufacturer's instructions (Annex 2).

Operational Definitions

Anemia - Hemoglobin (Hgb) <11 g/dL in children whose age is 6–59 months.¹⁴

Mild anemia: The Hgb value is 10–10.9 g/dL.¹⁴

Moderate anemia: Hgb value 7–9.9 g/dL for children 6–59 months.¹⁴

Severe anemia: Hgb value <7 g/dL for children aged 6–59 months.¹⁴

Low income: family monthly income less than 750 birr (ETB).¹⁵

Moderate income: family monthly income from 750–1500 ETB¹⁵

High income: family monthly income greater than 1500 ETB¹⁵

Recent infection: Children diagnosed with helminthic, intestinal protozoa and malaria within the last two weeks.

Data Quality Control

To assure data quality, 5% of the estimated sample was pretested at the Bule Hora health center before the data collection to determine whether the questionnaires were simple and understandable. After every data collection, the completeness and consistency of the questionnaire were checked. Data collector training and daily supervision were performed before and during the data collection period.

During blood sample analysis, the standard operating procedures (SOPs) and manufacturers' instructions were strictly followed for all laboratory activities. The sample was checked for hemolysis, clotting and enough volume before running the test. When the machine passed the control, the samples were analyzed. Finally, the laboratory test result was recorded, and the specimens were managed properly.

Data Processing and Analysis

Data were entered, sorted and categorized. Data cleaning was performed to check for completeness, accuracy, and missed values, and any errors identified were corrected. Then, the data were analyzed using SPSS version 22. Descriptive statistics (mean, frequency) were carried out to describe the sociodemographic status of the participants presented by table, pie chart and graph. A binary logistic regression model was fitted to identify factors associated with anemia. Variables with a P value ≤ 0.25 in the bivariate analysis were considered candidates for the multivariate analysis. Multivariate logistic regression was performed to control for possible confounding and identify the true effect of the selected predictor variables. The model fitness was checked with the Hosmer–Lemeshow test. The extant association between the different variables related to anemia was measured using AOR at 95% CI. A P value ≤ 0.05 was considered statistically significant.

Results

Sociodemographic and Economic Status

Of the 392 children under the age of five, 375 participated in this study, with a 95.7% response rate. The participants' ages ranged from 6 to 59 months, with a mean (SD) age of 3.3 (± 2.195) months. Among 375 children under the age of five, 54.4% (204) were female, and the majority 39.7% (149) of children's mothers/caregivers were farmers. More than half, 54.4% (204), were from rural areas. Approximately 29.4% (110) of child caregivers were unable to read and write. Out of 375 children under the age of five, approximately 55.9% (210) were protestants. Almost half of them (44.3%, 166) were living in a household with 3 family members, and the majority of households (36.8%, 138) had incomes less than 750 Ethiopian birr (Table 1).

Table 1 Sociodemographic and Other Selected Characteristics of Under Five Year Old Children Attended Bule Hora General Hospital

Variables	Category	Frequency	Percent
Age group	6–14 months	50	13.3
	15–23 months	88	23.5
	24–32 months	61	16.3
	33–41 months	38	10.1
	42–50 months	66	17.6
	51–59 months	72	19.2
Sex of child	Male	171	45.6
	Female	204	54.4
Religious	Orthodox	81	21.7
	Muslim	81	21.7
	Protestant	210	55.9
	Others	3	0.7
Sex of care giver	Male	204	54.4
	Female	171	45.6
Marital status of care giver/ mothers	Married	281	75.0
	Divorced	33	8.8
	Widowed	11	2.9
	Single	50	13.2
Educational level of care giver/mothers	Unable to read and write	110	29.4
	Able to read and write	72	19.1
	Grade 1–8	88	23.5
	Grade 9–12	28	7.4
	College and above	77	20.6

(Continued)

Table 1 (Continued).

Variables	Category	Frequency	Percent
Occupational of care giver	Herder	0	0.0
	House wife	77	20.6
	Merchant	39	10.3
	Farmer	149	39.7
	Private employee	44	11.8
	Government employee	66	17.6
Sex of household head	Male	292	77.9
	Female	83	22.1
Family size	1–2	88	23.5
	3	166	44.3
	4	83	22.1
	5≤	38	10.1
Residence	Urban	171	45.6
	Rural	204	54.4
Income of care giver	< 750 ETB	138	36.8
	750–1500 ETB	127	33.9
	> 1500 ETB	110	29.3

Feeding-Related Factors

Regarding feeding practices, among 375 under five children 69.1% (259) dietary diversity practice, 79.4% (298) introduction of complementary food after 6 months, 61.8% (232) mothers/caregivers do not have nutritional knowledge, 23.5% (88) faces food insecurity within four weeks, 69.2% (259) had meal three times feed per day while majority 80.8% (303) breastfeeding practice at 6–12 months ([Table 2](#)).

Table 2 Feeding-Related Factor Characteristics of Children Attended Bule Hora General Hospital

Variables	Category	Frequency	Percent
Dietary diversity Practice	Yes	259	69.1
	No	116	30.9
Product use in dietary diversity	Animal product	11	2.9
	Plant product	0	0.0
	Both	364	97.1
Introduction of complementary foods	≤6 months	77	20.6
	> 6 months	298	79.4

(Continued)

Table 2 (Continued).

Variables	Category	Frequency	Percent
Duration of breast feeding practice	< 6month	28	7.5
	6–12month	303	80.8
	1 <	44	11.7
Have nutritional knowledge	Yes	143	38.2
	No	232	61.8
Food insecurity in past four weeks	Yes	88	23.5
	No	287	76.5
Animal products use	Yes	303	80.9
	No	72	19.1
Meal frequency	One times	11	2.9
	Two times	66	17.6
	Three times	259	69.2
	Four and above times	39	10.3

Health Care and Disease Characteristics

Out of 375 under five children enrolled in the study, 17.6% (66) had intestinal protozoa followed by soil-transmitted helminthic infection 16.2% (61) and malaria infection 14.7% (55) (Table 3).

Prevalence of Anemia

Based on the hemoglobin cutoff value, less than 11 g/dL was categorized as anemic, and Hgb values of 10–10.9 g/dL, 7–9.9 g/dL, and less than 7 g/dL were determined to be mild, moderate and severe, respectively.¹⁶ The overall prevalence of anemia was 13.2% (50) (Figure 1). Among anemic children under five years of age, 12% (6) were mild, 32% (16) were moderate and 56% (28) were severe (Figure 2).

Table 3 Clinical Characteristics of Children Who Attended Bule Hora General Hospital

Variables	Category	Frequency	Percent
Recent acute blood loss	Yes	28	7.4
	No	347	92.6
Recent blood transfusion reaction	Yes	6	1.5
	No	369	98.5
Recent surgical procedure	Yes	0	0.0
	No	375	100.0
Recent accident injury	Yes	22	5.9
	No	353	94.1

(Continued)

Table 3 (Continued).

Variables	Category	Frequency	Percent
Recent intestinal protozoa infection	Yes	66	17.6
	No	309	82.4
Recent helminthic infection	Yes	61	16.2
	No	314	83.8
Recent malaria infection	Yes	55	14.7
	No	320	85.3
Recent epistaxis	Yes	22	5.9
	No	353	94.1
Chronic diseases	Yes	11	2.9
	No	364	97.1

Note: Statistical significance at $P<0.001=***$, $P<0.01=**$ and at $P<0.05=*$.

Abbreviations: COR, crude OR; AOR=adjusted OR; CI, confidence interval.

Factor Associated with Prevalence of Anemia

In bivariate analysis, variables such as child age group, child sex, family size, income of caregiver, dietary diversity practice, breastfeeding practice, nutritional knowledge, meal frequency, recent intestinal protozoan infection, recent helminthic infection, and recent malaria infection had a p value of <0.25 and were considered candidates for multivariate analysis. In multivariate analysis, the chance of having anemia was approximately 2 times higher among children under the age of five who had recent intestinal protozoan infection [AOR= 2.13, 95% CI =1.35–9.270] than among their counterparts. Additionally, children who had recent soil-transmitted helminths infections were 6 times more likely to have anemia [AOR=6.09, 95% CI=2.047–27.54] than their

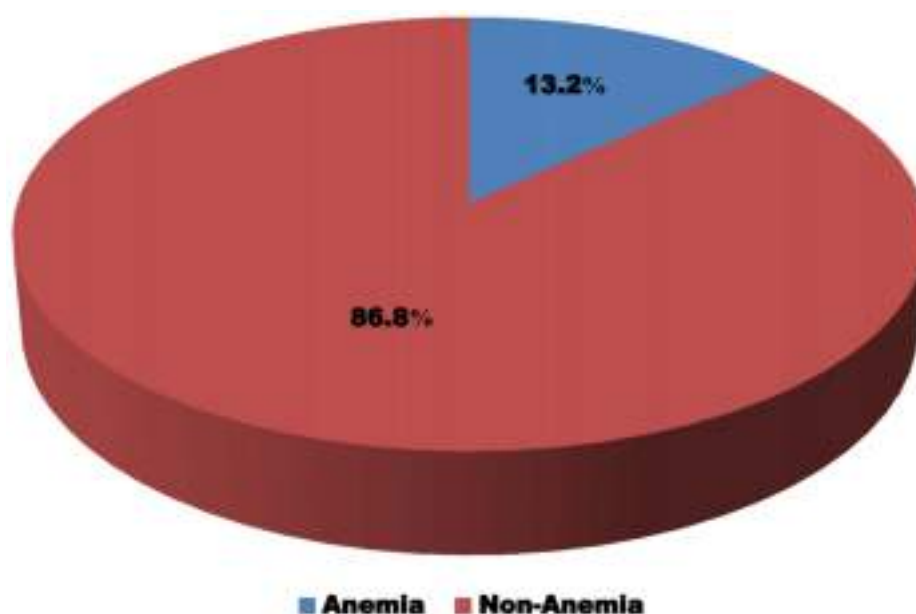


Figure 1 This figure shows the anemia among under five year old children attended Bule Hora General Hospital. Anemia is children scores hemoglobin less than 11 g/dL and non-anemia children score hemoglobin greater than or equal to 11 g/dL.

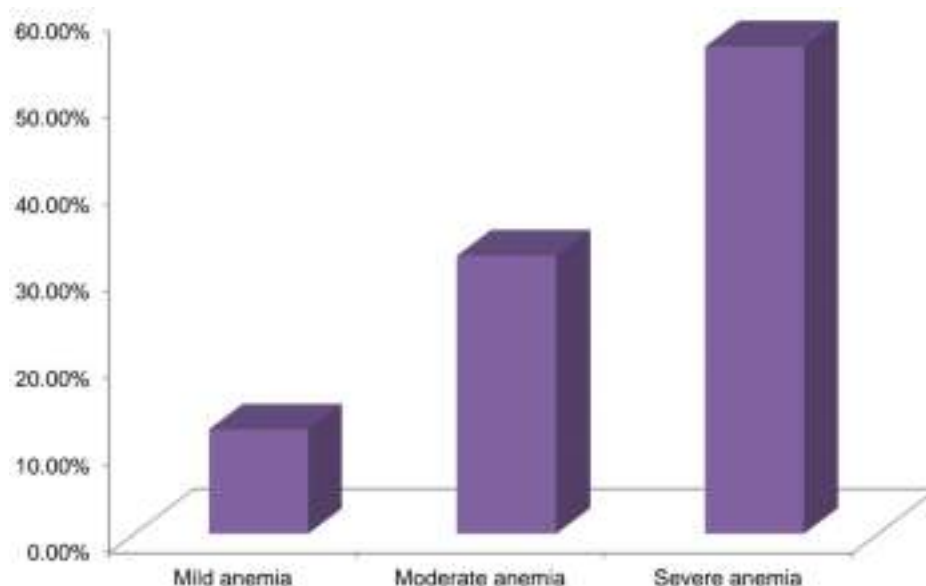


Figure 2 Anemic level among under five year old children attended Bule Hora General Hospital. Anemic children scores hemoglobin values of 10–10.9 g/dl were mild anemia, 7–9.9 g/dl were moderate anemia, and less than 7 g/dL were determined severe anemia.

counterparts. Similarly, the children who had recent malaria infection were nearly 5 times more likely [AOR=5.42, 95% CI=0.307–11.034] to have anemic than those who did not have recent malaria infection (Table 4).

Table 4 Bivariate and Multivariate Analyses of Factors Associated with the Prevalence of Anemia in Among Under five Children Attended at Bule Hora General Hospital, Southern Ethiopia

Variables	Category	Anemic	Non Anemic	COR(95% CI)	AOR(95% CI)
Child Age-group	6–14 months	11(22%)	39(78%)	1.04(0.143–7.527)	1.54(0.773–8.504)
	15–23 months	12(13.6%)	76(86.4%)	0.01(0.00–1.002)	0.73(0.102–2.112)
	24–32 months	11(18%)	50(82%)	1.39(0.118–6.227)	1.62(0.433–17.256)
	33–41 months	6(15.8%)	32(84.2%)	2.54(0.230–5.021)*	3.04(0.230–6.1021)
	42–50 months	6(9.1%)	60(90.9%)	0.81(0.108–6.036)	1.21(0.312–8.176)
	51–59 months	4(5.6%)	68(94.4%)	1	1
Child sex	Female	28(13.7%)	176(86.3%)	1.08(0.257–4.324)	1.22(0.341–6.932)
	Male	22(12.9%)	149(87.1%)	1	1
Family size	1–2	16(18.2%)	72(81.8%)	0.61(0.231–5.008)	0.42(0.034–4.678)
	3	23(13.9%)	143(86.1%)	0.87(0.178–4.210)	0.57(0.948–6.154)
	4	6(7.2%)	77(92.3%)	2.80(0.297–26.400)*	2.51(0.255–8.050)
	5≤	5(13.2%)	33(86.8%)	1	1

(Continued)

Table 4 (Continued).

Variables	Category	Anemic	Non Anemic	COR(95% CI)	AOR(95% CI)
Income of care giver	< 750 ETB	28(20.3%)	110(79.7%)	0.71(0.147–3.395)	0.53(0.074–2.115)
	750–1500 ETB	6(4.7%)	121(95.3%)	3.88(0.370–4.709)	2.98(0.130–4.321)
	> 1500 ETB	16(14.5%)	94(85.5%)	I	I
Dietary diversity practice	Yes	39(14.9%)	220(85.1%)	1.66(0.315–8.776)*	2.03(0.510–10.060)
	No	11(9.5%)	105(90.5%)	I	I
Duration of breast feeding practice	< 6month	6(21.4%)	22(78.6%)	I	I
	6–12month	38(12.5%)	265(87.5%)	0.42(0.034–5.317)*	0.48(0.055–6.722)
	I <	6(13.6%)	38(86.4%)	3.88(0.370–21.709)**	5.21(0.109–12.840)
Have nutritional knowledge	Yes	17(11.5%)	126(88.5%)	I	I
	No	33(14.3%)	199(85.7%)	1.23(0.290–5.621)	0.95(0.064–5.008)
Meal frequency	One times	6(54.5%)	5(45.5%)	1.66(0.315–8.776)	1.02(0.023–6.222)
	Two times	11(16.7%)	55(83.3%)	0.83(0.062–11.277)	0.40(0.102–4.296)
	Three times	27(10.4%)	232(89.6%)	1.79(0.171–18.822)	2.02(0.541–9.807)
	Four and above	6(15.4%)	33(84.6%)	I	I
Recent intestinal protozoa infection	Yes	11(16.7%)	55(83.3%)	1.38(1.129–7.975)**	2.13(1.35–9.270)**
	No	39(12.6%)	270(87.4%)	I	I
Recent helminthic infection	Yes	22(36.1%)	39(63.9%)	5.76(0.816–21.18)**	6.1(2.047–27.54)*
	No	28(8.8%)	286(91.2%)	I	I
Recent malaria infection	Yes	22(40%)	33(60%)	6.95(1.03–14.75)***	5.4(0.307–11.034)*
	No	28(8.8%)	292(91.2%)	I	I

Discussion

In this study, the overall prevalence of anemia among under five children attending Bule Hora General Hospital was 13.2% (50) (95% CI=5.2–21.2%). According to the WHO definition, anemia can be defined as a mild, moderate and severe public health problem when the prevalence is 5–19.9%, 20–39.9% and greater than 40%, respectively. Therefore, the prevalence of anemia in this study is considered a mild public health concern.¹⁷

This is lower than the previous study conducted in Western China (51.2%),¹⁸ Eastern Sudan (86%),¹⁹ Cape Verde West Africa (51.8%),²⁰ Nigeria (70.5%),²¹ Tanzania (77.2%),²² Gonder town Ethiopia (66.8%),²³ Gonder, Ethiopia (58.6%),²⁴ Wagmra zone, Ethiopia (66.6%),²⁵ and South Wollo, Northeast Ethiopia (41.1%).¹⁵ The difference in prevalence might be due to variations in the number of participants, sample analysis equipment, hemoglobin cutoff points, and cultural, geographical and behavioral characteristics of the community. In contrast, this finding was higher than reports from Brazil (10.2%)²⁶ and Sub-Sahara Africa (9.7%).²⁷ The possible reason might be due to the age difference of the study participants. In our study, the study participants were aged from 6–59 months, but in Sub-Saharan Africa, the study participants were aged below 36 months.

Multivariate analysis showed that having a previous intestinal protozoan infection was significantly associated with the prevalence of anemia among under five children. This finding is in line with a study conducted in Pawe town, Benishangul

Gumuz, region²⁸ and Gonder, Ethiopia.²⁴ Similarly, children who had previous malaria infection were significantly associated with anemia among under five children. This finding agrees with a study conducted in Ghana.²⁹

In addition, anemia was 6 times more likely among children with previous soil-transmitted helminthic infections than among their counterparts. This finding is consistent with a study conducted in Gonder, Ethiopia.²³ Possible justification could be due to intestinal wall bleeding, erythrocyte lysis, reduction of iron and damaged organs involved in hematopoiesis.

Out of 375 children under five years of age involved in the study, nearly half (48.5%, 182) were infected with at least one of the following species of parasites: *Plasmodium spp.*, *Entamoeba histolytica*, *Giardia lamblia*, *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Trichuris trichuria*, *Enterobius vermicularis* and *Hookworm*. Of those children infected with recent malaria, 22 (40%), soil-transmitted helminths, 22 (36.1%) and intestinal protozoa 11 (16.7%) were found to be anemic. The parasite species may deplete red blood cells through loss of blood in the time of diarrhea and gastrointestinal bleeding.

Strength of the Study

This research could elaborate the important part of health accessibility issues, especially in child health, and identified associated factors of anemia.

Limitations of the Study

As a hospital-based study with convenience sampling technique was applied, and a small sample size was used, the result cannot be extrapolated to a larger community. Additionally, this study does not differentiate the types of anemia such as hemolytic, sickle cell, thalassemia and so on.

Conclusion

The present study demonstrated a 13.2% overall prevalence of anemia among children under the age of 5 who attended Bule Hora General Hospital. This finding was low compared with previous studies conducted in different parts of Ethiopia. The factors significantly associated with anemia were recent intestinal protozoan infections, soil-transmitted helminths, and malaria infection. Therefore, early diagnosis, treatment and prevention of parasite infection should be important in the reduction of anemia among under five children.

Abbreviations

CBC, complete blood count; EDTA, ethylene diamine tetraacetic acid; HGB, hemoglobin; g/dL, gram per deciliter; SOPS, standard operation procedures; AOR, adjusted odds ratio; COR, crude odds ratio; CI, confidence interval; WHO, World Health Organization; EDHS, Ethiopian Demographic Health Survey; SPSS, Statistical Package for Social Science; ETB, Ethiopian Birr.

Data Sharing Statement

Data essential for the conclusion are included in this manuscript. Additional data can be obtained from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

The study was conducted after ethical approval from the Bule Hora University Research and Ethical Review Committee. The official letter was written to Bule Hora General Hospital. Informed, voluntary, written signed consent was obtained from the study participant, parent/caregiver and institution. The children's parents/caregivers were informed about the purpose of the study, and written informed consent was obtained before the questionnaire was administered based on Helsinki declaration. Then, blood samples were collected from the study participant. Participation in the study was voluntary. The participants were informed of their right to quite/refuse their participation at any stage of the study if they did not want to participate. To ensure confidentiality of participant information, codes were used, and any identifier of participants was not written on the questionnaire on the test tube. Any abnormal test results of participants were communicated to the concerned body.

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Author Contributions

All authors made a significant contribution 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.

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We declare that we do not have any conflicts of interest related to this work or any activities pertaining to this research work.

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Oxygen Saturation in Primary Teeth of Individuals With Sick Cell Disease and Sick Cell Trait

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Purpose: To determine oxygen saturation in the pulp of primary teeth in children with sickle cell disease (SCD) and sickle cell trait (SCT) for establishing the usefulness of pulse oximetry in screening and monitoring of SCD or therapy.

Materials and Methods: A cross-sectional study among 30–60 months children with sickle cell disease (SCD) and sickle cell trait (SCT) compared with healthy children (HbAA). A pulse oximeter (BCI 3301) recorded oxygen saturation on six anterior primary maxillary teeth and on index fingers. Data were analyzed using SPSS version 20.0. Mean oxygen saturation for teeth and fingers was calculated. Comparison of Mean across groups was done using post hoc analysis in one-way ANOVA (Bonferroni test). Pearson correlation coefficient was calculated for mean oxygen saturation on fingers and teeth. Level of significance was set at 0.05.

Results: Altogether 360, 102, and 96 teeth were examined from children with SCD, SCT, and HbAA respectively. 53% of participants were girls. The mean age of participants was 46.3 months $\pm$ 9.4 SD. Low mean oxygen saturation (77.5%) was recorded from teeth of children with SCD relative to those with SCT and HbAA ($>86\%$; $P = 0.00$). There was no statistically significant difference in oxygen saturation on teeth between children with SCT and HbAA. The mean oxygen saturation on fingers was found to be above 97.2% regardless of sickle cell status. There was no correlation between oxygen saturation on teeth and fingers.

Conclusion: Pulse oximeter detected a lower oxygen saturation in dental pulp of primary teeth of participants with SCD (HbSS) relative to those with SCT (HbAS) and HbAA. Oxygen saturation on fingers remained unaffected regardless of sickle cell disease status. Although more studies are needed, our study shows that when other conditions affecting peripheral tissue oxygen delivery are ruled out, the low pulse oximetry in primary teeth may be indicative of SCD. The oximeter may also be useful in monitoring response to SCD therapy targeted at improving oxygen carrying capacity and delivery.

Keywords: pulse oximeter, dental pulp, microcirculation, oximetry

Introduction

Sickle cell disease (SCD) is a genetic disorder due to the inheritance of mutant hemoglobin when glutamic acid at position six in the beta-globin chain is substituted by valine.¹ Erythrocytes with mutant hemoglobin become distorted, rigid, and more adherent to vascular endothelium following polymerization during low oxygen tension.² Sickled erythrocytes block capillaries and restrict blood flow to various organs and systems causing ischemia and infarction leading to multiple tissue and organ damage.³ Sickled erythrocytes are also inefficient in oxygen transportation through microcirculation resulting in low peripheral tissue oxygen saturation.⁴ Tissue oxygen saturation of 90–100% in healthy (HbAA) individuals is considered to be within the normal range.⁵

Pulse oximetry is a well-accepted and widely used non-invasive technique for monitoring oxygen saturation and real-time pulsation. A pulse oximeter utilizes red and infrared light at the wavelengths of 660 nm and 900–940 nm, respectively, to measure the differential level of blood oxygenation. In dentistry the tool is known for its use in assessing

dental pulp vascularity and vitality;^{6–9} it is relatively inexpensive, readily available and highly accepted, especially for pediatric patients compared with traditional tests such as electric and thermal pulp tests which are associated with discomfort and/or pain. To date, there is no commercially available pulse oximeter for dental use, so small-sized probes with or without modifications have been successfully adopted for the same.^{8,10,11} The device has been employed in assessing oxygen saturation in teeth of healthy individuals (HbAA) but has been used less in individuals with SCD, mostly in permanent teeth. The mean oxygen saturation in permanent teeth in HbAA in most reports is comparably lower than general body saturation recorded from fingers, but above 85% (Bruno et al, 2014; Pozzobon et al, 2011; Calil et al, 2008). Contrary to most studies, Kong and co-workers report as high as 97–100% pulpal oxygen saturation in HbAA which is similar to general body oxygen saturation.¹³ Immature permanent teeth with the open apex on the other hand have been shown to register a higher mean pulpal oxygen saturation than mature permanent teeth, suggesting a decrease in pulp blood flow with dental maturity in HbAA.¹⁴

Though there are limited data in subjects with SCD, Souza et al report an even lower (78%) mean pulpal oxygen saturation in individuals with SCD (HbSS) than HbAA counterparts.¹⁵ Low pulpal oxygen saturation on teeth is partly attributed to morphological barriers of teeth to red and infrared light of the oximeter, while the even lower saturation in SCD may be contributed to by the disease itself.

Although most reports are on permanent teeth, primary teeth, on the other hand, are less mineralized, have a wider pulp chamber, thinner and porous enamel, a thinner dentine layer, and a more pronounced cervical constriction,^{16,17} thus light from a pulse oximeter would easily be absorbed and less diffracted allowing better oximeter readings.

This study investigated the oxygen saturation in primary teeth of children with SCD, SCT, and HbAA using a pulse oximeter to establish its usefulness in screening and monitoring SCD therapy as an affordable, non-invasive and readily available addition to the available diagnostic and screening tests for SCD such as hemoglobin electrophoresis and DNA testing which are invasive, expensive, and not readily available.

Materials and Methods

This was a cross-sectional study that investigated 558 primary maxillary anterior teeth from 93 children aged 30–60 months. Participants were enrolled into three groups namely SCD, SCT, and HbAA based on hemoglobin electrophoresis. Participants with SCD were recruited from sickle cell clinics in Dar es Salaam whereas those with SCT and HbAA were recalled by age from Muhimbili sickle cell program database. Data collection was done using a questionnaire and a hand-held pulse oximeter (BCI 3301) with a finger probe and a universal (Y) sensor for measuring oxygen saturation. A small universal probe (BCI 3043) was selected for its design that enables close adaptation to the tooth surface. The BCI 3301 hand-held pulse oximeter ensures an accuracy in its measurements of upto 99% with readings range of 0–99% indicating a functioning device, whereas 0% reading is for non-vital teeth. The oximeter was tested on 60 teeth among 10 subjects before the actual data collection for functionality and accuracy. A dental surgeon was trained in positioning the patient and the sensor on the tooth during data collection. An assistant nurse was present for recording the readings.

Questionnaire

A questionnaire was used to record age, sex, level of hemoglobin, hemoglobin phenotype, and genotype, and if the participant is taking any medication including hydroxyurea, folic acids, and penicillin. Participants with a hemoglobin level less than 6 g/dl, those with dental caries, deep restorations, severe periodontal disease, tooth mobility, developmental defects, severe sickle cell disease symptoms, chronic pulmonary illness (asthma and bronchitis), and heart disease were excluded from the study.

Clinical Examination

A handheld pulse oximeter (BCI 3301) was used to measure oxygen saturation from the index finger and primary (deciduous) maxillary anterior teeth. Before measurements, teeth were cleaned and dried with sterile gauze. For easy attachment of the probe on teeth, participants were supine positioned and the back supported at 45 degrees. The light-emitting diode of the sensor was placed palatally and the photoreceptor on the labial surface parallel to each other at the centre of the tooth crown. The sensor and receptor were held in place and stabilized by an incisal and buccal/palatal

finger rest until stable readings were detected on the oximeter display before recording the data. Six teeth (right and left central incisors (FDI teeth 51 and 61), right and left lateral incisors (FDI teeth number 52 and 62) and right and left canines (FDI teeth number 53, 63)) were serially investigated from each participant.

After examining the teeth, the participant's index finger was inserted into a finger probe and the oximeter used to take stable oxygen saturation; in both teeth and index finger readings, measurements without changing for a minute or more were considered stable and recorded. The finger probe and the universal sensor were disinfected with an alcohol-based disinfectant between participants. Handwashing and gloving for examiners were ensured.

Data Analysis

Data were analyzed using Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, version 20.0 Armonk, NY: IBM Corp.). Age, sex, hemoglobin level, hemoglobin genotype, and medications were considered independent variables whereas oxygen saturation from teeth and index finger as dependent variables. Age and hemoglobin levels were summarized as mean, sex as the frequency distribution of boys and girls, and hemoglobin genotype as HbSS, HbAS, HbAA. Oxygen saturation on teeth was summarized as a mean percentage for groups of teeth (central incisors, lateral incisors, and canines) based on sickle cell genotype and as mean oxygen saturation for each study group [SCD (HbSS), SCT (HbAS), and HbAA] and oxygen saturation on fingers as a mean percentage based on a study group. Comparison of mean was done using post hoc analysis in one-way ANOVA (Bonferroni test calculated). The Pearson's correlation coefficient was calculated for mean oxygen saturation on teeth and index fingers for both groups. The level of statistical significance was set at $P < 0.05$.

Ethical Consideration

Ethical approval for this study (MUHAS-REC-07-2020-317) was provided by the Research and Ethics Committee of the Muhimbili University of Health and Allied Sciences (MUHAS). Written informed consent to participate in the study was obtained from parents before participation. Tailored oral health information, advice, and oral hygiene instructions were provided to escorts and children after an examination. All activities done in this research comply with the Declaration of Helsinki.

Results

We investigated 558 primary maxillary anterior teeth, comprising 360, 102, and 96 teeth from children with SCD, SCT, and HbAA respectively. A total of 93 children (30–60 months) were studied, 53% of which were girls. The participants' mean age was $46.3 \text{ months} \pm 9.4 \text{ SD}$. Only 3% of participants with SCD were on hydroxyurea and 88.3% of them were on either folic acid or penicillin or both.

We found a low (75.2–80.4%) mean oxygen saturation for a group of teeth from children with SCD compared with SCT and HbAA. Canines recorded slightly higher oxygen saturation in both study groups. A mild difference in mean oxygen saturation for teeth between children with SCT and HbAA was observed but the difference was not statistically significant (Figure 1).

When pulled together, the overall mean oxygen saturation on teeth was found to be 77.5% for children with SCD compared with 88.2% for SCT and HbAA. The observed difference between mean oxygen saturation on teeth from children with SCD and the other groups are statistically significant ($P = 0.00$). For index fingers, the mean oxygen saturation was found to be above 97% with no statistically significant difference across the study groups (Table 1).

There was no correlation between teeth and finger oxygen saturation regardless of sickle cell status. Although there were more participants with SCD than the rest, it was found that the majority of participants in both groups had oxygen saturation above 80%, and there was a weak positive correlation of oxygen saturation to hemoglobin levels (Figure 2A and B).

Discussion

The current study found a low oxygen saturation from the dental pulp in primary teeth of children with SCD, findings which agree with another similar study in permanent teeth.¹⁵ Other studies^{11,12,18,19} have reported low mean oxygen saturation from teeth in HbAA; our study recorded an even lower oxygen saturation from primary teeth in individuals

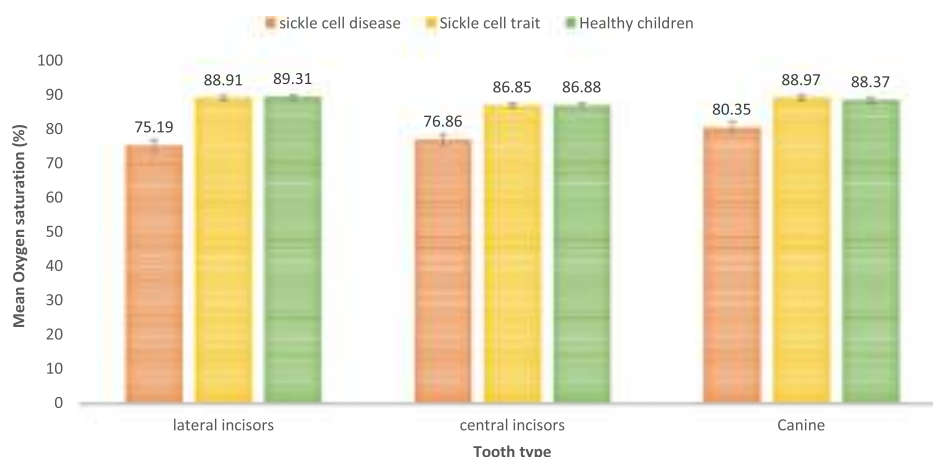


Figure 1 Summary of mean oxygen saturation per tooth type based on sickle cell status.

with SCD. These findings could be attributed to the fact that in addition to diffraction of red and infrared light from the pulse oximeter by enamel and dentine surrounding the dental pulp,³ SCD reduces resilience and oxygen-carrying capacity of erythrocytes and ultimately oxygen delivery through the microvasculature. Analysis of grouped teeth consistently showed low mean oxygen saturation in SCD with canines displaying a slight high oxygen saturation. The slightly high saturation in canines may be explained by a relatively wider pulp space with comparably bulk pulp tissue to central and lateral incisors. There was no statistically significant difference in oxygen saturation for teeth between participants with SCT and HbAA, signifying that there is a less clinical manifestation of structural and functional changes in SCT at optimal oxygen condition as reported in other studies elsewhere.^{20,21} Although, a pulse oximeter is shown from a previous study by Karayılmaz and Kırzio to be ineffective in assessing the effect of physiological root resorption on oxygen saturation, laser Doppler flowmetry in the same study reports an increase in oxygen saturation with increase in level of resorption.²² In our study the age for inclusion was 30–60 months, at which the physiological root resorption for anterior teeth is still minimal and the fact that presence of tooth mobility which usually in absence of trauma or periodontal disease is indicative of a high level resorption was an exclusion criteria, narrows down the effect of resorption in our findings. Low oxygen saturation is also reported in irreversibly damaged but vital pulp in HbAA;⁹ our study investigated otherwise healthy teeth from both groups of subjects.

However, oxygen saturation on fingers was found to remain unaffected regardless of sickle cell status. The possible explanation of these findings could be the presence of collateral blood supply on fingers contrary to teeth, whereby, in addition to diffraction of red and infrared light from a pulse oximeter, the vascular tissue (pulp) is confined within

Table 1 Comparison of Mean Oxygen Saturation on Teeth and Fingers Based on Sickle Cell Status (Bonferroni Test)

SCD (Mean % ± SD)	Comparison Sickle Cell Status	(Mean % ± SD)	P-value
Oxygen saturation in dental pulp			
77.5±7.5	SCT	88.2±3.9	0.000 ^a
	HbAA	88.2±3.8	0.000 ^a
Oxygen saturation in index fingers			
97.4±3.4	SCT	97.2±3.1	0.831
	HbAA	98.3±0.8	0.283

Note: ^aStatistically significant (P<0.05).

Abbreviations: SD, standard deviation; SCD, Sickle Cell Disease; SCT, Sickle Cell Trait; HbAA, Hemoglobin AA.

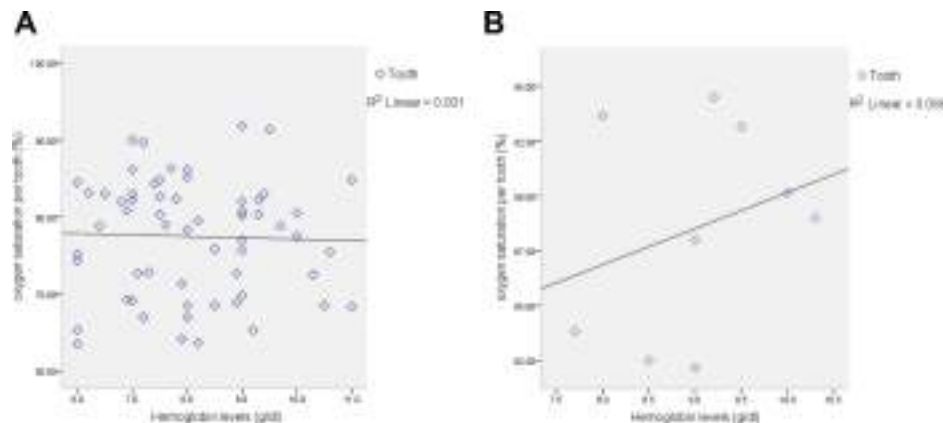


Figure 2 (A) Scatter plot showing mean oxygen saturation to hemoglobin levels for teeth among participants with SCD. **(B)** Scatter plot showing mean oxygen saturation to hemoglobin levels for teeth among participants with SCT.

surrounding hard tissue (the enamel, and dentine) which does not allow sufficient vascular elasticity, which when coupled with the lack of collateral blood supply²³ may lead to poor oxygen delivery and more sensitivity of teeth to oximetry.

A recent review showed only a limited number of other studies on primary teeth.¹¹ Our study has been able to shed light that the dental pulp which is known to lack collateral blood supply and is encased within less mineralized hard tissue of primary teeth is readily affected by disorders that affect peripheral tissue oxygen delivery such as SCD and the pulp is more sensitive to pulse oximetry, such that it can be useful in detecting such disorders before further confirmatory tests can be done.

We recommend further exploration with more advanced tools such as Doppler flowmetry studies to benchmark and validate our findings for the possible use of an oximeter as a screening tool and/or for monitoring improvement in oxygen saturation during SCD therapy. Further studies on oxygen saturation at other than optimal sickle cell disease status are needed to assess the reliability of oxygen saturation on monitoring SCD conditions and therapy.

Conclusion

Our study shows that children with SCD register a low oxygen saturation from their primary teeth relative to those with SCT and those with HbAA. A pulse oximeter detected no difference in oxygen saturation on fingers regardless of sickle cell disease status. The confined nature of the dental pulp and the lack of collateral blood supply may have made the tooth more sensitive to vascular conditions affecting tissue oxygen delivery such as SCD. Although more studies are needed, the current findings show that the low oxygen saturation on teeth measured with a pulse oximeter may be indicative of SCD, thus a pulse oximeter may be a useful adjunct tool for screening conditions that affect oxygen delivery to the peripheral tissues such as SCD and for monitoring therapy that targets improvement in tissue oxygenation such as hydroxyurea in SCD.

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Disclosure

The authors report no conflicts of interest in this work.

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Red Blood Cell Distribution Width as Novel Biomarker in Cardiovascular Diseases: A Literature Review

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Red Blood Cell Distribution Width as Novel Biomarker in Cardiovascular Diseases: A Literature Review

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Abstract: Red blood cell distribution width (RDW) is a measure of the change in size of red blood cells and it is used in combination with other hematological parameters for the differential diagnosis of anemias. Recent evidence suggested that the change in RDW level may be a predictive biomarker of morbidity and mortality in cardiovascular diseases (CVDs). Cardiovascular diseases are the most common cause of death globally as compared to cancer and communicable diseases. Early diagnosis and prompt intervention of these diseases are very important to minimize their complications. Nowadays, the diagnosis of most cardiovascular diseases majorly depends on clinical judgment, electrocardiography and biochemical parameters. Red blood cell distribution width as a new predictive biomarker may play a pivotal role in assessing the severity and progression of CVDs. However, the underlying mechanisms for the association between RDW and CVDs are not clear. A deeper understanding of their association could help the physicians in more careful identification, early prevention, intervention, and treatment to prevent adverse cardiovascular events. This review aims to elaborate on the recent knowledge on the association between RDW and cardiovascular diseases and some possible pathophysiological mechanisms.

Keywords: biomarkers, cardiovascular diseases, inflammation, red blood cell distribution width, prognosis

Introduction

Red blood cells (RBCs) are non-nucleated blood cells characterized by having a typical oval biconcave shape, with a diameter of 6 to 8 μm and a thickness of 2 μm . The normal volume of RBCs ranges from 80 to 100 femtoliter (fL), but different physiological and pathological conditions may increase the degree of anisocytosis. The red blood cell distribution width (RDW) is a quantitative measure of variation in the size of circulating RBCs, which is receiving increasing interest as a diagnostic and prognostic marker in a vast array of human disorders.¹ The RDW has been introduced as a novel inflammatory predictor in various disorders including functional bowel conditions,² autoimmune diseases,^{3,4} malignancy,⁵ COVID-19 and multiple hospital admissions in subjects with chronic conditions.⁶

Red blood cell distribution width is calculated automatically by hematological analyzers by simply dividing the standard deviation (SD) of the mean corpuscular volume (MCV) by the MCV and multiplying by 100 to yield a percentage value. Since the methods of measuring the RBC size, the instrument, and statistical methods are varying in many laboratories, there is no established common reference range till now.⁷ The normal reference range of RDW most laboratories used is roughly ranging from 11% to 15%.

An RDW value below the reference range has been considered without clinical relevance, whereas an increased RDW value reflects a greater difference in the size of RBCs, which can be due to the presence of smaller or larger RBCs, or both. An elevated RDW usually results from increased or ineffective production of RBCs and excessive fragmentation or destruction of RBCs.⁸ Thus, RDW is increased in patients with iron deficiency anemia, megaloblastic anemia, myelodysplastic syndrome, hemolytic anemia, liver failure, sickle cell disease, and blood transfusions.⁹ Red blood cell

distribution width together with MCV has been almost exclusively used for the differential diagnosis of anemias.¹ However, it is almost an unknown parameter to which very few clinicians pay attention when examining blood cell count.

In addition to RBC disorders, various cardiovascular diseases (CVDs) such as heart failure (HF), ischemic cerebrovascular disease (including stroke), acute coronary syndrome (ACS), peripheral artery disease (PAD), hypertension, and atrial fibrillation (AF) are often associated with a high degree of anisocytosis.¹⁰ Since both RDW and cardiovascular conditions are associated with inflammation, RDW could also be associated with CV diseases. Cardiovascular diseases are a diverse group of disorders that affect the heart and blood vessels, and they are mostly interrelated with the underlying pathology of atherosclerosis.¹¹ Compared to cancer and communicable diseases, CVD continues to be the most common cause of death globally (17.3 million death). Proper risk assessment and stratification, as well as prognosis evaluation for patients with CVDs, are critical to cardiologists. The diagnosis of CVDs mainly depends on clinical judgment, imaging, and biochemical parameters (Troponin, C-reactive protein, B type natriuretic peptide, myoglobin), but biomarkers that could be commonly used in different clinical practices are finite.¹² In addition, noninvasive anatomical and electrophysiological (electrocardiography) cardiac biomarkers are commonly used as surrogate markers of CVDs, especially in its acute phase.^{13,14}

The biomarker is defined as a characteristic objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention. Ideal diagnostic biomarkers should be highly specific, sensitive, rapidly available, inexpensive, and noninvasive.¹⁵ Clinicians have been trying for years to search for such a biomarker to aid the identification, early prevention, intervention, and therapy to prevent adverse cardiovascular events. One candidate is RDW, which is widely investigated and routine laboratory value reported as a component of standard complete blood count (CBC) without the need for extra procedures. Nowadays, scientific evidence has shown that RDW may provide valuable information for the diagnosis of different diseases and establishing the short- and long-term prognosis in patients with CVDs.¹⁶ Hence, recent evidence suggested that the change in RDW level may be a predictive marker of morbidity and mortality in CVDs.¹⁷ However, the underlying mechanisms of whether the increased heterogeneity is the cause or consequence of other pathophysiological conditions exceedingly remain unclear. This review aims to elaborate on the recent knowledge on the association between RDW and cardiovascular diseases and some possible pathophysiological mechanisms.

This review was conducted based on the relevant articles published in the English language on the current topic retrieved from electronic databases like PubMed, PubMed Central, ScienceDirect, Google Scholar, and google. Relevant articles were searched by the following words: red blood cell distribution width, cardiovascular diseases, coronary artery disease, myocardial infarction, acute coronary syndrome, heart failure, hypertension, and stroke, separately and in combination. Additionally, reference lists of publications in this field were searched to identify any studies for inclusion and the current review includes original peer-reviewed articles. Also, articles such as reviews, systematic reviews, and meta-analyses were considered.

Red Blood Cell Distribution Width and Cardiovascular Disease

Currently, a number of articles have been published regarding the role of RDW in clinical practice and the risk stratification of patients with CVDs. In this review, the most important and appropriate studies investigating the role of RDW in patients with CVDs are discussed here under and summarized in [Table 1](#).

RDW in Myocardial Infarction

In addition to its routine index in clinical practice, the increased value of RDW has been proposed to be associated with the adverse outcomes of myocardial infarction. A study was conducted by Cemin et al,¹⁸ including 1971 patients admitted due to chest pain with suspected cardiac origin at the emergency department. The authors found that RDW was a significant predictor of acute myocardial infarction (AMI), showing the area under the curve (AUC) of 0.61 (95% CI, 0.54–0.68). The specificity and sensitivity of RDW at the 13.7% cut-off value were 52% and 75%, respectively.¹⁸ In addition, the diagnostic value of joint detection of RDW and homocysteine (HCY) variable coefficient on AMI were investigated by recruiting 400 study subjects (100 healthy and 300 patients with coronary heart disease). Among patients,

Table I Summary of Studies Exploring Association Between RDW and CVDs

Author, Year	Study Design	Study Population	Major Findings	References
Felker et al, 2007	Retrospective cohort	2679 chronic HF patients	Higher RDW in patient with CV event than without CV events (15.2 vs 14.4). HR for morbidity and mortality (1 SD increment of RDW): 1.17 (95% CI, 1.10–1.25, $p<0.001$)	[21]
Dai et al, 2014	Cross-sectional study	521 patients with acute HF	Higher RDW (16.2% vs 14.4%) in acute HF patients at admission were associated with worse short- and long-term outcomes and RDW values were more prognostically relevant than Hgb levels	[22]
Jenei et al, 2014	Prospective cohort study	195 patients with stable chronic HF	RDW $>14.5\%$ was independent predictor of 5-year mortality (HR 1 SD increment 1.46, 95% CI 1.221–1.733, $p<0.001$)	[23]
He et al, 2014	Prospective cohort	128 patients with acute HF	Both RDW and NT-proBNP are strong independent predictors of cardiovascular events	[24]
Liu et al, 2016	Retrospective study	179 chronic HF patients	RDW was markedly elevated in the mortality group compared with the survival group (15.8 ± 1.8 vs 13.7 ± 1.7 , $p<0.01$). RDW was an independent risk factor for mortality (OR=2.531, 95% CI: 1.371–4.671) during hospitalization with AUC = 0.837	[25]
Huang et al, 2014	Meta-analysis of 17 studies	18,288 patients with HF	RDW on admission and discharge, as well as its variation during treatment are prognostic markers in HF patients. In particular, each 1% increase in baseline RDW was associated with a 10% increased risk of all-cause mortality (OR, 1.10; 95% CI, 1.07–1.13).	[26]
Cemin et al, 2011	Prospective study	1971 patients admitted due to chest pain of suspected cardiac origin	Higher RDW value was obtained in patient with AMI compared to without AMI (14.4 vs 13.7) and RDW cut-off value of 13.7% showed a sensitivity and specificity of 75% and 52%, respectively	[18]
Hu et al, 2017	Case-control study	100 healthy and 300 patients with coronary heart disease	Stenocardia 121 cases, HF 65 cases and acute MI 114 cases were found. The result revealed that the RDW and HCY were both significantly higher in acute MI groups than in the 3 other groups	[19]
Lippi et al, 2009	Prospective study	2304 adult patients admitted for chest pain suggestive of ACS	The combined measurement of cardiac troponin T and RDW increases diagnostic sensitivity to 99% in diagnosing ACS (diagnostic sensitivity of cardiac troponin T alone was 94%).	[20]
Söderholm et al, 2015	Prospective study	26,879 participants without a history of coronary events or stroke	High RDW was associated with increased incidence of total stroke (HR for stroke 1.31 (1.11–1.54 the highest quartile compared to the lowest)	[29]
Ramírez et al, 2013	Case-control study	224 patients with ischemic stroke and 224 control subjects	Subjects who have RDW $>14.61\%$ were more likely to have a stroke compared with patients with RDW $<13.27\%$, [OR 4.50; 95% CI: 2.50–8.01, $P<0.0001$]	[30]
Wan et al, 2015	Prospective study	300 patients with AF	RDW was independently associated with all-cause mortality (HR: 1.024; 95% CI: 1.012–1.036, $P<0.001$) and major adverse events (HR: 1.012; 95% CI: 1.002–1.023, $P=0.023$).	[31]

(Continued)

Table 1 (Continued).

Author, Year	Study Design	Study Population	Major Findings	References
Ertas et al, 2012	Retrospective	132 patients with no histories of AF undergoing coronary artery bypass grafting	Preoperative RDW levels were significantly higher in patients who developed AF than in those who did not (13.9 ± 1.4 vs 13.3 ± 1.2 , $p=0.03$). Using a cut point of 13.45, the preoperative level correlated with the incidence of AF with a sensitivity of 61% and specificity of 60%.	[32]
Eryd et al, 2014	Prospective study	27,124 subjects from the general population without history of CVDs	HR for incidence of AF was 1.33 [95% CI 1.16–1.53] for the fourth versus first quartile of RDW $P < 0.001$.	[33]
Osadnik et al, 2013	Retrospective	2550 consecutive patients with stable coronary artery disease	4-fold increase (4.3% vs 17.1%, $p < 0.0001$) in mortality between the group of patients with RDW values < 13.1 vs > 14.1 %. RDW is an independent predictor of mortality in patients with stable coronary artery disease	[34]
Ye et al, 2011	Prospective study	13,039 consecutive outpatients with PAD	Subjects in the highest quartile of RDW (> 14.5 %) had a 66% greater risk of mortality compared to those in the lowest quartile (RDW < 12.8 %; $P < 0.0001$). A 1% increment RDW was associated with a 10% greater risk of all-cause mortality (HR: 1.10; 95% CI, 1.08–1.12)	[36]

121 were stenocardia cases, 65 were HF cases and 114 were AMI cases.¹⁹ In this study, the authors found that RDW and HCY were increased in AMI groups than in the three other groups ($P < 0.05$).

Furthermore, a study done by Lippi et al²⁰ showed that RDW had a diagnostic value in patients admitted to the ICU for chest pain suggestive of ACS. On admission, the sensitivity and specificity of troponin T were 94% (25 false-negative results) and 100%, respectively. With a 14% cut-off value, the sensitivity and specificity of RDW were 79% and 50%, respectively. The measurements of troponin T and RDW help to diagnose MI with greater sensitivity (99%) than the analysis of troponin T alone. This implicated that RDW and other parameters like HCY and troponin T joint detection of AMI has a higher sensitivity and specificity, which may highly improve the accurate diagnosis of MI. However, homocysteine is expensive and not widely available. If the use of RDW in resource poor settings are considered, homocysteine–RDW combination may not be useful.

RDW in Heart Failure

As mentioned earlier, the RDW and anemia relationship has been studied since the sixties. The RDW has also been suggested as a useful tool for predicting morbidity and mortality in patients with acute and chronic heart failure.^{21–25} In 2007 Felker et al²¹ reported for the first time in two cohorts ($n = 2679$) of patients with chronic HF. They demonstrated that the RDW was the most powerful predictor of adverse follow-up outcomes, both in cardiovascular mortality and in hospitalizations due to HF. Hazard ratio (HR) for morbidity and mortality (RDW increased in 1 SD): was 1.17 (95% confidence interval [CI], 1.10–1.25, $P < 0.001$).²¹ A recent meta-analysis reported by Huang et al²⁶ showed that RDW is a prognostic marker in HF patients on admission, discharge, and during treatment. A 1% higher RDW in baseline was associated with a 10% increased risk of future mortality events (HR, 1.10; 95% CI, 1.07–1.13). This indicates that 1% increment in RDW during admission can predict the patient's future outcome. Several studies have shown a positive relationship between RDW and different markers that have been applied over the last decade in predictive models of survival for patients with HF including C-reactive protein, in-hospital mortality,²² hospital readmissions,²³ and type B natriuretic peptide (BNP).²⁴

Regarding the latter blood marker highly studied in HF, He et al²⁴ conducted a prospective study to evaluate the predictive value of RDW for a cardiovascular event during hospitalization of acute HF patients and compared it with that

of NT-proBNP. The main result of this study was a significant weak positive correlation existed between the RDW and NT-proBNP levels ($r = 0.217$; $P < 0.05$), the cutoff point for NT-proBNP was 1471.5 pg/mL with a 93.5% sensitivity and 32.9% specificity. For RDW, the cutoff point was 14.1%, with 87.0% sensitivity and 54.9% specificity and survival analysis revealed that patients with an RDW level $>14.5\%$ and NT-proBNP >1471.5 pg/mL were at the highest risk for cardiovascular event ($P < 0.001$). This investigation suggests that the combination of RDW and other validated biomarkers such as NT-proBNP can help cardiologists improve the accurate rate of diagnosis and reduce misdiagnosis.²⁴

RDW in Ischemic Cerebrovascular Disease

A piece of evidence showed that the RDW value is increased in patients with stroke compared to non-stroke patients and is also a significant and independent predictor of both cardiovascular and all-cause mortality in stroke patients.²⁷ In addition, RDW is associated with the risk of stroke in patients with HF²⁸ and the general population in the community.²⁹ Kaya et al compared HF patients with and without stroke, they found that significantly increased basal RDW (16.9 ± 1.14 vs 14.6 ± 1.4 , $P < 0.001$). An RDW $\geq 15.2\%$ measured on admission had 87% sensitivity and 74% specificity in predicting stroke in patients with HF (AUC: 0.923, 95% CI: 0.852–0.994, $P < 0.001$).²⁸

A population-based study conducted involving 26,879 participants without a history of coronary events or stroke²⁹ showed that increased RDW was associated with a higher incidence of total stroke. Study participants grouped in the highest quartile of RDW showed a significantly higher incidence of stroke (HR, 1.31; 95% CI, 1.11–1.54). Ramírez et al³⁰ conducted a case–control study, found that RDW was higher in the stroke group as compared to controls 14.48 ± 1.76 vs 13.9 ± 1.43 , $P = 0.001$). Patients in the fourth quartile (RDW $>14.61\%$) were significantly more likely to have a stroke compared with the lower quartile [RDW $< 13.27\%$, odds ratio (OR) 4.50; 95% CI: 2.50–8.01, $P < 0.0001$]. In summary, these data suggest RDW may be a predictive marker in stroke.

RDW in Atrial Fibrillation

Likewise in patients with other CVDs, an increased RDW is an independent predictor of unfavorable clinical outcomes, such as all-cause mortality and the incidence of major adverse events in patients with AF.^{31–33} In brief, Wan et al³¹ followed 300 patients with AF and they found that those study subjects in the higher RDW quartile (RDW $\geq 14.4\%$) had a 13.77% increased risk of mortality as compared to the lowest quartile (RDW $< 12.8\%$, $P < 0.001$). In addition, RDW was independently associated with all-cause mortality and major adverse events (HR: 1.024; 95% CI: 1.012–1.036, $P < 0.001$) and (HR: 1.012; 95% CI: 1.002–1.023, $P = 0.023$), respectively. The analysis of receiver operating characteristics (ROC) showed that RDW predicted both mortality and major adverse events with AUC of 0.682 and 0.617; the best cutoff points were 13.85% with a sensitivity of 63.3% and a specificity of 70.4%. And 13.55%, with a sensitivity of 62.0% and a specificity of 60.6%, respectively.³¹

RDW in Coronary Artery Disease

Several studies have shown that the value of RDW is higher in patients with coronary artery disease.^{34,35} The study by Osadnik et al³⁴ analyzed 2550 consecutive patients with stable coronary artery disease (CAD). The patients were grouped by their RDW values at the lower quartile ($<13.1\%$) and higher quartile ($>14.1\%$) and followed for a mean of 2.5 years. During the follow-up, there was an almost fourfold increase (4.3% vs 17.1%, $P < 0.0001$) in mortality between the group. Interestingly, RDW remained significantly associated with mortality in the entire cohort (HR=1.23 [95% CI (1.13–1.35), $P < 0.0001$]) after adjusting for other variables. Additionally, Lapp et al³⁵ studied 1489 patients with CAD and 449 healthy controls. They reported that RDW strongly predicts all-cause mortality in patients with CAD and the control population (HR=1.37 per quintile; 95% CI=1.29, 1.46; $P < 0.001$) and (HR=1.33 per quintile, CI=1.15, 1.55; $P < 0.001$), respectively. However, highly sensitive C-reactive protein (hs-CRP) did not predict mortality in the control group. Thus, the association of RDW with mortality in patients with CAD may provide potentially useful prognostication.

RDW in Peripheral Artery Disease

Recent studies have shown that the value of RDW is increased in patients with peripheral arterial disease. Ye et al³⁶ employed an epidemiological study to investigate the prognostic role of RDW in patients with peripheral artery disease

(PAD). In this study, the authors reported that those patients in the upper quartile of RDW ($>14.5\%$) had a 66% higher risk of mortality compared to those in the lower quartile ($\text{RDW} < 12.8\%$; $P < 0.0001$). One percent increment in RDW was associated with a 10% higher risk of all-cause mortality (HR: 1.10; 95% CI, 1.08–1.12).

RDW in Hypertension

It has been reported that the value of RDW is increased in hypertensive patients. Tanindi et al³⁷ investigated the role of RDW in apparently healthy individuals ($n = 36$), prehypertensive patients ($n = 74$), and hypertensive patients ($n = 128$). Following adjusting for confounding variables, they found the mean value of RDW was 15.26 ± 0.82 in prehypertensive, 16.54 ± 0.91 in hypertensive, and 13.87 ± 0.94 in control groups ($P < 0.05$). Additionally, RDW showed a strongly positive and statistically significant correlation with systolic and diastolic blood pressures ($r = 0.848$ and $r = 0.748$, respectively, $P < 0.01$), independent of confounders such as age, inflammatory status, and anemia. Another comparative cross-sectional study conducted by Enawugaw et al³⁸ showed that RDW increased significantly in hypertensive groups compared to normotensive individuals ($P < 0.001$).

Pathophysiological Mechanisms

Despite the numerous clinical evidence available, that shows the increase in RDW as a potential biomarker in CVD to be taken into account in the coming years, the underlying pathophysiological mechanisms have not been studied and can generate strong attention in the medical and biomedical community. The main question we can ask ourselves is how a greater heterogeneity of RBC in the blood can directly cause CVD or if this greater heterogeneity is simply an epiphenomenon, secondary to other deleterious processes, previously activated in these pathologies. The existing hypothesis mainly includes the rheological properties of RBCs, microparticles derived from RBCs, anemia, oxidative stress, and inflammatory cytokines (Figure 1).

Rheological Properties of Red Blood Cells

Red blood cells play a major role in humans and are responsible for the transportation of oxygen and carbon dioxide between blood and tissues. The red blood cells perform this activity because of their ability to deform and flow in the vascular system. High deformability that allows red blood cells to flow through microcapillaries that are smaller in size than the cells themselves is attributed to several factors, including cell shape, the viscosity of the intracellular fluid, and rheological properties of the cell membrane.³⁹ There are many morphological abnormalities that affect the deformability and elasticity of RBCs including drepanocyte (sickle shape RBC), spherocyte and acanthocyte. Sickle cell anemia is a type of inherited hemolytic anemia due to hemoglobin S (Hgb S) variant. In the presence of Hgb S, the morphology of RBCs can be affected which results in less deformable sickle-shaped RBCs.⁴⁰ Cardiovascular diseases are common in sickle cell anemia, including cardiac enlargement, myocardial infarction, stroke, and pulmonary hypertension.⁴¹

Moreover, change in osmolality under some pathological and physiological conditions can affect the deformability of RBCs and microvascular blood flow and result in CVDs eventually.⁴² One study shows that RDW values above 14.0% were significantly associated with decreased RBC deformability, which can compromise blood flow through microcirculation.⁴³ From the perspective of hematology, the predictive role of osmolality in cardiovascular prognostication may be evaluated by the deformability of RBCs, which is assessed by the value of RDW and the resulting hypoxia may be used to explain the higher risk for CVD events related to higher RDW.

In addition, an increased cholesterol erythrocyte membrane level is another cause of decreased membrane fluidity and deterioration of erythrocyte deformability. This can affect the microcirculation, resulting in short survival of circulating erythrocyte, and higher RDW values. In recent studies, a strong and direct relationship was observed between the RDW value and the cholesterol content of erythrocyte membranes (CEM), and the CEM is also positively and independently associated with clinical instability in patients with CVDs.^{44,45}

The excess free cholesterol within the necrotic core of atherosclerotic plaque can be derived from different cellular sources, including RBCs.⁴⁶ The RBCs entrapped within the atherosclerotic plaque core and directly participate in the accelerated atherogenesis and CVDs through a variety of mechanisms.⁴⁷ First, the assembly of free and crystallized cholesterol originating from the RBC membrane promotes the expansion of the lipid core. Second, injury of RBCs

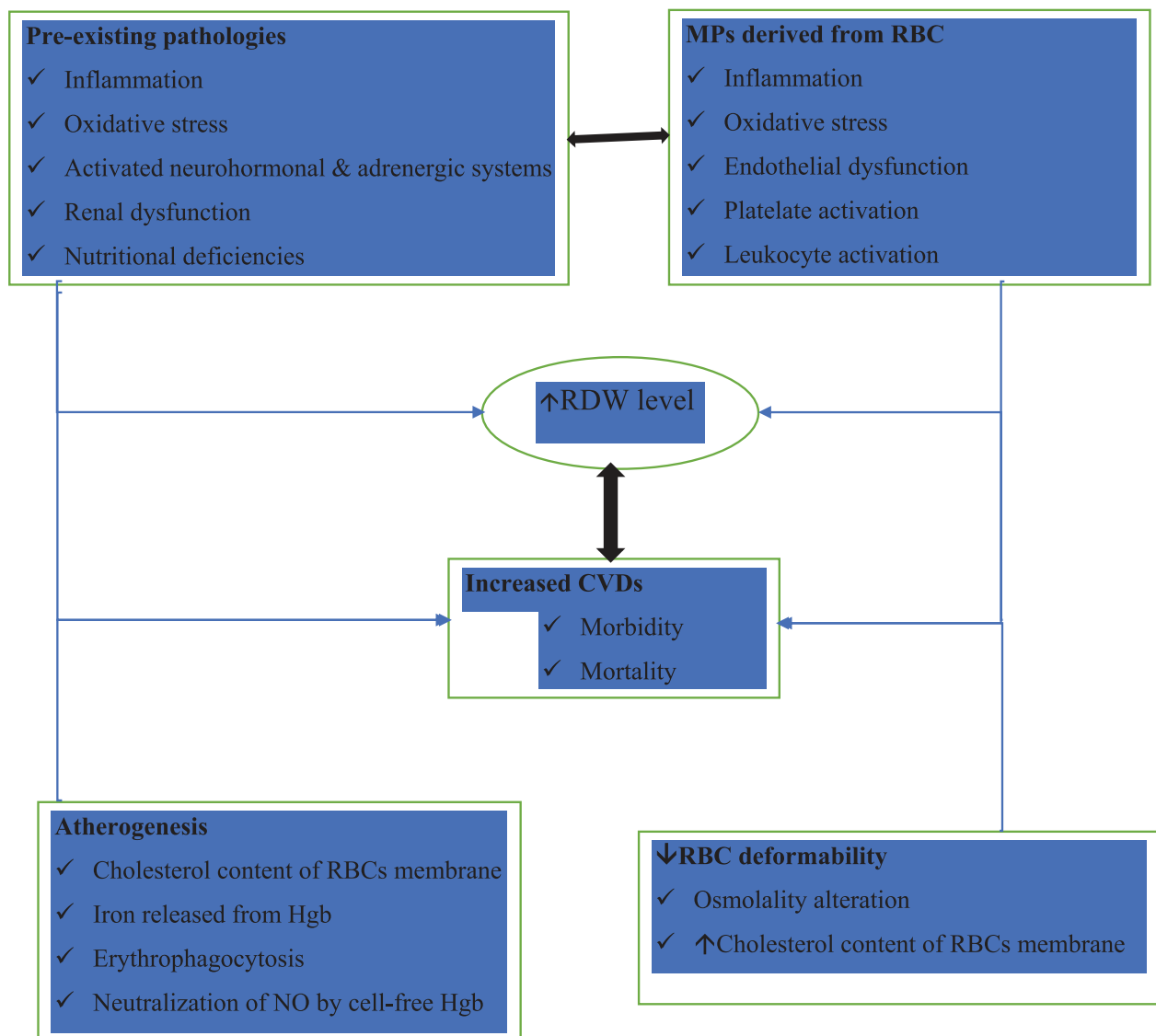


Figure 1 Hypothetical model that would explain the mechanisms for increase in RDW in patients with cardiovascular diseases. All these mechanisms as a whole and separately, would perpetuate deleterious processes that would spread and generate cardiovascular diseases by having a high RDW.

Abbreviations: CVDs, cardiovascular diseases; RBCs, red blood cells; RDW, red blood cell distribution width; Hgb, hemoglobin; NO, nitric oxide; ↓, decrease; ↑, increase.

within the atherosclerotic plaque results in release of iron found in the hemoglobin content that triggers the generation of reactive oxygen species, tissue injury, and activation of many pro-inflammatory cytokine pathways. Third, erythrophagocytosis mediated by the interaction of erythrocytes with scavenger receptors on macrophages and other phagocytes may also amplify the formation of foam cells and facilitate the excrescence of the atherosclerotic plaque. Fourth, the neutralization of nitric oxide by cell-free hemoglobin released from injured erythrocytes inside the necrotic core of the atherosclerotic plaque may also contribute to inhibiting endothelium-dependent nitric oxide-mediated vasodilation.^{1,47}

Microparticles Derived from Red Blood Cells

Microparticles (MPs) are active subcellular structures (0.1 to 1 μm), derived from the plasma membrane of different types of cells, mainly platelets, RBCs, monocytes, lymphocytes, neutrophils, and endothelial cells during the processes of cellular activation or apoptosis. Microparticles have various receptors and molecules inside, allowing them to activate

mainly negative processes in other cell groups such as endothelial cells, platelets, and white blood cells.⁴⁸ In comparison to healthy individuals, blood levels of MPs derived from RBCs are elevated in patients with CVDs; secondary to nutritional deficiency, pro-inflammatory, and oxidative states, where its excessive increase has been associated with different cardiovascular pathologies.⁴⁹ Several research groups have shown that the deleterious participation of MPs in CVDs would be mainly related to their high proinflammatory, prooxidant and prothrombotic potential.^{48–50} It is possible to propose that there are increased MPs derived from RBCs due to nutritional deficiency, pro-inflammatory and oxidative states, and these MPs once released into the blood can interact directly with processes and cells generating a greater proinflammatory, prothrombotic and oxidative state, which could increase the heterogeneity of RBCs, resulting in a higher RDW, which could be the potential factor in the pathogenesis and poor prognosis in patients with CVDs.

Anemia and Iron Metabolism

Anemia has been recognized as a well-documented risk factor for an increased mortality rate in patients with CVDs including chronic HF and ACS. The potential mechanisms for the development of anemia are multifactorial including inflammation, decreased production of erythropoietin, ineffective erythropoiesis, nutritional deficiencies, and comorbidities (chronic kidney disease, diabetes mellitus). Because many of these mechanisms can cause variation in the size of the erythrocyte, the association between these mechanisms and outcomes can be demonstrated by RDW in an integrated fashion.⁵¹ Most chronic anemias are associated with left ventricular hypertrophy and heart failure. Patients with chronic anemia and hemoglobin <10 g/dL show some hemodynamic compensatory responses like decreased systemic vascular resistance, increased cardiac output, water, and sodium retention, and low renal blood flow and GFR. These responses may cause an increased cardiac workload to maintain sufficient oxygen supply because of an increase in blood volume and consequent left ventricular remodeling.⁵²

Red blood cell distribution width is affected by iron metabolism. It is noted that impaired iron metabolism is characterized by the presence of higher RDW, low hemoglobin, low MCV, high erythropoietin, normal iron-binding capacity, and ferritin. It is understood that the ability to mobilize and use stored iron may be impaired even in the presence of sufficient total body iron. This condition is known as “reticuloendothelial block” mediated by overproduction of hepcidin (the master regulator of iron metabolism) in response to inflammatory cytokine (IL-6), which is strongly associated with higher RDW. Also, IL-6 increases the expression of ferritin and iron retention within macrophages, leading to a decreased iron level in the circulation and limited availability of iron for erythroid cells.⁵³ Studies indicated that increased baseline RDW and MCV has been associated with the formation of anemia during hospitalization in patients with AMI and with normal hemoglobin levels at admission.⁵⁴ Furthermore, increased RDW levels increase mortality in patients with cardiovascular disease regardless of the baseline hemoglobin value and RDW has a good prognostic role in non-anemic patients.³⁴

Oxidative Stress

Oxidative stress is a condition developed from an imbalance between the formation of free radicals and body antioxidant defenses, resulting in damage to macromolecules (nucleic acids, proteins, and lipids) and tissue injury.⁵⁵ It is commonplace in most chronic diseases such as cancer, diabetes mellitus, CVDs, inflammation, liver failure, and chronic kidney disease.⁵⁶ Oxidative stress exerts a profound effect on RBC's homeostasis and survival. Some studies show a close association between the increase in RDW and biomarkers of oxidative stress.^{57,58} In brief, Az et al showed that the addition of vitamin C (500 mg/day) to 22 soccer players under 25 years of age generated a decrease in RDW levels compared to another group that did not receive vitamin C.⁵⁷

Additionally, Semba et al have reported that a decrease in plasma levels of selenium and carotenoids (potent antioxidants in the blood) was associated with the increase in RDW in 786 women.⁵⁸ These studies allow the preliminary hypothesis that oxidative stress can generate changes in the size of the RBCs, increasing its heterogeneity and therefore the RDW. Moreover, smoking can result in increased oxidative stress and higher RDW is correlated with the number of cigarettes smoked per day and the duration of smoking.⁵⁹ In addition, it has been recognized that smoking is a well-known risk factor in the development of CVDs including myocardial infarction. Some reports showed a stronger relation

between RDW and myocardial infarction in smokers, which supports the hypothesis that RDW value can reflect inflammation conditions.⁶⁰

Inflammatory Cytokine

Some studies have reported a univariate and multivariate statistical relationship between the RDW with various established systemic inflammatory parameters.^{51,61} Lippi et al published for the first time in a cohort of 3845 subjects who were monitored with routine blood count for 3 years, that hsCRP predicted the increase in RDW levels regardless of age, gender, MCV, hemoglobin, and ferritin.⁶¹ Inflammation can increase RDW levels by altering iron metabolism, decreasing RBCs' survival, impairing RBC maturation, and causing immature RBC to enter the peripheral circulation. The production of erythrocytes in bone marrow can be inhibited by proinflammatory cytokines as a result of the blockage of erythroid progenitor cell proliferation and proerythroblast maturation.^{51,62}

The chronic inflammatory state is one of the roots of atherosclerosis and resultant complications including CVDs.⁶³ Cardiovascular disease is accompanied by increased circulating pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, which can affect erythropoiesis, reflected by an increase of RDW.⁶⁴ The process of such conditions mainly resulted from desensitizing erythroid progenitor cells to erythropoietin (EPO), blocking antiapoptotic and maturation effects of EPO, and decreased renal EPO synthesis by inflammatory cytokines. Additionally, the synthesis of EPO is lower in patients with heart failure and impaired kidney function.⁶⁴ An increased RDW value highlights the presence of immature RBC in the peripheral circulation, which could be due to reduced functional iron availability or inhibition in the synthesis or activity of EPO. So RDW may be a representative of an integrative indicator of different pathological processes in CVDs.

Conclusions and Future Directions

Currently, a piece of evidence that supports a higher degree of heterogeneity is common in patients with different CVDs such as stroke, PAD, hypertension, ACS, HF, and AF. A higher RDW value significantly and independently predicts a worse prognosis in patients with CVDs and the general population. As compared to other biochemical parameters in CVDs, RDW is an easily available biomarker as is a rapid and inexpensive test without requiring specialized skills. However, the specificity of RDW is not very high, its high sensitivity and correlation with other biomarkers like NT-proBNP, troponin, HCY, and CRP can support cardiologists in the diagnosis and management of CVDs. Generally, the scientific evidence available so far indicated that the role of RDW may be expanded beyond the routine boundaries of RBC dysfunctions, especially it helps in the diagnosis and prognosis of patients with CVDs. However, an interesting and yet unanswered question exists is the relationship between increased red cell distribution width and CVDs is causal or occurs as a result of the underlying pathophysiological state. As, the data reviewed in this manuscript are preliminary and observational in nature, they cannot investigate the underlying mechanisms. If RDW is considered as a new biomarker and risk factor, it must meet a series of requirements to be recognized such as being modified by the effect of therapies. Future studies should focus on investigating the most precise biological mechanisms that explain this increase seen in clinical practice, how to standardize its determination in a more unified way, and also the evaluation of the RDW and its potential capacity to be modified by the use of therapies.

Abbreviations

ACS, acute coronary syndrome; AF, atrial fibrillation; AMI, acute myocardial infarction; AUC, area under the curve; CAD, coronary artery disease; CBC, complete blood cell count; CEM, cholesterol content of erythrocyte membranes; CI, confidence interval; CVDs, cardiovascular diseases; EPO, erythropoietin; HCY, homocysteine; HF, heart failure; HR, hazard ratio; hsCRP, highly sensitive C-reactive protein; IL-1 β , interleukin 1 beta; IL-6, interleukin 6; MCV, mean corpuscular volume; MPs, microparticles; NO, nitric oxide; NT-proBNP; N-terminal pro-B-type natriuretic peptide; OR, odds ratio; PAD, peripheral artery disease; RBC, red blood cell; RDW, red blood cell distribution width; ROC, receiver operating characteristic; SD, standard deviation; TNF- α , tumor necrosis factor-alpha.

Compliance with Ethics Guideline

This review is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

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Low Rate of Clinically Evident Extravascular Hemolysis in Patients with Paroxysmal Nocturnal Hemoglobinuria Treated with a Complement C5 Inhibitor: Results from a Large, Multicenter, US Real-World Study

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Purpose: Most patients with paroxysmal nocturnal hemoglobinuria (PNH) treated with a complement protein 5 (C5) inhibitor achieve full control of terminal complement activity and intravascular hemolysis. The minority remains anemic and transfusion dependent despite this control. Etiology for ongoing anemia is multifactorial and includes bone marrow failure, breakthrough hemolysis, extravascular hemolysis (EVH) and nutritional deficiencies.

Patients and Methods: To evaluate the potential etiologies of hemoglobin levels <10 g/dL despite receiving C5 inhibitor therapy, we performed a retrospective US chart review of adult patients with PNH and treated for at least 12 months with eculizumab (n=53), ravulizumab (n=32), or eculizumab followed by ravulizumab (n=15). Clinically evident EVH was defined as at least one transfusion, reticulocyte count $\geq 120 \times 10^9/L$ and hemoglobin level ≤ 9.5 g/dL. Safety data were not collected. Mean treatment duration was 26.5 ± 17.2 months.

Results: Treatment with C5 inhibitors significantly improved hemoglobin, lactate dehydrogenase, and number of transfusions versus baseline. Among the patients with hemoglobin <10 g/dL during the last 6 months of treatment (n=38), one patient (eculizumab) had clinically evident EVH, and 10 patients had active concomitant bone marrow failure. Bone marrow failure was a major contributor to hemoglobin <10 g/dL and transfusion dependence; clinically evident EVH was uncommon.

Conclusion: A range of hematologic causes need to be considered when evaluating anemia in the presence of treatment with a C5 inhibitor.

Keywords: real-world, anemia, intravascular hemolysis, bone marrow failure

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired, hematologic disorder caused by somatic mutations in the *PIGA* gene in bone marrow stem cells, which disrupt glycosylphosphatidylinositol (GPI) biosynthesis.¹ In the absence of functional GPI, the terminal complement pathway becomes uncontrolled on the cell surface of erythrocytes, rendering them particularly susceptible to lysis by the membrane attack complex (C5b-9).¹⁻⁵ In addition, activity of the membrane attack complex on PNH white blood cells and platelets, as well as the accompanying release of complement protein (C) 5a, promotes increases in inflammatory mediators and a prothrombotic state.² In this context, uncontrolled terminal complement activation causes intravascular hemolysis (IVH), increased risk of thromboembolic events, organ damage, and eventually death.^{2,6,7}

First approved in 2007, eculizumab, a C5 inhibitor, has been utilized for the treatment of PNH for more than 15 years;⁸ it inhibits terminal complement pathway activation thereby preventing the formation of C5a and the membrane attack complex. Ravulizumab-cwvz (ravulizumab), the first long-acting C5 inhibitor, was approved in the USA in 2018 and has become the standard of care in countries where it is available.⁹ In large, phase 3, randomized controlled trials and in real-world studies, treatment with either C5 inhibitor has been shown to reduce IVH, thromboembolism, organ damage, and mortality.^{10–22}

However, some patients who receive ongoing treatment with a C5 inhibitor remain anemic and transfusion dependent. Etiologies for hemoglobin <10 g/dL include bone marrow failure, breakthrough hemolysis, extravascular hemolysis (EVH) and nutritional deficiencies in iron/folate. EVH is a mechanistic consequence of treatment with C5 inhibitors and is believed to be caused by ongoing C3 deposition on surviving yet defective red blood cells, which renders them susceptible to phagocytosis in the liver or spleen as they are no longer destroyed by IVH.^{23–28} As a result, lactate dehydrogenase (LDH) is not released in the blood and cannot be used to assess EVH. In eculizumab-treated patients, C3 deposition on red blood cells has been reported to occur in most patients to varying degrees.^{25,26} However, the clinical impact of C3 deposition remains unclear because the amount of C3 deposition does not correlate with levels of hemoglobin or transfusion dependence.^{25,29} The primary objective of this retrospective chart review was to assess the real-world incidence of clinically evident EVH or EVH requiring an intervention, in patients treated for PNH with eculizumab or ravulizumab.

Materials and Methods

In this retrospective, non-interventional study of community-based medical practices in the USA, 796 hematologists and oncologists who were part of the Cardinal Health Specialty Solutions Oncology Provider Extended Network were invited electronically to participate in a chart review of adult patients with diagnosed PNH and treated with a C5 inhibitor (eculizumab or ravulizumab) for at least 12 months. To minimize selection bias, providers were asked to identify all eligible patients starting with the first chronological patient meeting eligibility criteria and then randomly selecting up to 10 patients from that point forward. Chart data were entered by providers into electronic case report forms for analysis. The study was conducted in accordance with International Society for Pharmacoepidemiology Guidelines for Good Pharmacoepidemiology Practices and applicable regulatory requirements. A waiver from the central institutional review board was received prior to the start of the study. Patient-protected health information was kept confidential at all times and in accordance with the Health Insurance Portability and Accountability Act of 1996; no data that could directly identify patients were extracted. As such, patient informed consent was not needed. Providers were compensated at fair market value for the time needed to complete data abstraction and quality control procedures.

Patient records could be included if the patient was an adult who was being managed and treated by the provider and if the patient had a PNH diagnosis with a known percentage of GPI-deficient granulocytes or monocytes confirmed via flow cytometry. Treatment of PNH needed to have been ongoing for at least 12 consecutive months (unless deceased) and needed to have been initiated during the index period: between January 1, 2014 and November 1, 2019 for eculizumab and between December 21, 2018 and November 1, 2019 for ravulizumab. Ongoing treatment was defined based on the prescribing information with intervals between infusions of not more than 16 days for eculizumab and 63 days for ravulizumab.^{8,9} For patients who were switched from eculizumab to ravulizumab (the “switch” subgroup), eculizumab treatment needed to have been initiated between January 1, 2014 and November 1, 2019 and have been switched to ravulizumab between December 21, 2018 and May 1, 2020. Switch patients needed to have been treated with ravulizumab for at least 6 consecutive months. At least one routine clinic visit needed to have taken place during the last 6 months of eculizumab or ravulizumab therapy. In addition, laboratory values from at least three separate clinic visits (routine or acute) during treatment needed to be available. Records were excluded if the patient had been enrolled in a clinical trial investigating PNH treatment or if C5 inhibitor treatment was initiated prior to regulatory drug approval.

Laboratory results from hematological evaluations were analyzed at baseline and at follow-up. Baseline laboratory data were collected from tests performed within 14 days prior to the initiation of treatment with the C5 inhibitor (eculizumab in the switch subgroup). The follow-up period was defined as the window of time directly preceding data entry, treatment discontinuation, or patient death (Figure 1). For all variables other than transfusion, the follow-up period



Figure 1 Study design. In this retrospective US chart review of adults diagnosed with PNH, patients needed to have been treated with a C5 inhibitor (eculizumab or ravulizumab) for at least 12 consecutive months. For patients who were switched from eculizumab to ravulizumab, patients needed to have been treated with ravulizumab for at least 6 consecutive months. The follow-up period was defined as the window of time directly preceding data entry, treatment discontinuation, or patient death. For all variables except transfusion, the follow-up period was 6 months. Transfusion follow-up was assessed over 12 months in patients treated with eculizumab or ravulizumab only and over 6 months for patients in the switch subgroup. C5, complement protein C5; PNH, paroxysmal nocturnal hemoglobinuria.

was 6 months. Transfusion follow-up was assessed over 12 months in patients treated with eculizumab or ravulizumab only and over 6 months for patients in the switch subgroup.

Anemia was differentiated as hemoglobin levels <12 g/dL or <10 g/dL. Intravascular hemolysis was defined as LDH levels ≥ 1.5 times the upper limit of normal (ULN). Transfusion dependence was defined as at least one transfusion of packed red blood cells during follow-up. Breakthrough hemolysis (BTH) was defined as hemoglobin levels <10 g/dL and LDH elevation $>2 \times \text{ULN}$ when the previous measurement was $<1.5 \times \text{ULN}$.^{19,20} According to the protocol, BTH was evaluated only in eculizumab-treated patients. Clinically evident EVH was defined as at least one transfusion, reticulocyte count $\geq 120 \times 10^9/\text{L}$, and hemoglobin level ≤ 9.5 g/dL during follow-up and was evaluated in patients who had data for all three variables. Because no consensus definition for EVH has been established in guidelines,^{30–33} this definition was based on the phase 3 ALPHA trial that is assessing danicopan (factor D inhibitor) treatment in patients with clinically evident EVH while being treated with eculizumab or ravulizumab.³⁴ Concomitant active bone marrow failure was defined according to Camitta criteria as at least two of the following: hemoglobin level <10 g/dL, platelet count $<50 \times 10^9/\text{L}$, neutrophil count $<1.5 \times 10^9/\text{L}$.³² Reticulocytopenia was defined as a reticulocyte count $<60 \times 10^9$. Safety data, including treatment-related adverse events and cause of death (all-cause), were not collected. Data describing other reasons for anemia, such as folate, erythropoietin, and iron deficiency, were not collected.

This is a descriptive analysis of retrospectively collected patient-level data. Data were analyzed for the whole cohort as well as for each subgroup (eculizumab only, ravulizumab only, and switch). Categorical variables are presented as number of patients and percentage. Continuous variables are presented as means $\pm$ standard deviation. Where applicable, medians, interquartile range (IQR), and minimum–maximum range are presented. Baseline and follow-up laboratory values and the number of transfusions were compared using paired t-tests with a statistical significance set at $p \leq 0.05$. If multiple visits of the same type (routine or non-routine) occurred within the follow-up period, the arithmetic mean for the data point was taken. Sensitivity analyses for EVH were performed by varying the cut-offs for hemoglobin level (<10 g/dL) and absolute reticulocyte count ($\geq 100 \times 10^9/\text{L}$ and $\geq 150 \times 10^9/\text{L}$) and by removing transfusion as a variable from the definition. Because no a priori hypotheses were specified for this study, a sample size power calculation was not performed.

Results

A total of 18 physicians responded to the invitation; 15 of them submitted at least one electronic case report. Provider practices (11 community practices, two academic medical centers, two affiliated teaching hospitals) were located throughout the USA and in a range of settings (six urban, seven suburban, two rural). The mean number of patients with PNH was 8.1 ± 5.5 per physician in the year preceding this study (range 1–20).

Records for 100 patients were submitted and included 53 patients in the eculizumab subgroup, 32 patients in the ravulizumab subgroup, and 15 patients in the switch subgroup. The last day for data entry was February 22, 2021.

In the full cohort of 100 patients, 57.0% of patients were male (Table 1); mean ages at diagnosis and at C5 inhibitor treatment initiation were 54.3 ± 10.5 and 54.6 ± 10.6 years, respectively. The mean percentage of the GPI-deficient cells was $53.5 \pm 26.9\%$ for granulocytes and $54.1 \pm 26.8\%$ for monocytes. Fifteen percent of patients had a history of

Table I Demographics and Disease Characteristics Prior to the Initiation of Any C5 Inhibitor

	All N=100
Age at diagnosis (years)	
Mean $\pm$ SD	54.3 $\pm$ 10.5
Age at start of therapy (years)	
Mean $\pm$ SD	54.6 $\pm$ 10.6
Male sex, n (%)	57 (57.0%)
Race, n (%)	
White	68 (68.0%)
Asian	8 (8.0%)
Black/African American	23 (23.0%)
Other/not reported	1 (1.0%)
Ethnicity, n (%)	
Hispanic/Latino/Latina	7 (7.0%)
Non-Hispanic/Latino/Latina	93 (93.0%)
Comorbidities (>10% of patients), n (%)	
Hypertension	50 (50.0%)
Depression	19 (19.0%)
Diabetes	17 (17.0%)
Cardiovascular disease	15 (15.0%)
TE (arterial or venous)	15 (15.0%)
History of MDS or AA, n (%)	14 (14.0%)
MDS	7 (7.0%)
AA	7 (7.0%)
Active bone marrow failure*	
Yes, n (%)	24 (24.0%)
Data missing	3
Disease characteristics	
% GPI-deficient granulocytes	
Mean $\pm$ SD	53.5 $\pm$ 26.9
Median (IQR)	50.0 (35.0–77.3)
% GPI-deficient monocytes	
Mean $\pm$ SD	54.1 $\pm$ 26.8
Median (IQR)	53 (31.0–78.5)

(Continued)

Table I (Continued).

	All N=100
Data missing	1
% GPI-deficient total RBCs	
Mean $\pm$ SD	45.2 $\pm$ 27.1
Median (IQR)	40.6 (25.5–65.8)
Data missing	8
LDH, n (%)	
$\geq 1.5 \times \text{ULN}^\dagger$	76 (76.0%)
$< 1.5 \times \text{ULN}^\dagger$	20 (20.0%)
Data missing	4
Hemoglobin, n (%)	
< 6 g/dL	9 (9.0%)
6 to < 8 g/dL	57 (57.0%)
8 to < 10 g/dL	26 (26.0%)
10 to < 12 g/dL	4 (4.0%)
≥ 12 g/dL	1 (1.0%)
Data missing	3
Transfusion of pRBCs (at any time), n (%)	89 (89.0%)
Absolute reticulocyte count, n (%)	
$\geq 100 \times 10^9/\text{L}$	7 (7.0%)
$\geq 120 \times 10^9/\text{L}$	7 (7.0%)
$\geq 150 \times 10^9/\text{L}$	7 (7.0%)
Data missing	43
Coombs test, n (%)	
Positive	7 (7.0%)
C3+	5 (71.4%)
IgG+	2 (28.6%)
Negative	87 (87.0%)
Not performed	6 (6.0%)

Notes: For the switch subgroup, baseline was defined as prior to initiation of eculizumab treatment. *Defined according to Camitta criteria, ≥ 2 of the following criteria: hemoglobin level < 10 g/dL, platelet count $< 50 \times 10^9/\text{L}$, neutrophil count $< 1.5 \times 10^9/\text{L}$. $^\dagger 1.5 \times \text{ULN} = 369$ IU/L.

Abbreviations: AA, aplastic anemia; C3, complement protein 3; C5, complement protein 5; GPI, glycosylphosphatidylinositol; IgG, immunoglobulin G; IQR, interquartile range; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; pRBC, packed red blood cell; RBC, red blood cell; SD, standard deviation; TE, thromboembolic event; ULN, upper limit of normal.

thromboembolism and 14.0% had a documented history of bone marrow disease (7.0% aplastic anemia; 7.0% myelodysplastic syndrome).

Prior to initiating C5 inhibitor treatment, 60.0% of patients were taking medications to treat PNH or associated bone marrow failure. These included folic acid (41.0% of patients), steroids (26.0%), anticoagulants (17.0%), iron (15.0%), and immunosuppressive therapy (7.0%). Prior to initiating C5 inhibitor treatment, most patients were anemic (96.0%; hemoglobin level <12 g/dL; 92.0%; hemoglobin level <10 g/dL), had elevated LDH (76.0%; LDH level $\geq 1.5 \times \text{ULN}$), were transfusion dependent (89.0%), or had active bone marrow failure (24.0%; Camitta criteria; data missing in three patients). Eighty-seven percent of patients had a negative Coombs test at baseline (87/100; data missing for six patients). Of the seven patients with a documented positive Coombs test, five were C3+ and two were immunoglobulin G+. Baseline characteristics of patients by treatment subgroup are presented in [Supplemental Table 1](#).

In the full cohort, the most frequent reasons reported by the provider for initiating treatment with a C5 inhibitor ([Table 2](#)) were symptoms of hemolysis (86.0%), LDH level $\geq 1.5 \times \text{ULN}$ (83.0%), high percentage of GPI-deficient cells (75.0%), previous thromboembolic event (25.0%), and hemolytic crisis requiring hospitalization (19.0%). Mean duration of therapy (from initiation of C5 inhibitor to last encounter) was 26.5 ± 17.2 months (range 5.4–84.0). Treatment characteristics by treatment subgroup are presented in [Supplemental Table 2](#). In the switch subgroup (n=15), the most frequently reported reasons for switching from eculizumab to ravulizumab treatment were patient preference (n=11; 73.3%), reduced treatment frequency (n=10; 66.7%), and reduced time spent getting treatment (n=8; 53.3%).

Of the 100 patients included, 74 were actively receiving C5 inhibitor at the time of data entry. Among the 26 patients who were not receiving active treatment, 11 patients had died. The mean time from initiation of therapy until death for these patients was 15.0 months. The mean time from discontinuation of therapy to death was 0.3 months. Five patients were no longer taking therapy owing to allo-transplantation, 4 patients were alive with disease but not receiving active therapy or palliative care, 1 patient was categorized as having normal blood count, 1 patient had changed practices, and 4

Table 2 Treatment Characteristics

	All N=100
Reason for C5 inhibitor initiation^{*,†}, n (%)	
Symptoms of hemolysis	86 (86.0%)
LDH level $\geq 1.5 \times \text{ULN}^{\ddagger}$	83 (83.0%)
High GPI-deficient clone (any kind)	75 (75.0%)
Previous thromboembolic event	25 (25.0%)
Hemolytic crisis requiring hospitalization	19 (19.0%)
Prevent complications	3 (3.0%)
Proteinuria and CKD	1 (1.0%)
Prevent thrombosis	1 (1.0%)
Duration of C5 inhibitor therapy[§] (months)	
Mean $\pm$ SD	26.5 ± 17.2
Range (minimum–maximum)	5.4–84.0

Notes: ^{*}In the switch group, reason for initiating C5 inhibitor treatment was defined as the reason for starting eculizumab. [†]Multiple reasons possible. [‡] $1.5 \times \text{ULN} = 369 \text{ IU/L}$.

[§]Start of C5 inhibitor to last encounter. For patients in the switch subgroup, treatment duration was defined as the sum of the two treatment durations.

Abbreviations: C5, complement protein C5; CKD, chronic kidney disease; GPI, glycosylphosphatidylinositol; LDH, lactate dehydrogenase; SD, standard deviation; ULN, upper limit of normal.

patients were lost to follow-up/unknown. The mean time from discontinuation of therapy to last seen encounter for these patients was 12.3 months.

Compared with baseline, routine laboratory measurements collected during follow-up indicated improvement in hemoglobin ($p<0.001$), LDH ($p<0.001$), aspartate transaminase ($p<0.001$), alanine transaminase ($p<0.001$), bilirubin ($p<0.001$), indirect bilirubin ($p<0.001$), ferritin ($p=0.080$), D-dimers ($p=0.009$), platelet count ($p<0.001$), and number of transfusions ($p<0.001$) (Table 3). Results by treatment subgroup are presented in Supplemental Table 3.

During follow-up, 86 patients (86.0%; data missing for three patients) had an LDH level $<1.5\times\text{ULN}$. Among the 11 patients (11.0%) who had an LDH level $\geq 1.5\times\text{ULN}$, 10 patients were in the eculizumab subgroup and 1 patient was in the ravulizumab subgroup. Four patients in the eculizumab group (7.5%; 4/53) met the criteria for BTH. A Coombs test was performed during the last 6 months of follow-up in 46 patients. Results were negative in all patients. One patient who had a positive Coombs test at baseline was negative at follow-up (switch subgroup). Follow-up Coombs tests were not available for the six other patients with positive baseline tests. Reticulocyte counts were available in 58 patients. Eight of these patients had an absolute reticulocyte count $\geq 120\times 10^9/\text{L}$ (four in the eculizumab subgroup, three in the ravulizumab subgroup, one in the switch subgroup).

The percentage of patients who had hemoglobin levels $<12\text{ g/dL}$ decreased from 96.0% at baseline to 76.0% at follow-up; and the percentage of patients with hemoglobin levels $<10\text{ g/dL}$ decreased from 92.0% at baseline to 38.0% (Figure 2A). Hemoglobin levels by treatment subgroup are presented in Figure 2B–D.

Table 3 Change in Laboratory Values Between Baseline and Follow-Up (Routine Visits)

Laboratory Parameter	Baseline Mean	Follow-Up* Mean	Difference Mean	95% CI	p value [†]
Hemoglobin (g/dL), n=96	7.4	10.5	3.1	2.7, 3.5	<0.001
Absolute reticulocyte count ($\times 10^9/\text{L}$), n=51	91.6	203.5	111.9	−46.6, 270.4	0.162
Platelet count ($\times 10^9/\text{L}$), n=96	145.5	187.0	41.5	22.5, 60.4	<0.001
White blood cell count ($\times 10^9/\text{L}$), n=96	6.0	5.8	−0.2	−0.7, 0.4	0.560
Neutrophils ($\times 10^9/\text{L}$), n=72	3.4	2.9	−0.5	−1.0, 0.0	0.064
ALT (U/L), n=45	60.9	38.6	−22.3	−30.8, −13.7	<0.001
AST (U/L), n=45	61.6	40.7	−20.9	−30.3, −11.5	<0.001
Lactate dehydrogenase (IU/L), n=93	600.4	224.2	−376.2	−470.6, −281.8	<0.001
Bilirubin (mg/dL), n=93	3.1	1.4	−1.7	−2.0, −1.4	<0.001
Indirect bilirubin (mg/dL), n=32	2.9	1.1	−1.8	−2.2, −1.4	<0.001
Ferritin ($\mu\text{g/L}$), n=12	447.3	346.8	−100.6	−215.5, 14.3	0.080
D-dimers (ng/mL), n=8	601.3	173.2	−428.1	−710.8, −145.3	0.009
GPI-deficient granulocytes (%), n=11	47.2	33.2	−14.0	−32.4, 4.4	0.120
GPI-deficient monocytes (%), n=10	42.8	29.1	−13.8	−34.4, 6.9	0.166
GPI-deficient total RBCs (%), n=10	37.3	25.2	−12.1	−29.2, 5.0	0.145
Number of transfusions [‡] , n=100	3.1	1.0	−2.1	−2.7, −1.5	<0.001
Number of transfusions [‡] among patients with ≥ 1 transfusion at baseline and ≥ 1 transfusion during follow-up*, n=34	3.9	2.9	−1.0	−1.9, −0.1	0.032

Notes: *Follow-up was defined as the last 6 months of treatment for all variables except transfusion, which was assessed over 12 months in patients treated with eculizumab or ravulizumab only and over 6 months for patients in the switch subgroup. [†]Difference and p value calculated using a paired t-test. [‡]Transfusions of packed RBCs.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CI, confidence interval; GPI, glycosylphosphatidylinositol; RBC, red blood cell.

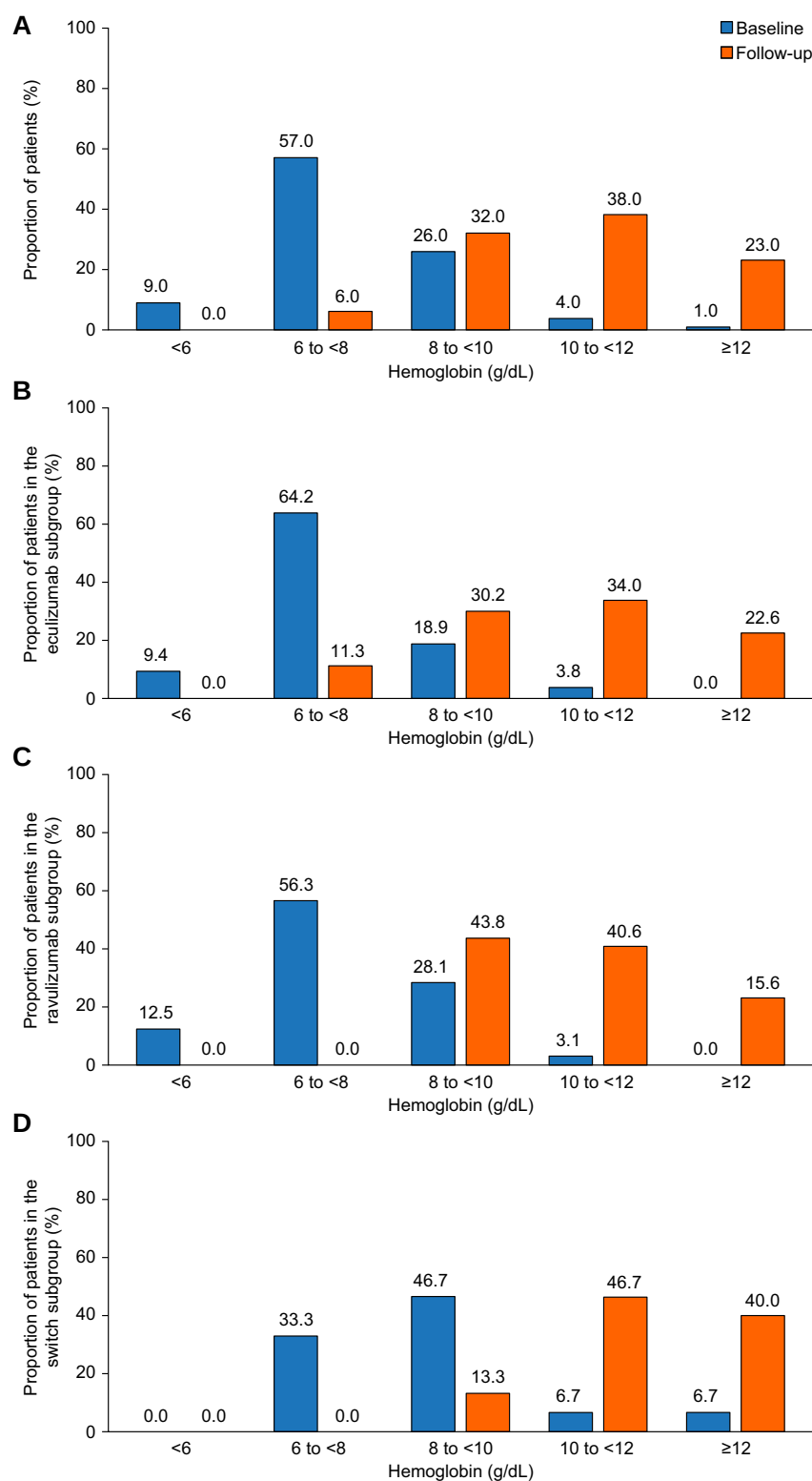


Figure 2 Improvement in hemoglobin levels with C5 inhibitor treatment. Percentage of patients by hemoglobin category at baseline and during follow-up (last 6 months of treatment with eculizumab or ravulizumab). Patients needed to have been treated with a C5 inhibitor (eculizumab or ravulizumab) for at least 12 consecutive months. For patients who were switched from eculizumab to ravulizumab, patients needed to have been treated with ravulizumab for at least 6 consecutive months. **(A)** All patients (N=100); data missing for three patients at baseline and one patient at follow-up. **(B)** Eculizumab subgroup (n=53); data missing for two patients at baseline and one patient at follow-up. **(C)** Ravulizumab subgroup (n=35). **(D)** Switch subgroup (n=15); data missing for one patient at baseline.

Among the 38 patients who had hemoglobin levels <10 g/dL during follow-up, 9 patients (23.7%) had an LDH level $\geq 1.5 \times \text{ULN}$ (all in the eculizumab subgroup) of whom 4 patients, as previously mentioned, met the BTH criteria. Ten out of 38 patients (26.3%) met the Camitta criteria; four patients had a hemoglobin level between 6 and <8 g/dL and six patients had a hemoglobin level between 8 g/dL and <10 g/dL. Only 3 of these 10 patients met the three criteria of having hemoglobin levels <10 g/dL, LDH level $\geq 1.5 \times \text{ULN}$ and the Camitta criteria; the remaining 7 patients had a hemoglobin level <10 g/dL and a neutrophil count $<1.5 \times 10^9/\text{L}$.

The percentage of patients who were transfusion dependent decreased from 89.0% at baseline to 35.0% during follow-up (24 patients [45.3%] in the eculizumab subgroup and 11 patients [34.4%] in the ravulizumab subgroup). Only one patient, who was not transfusion dependent at baseline, became transfusion dependent; this patient had neither aplastic anemia nor myelodysplastic syndrome. Among the 35 patients who were transfusion dependent during follow-up, 10 patients (28.6%) had an LDH level $\geq 1.5 \times \text{ULN}$ (all in the eculizumab subgroup) and 8 patients (22.9%) fulfilled Camitta criteria for active bone marrow failure. Among the 35 patients who were transfusion dependent during follow-up, mean reticulocyte count was $25.6 \pm 30.4 \times 10^9/\text{L}$ (median 13.9 [IQR: 8.5–33.9] $\times 10^9/\text{L}$) and was $<60 \times 10^9/\text{L}$ in 21 patients (60.0%; 21/35; data missing for 8 patients). Only 1 patient of 35 had a reticulocyte count $\geq 120 \times 10^9/\text{L}$; this patient had an LDH level $<1.5 \times \text{ULN}$. Coombs tests were either negative ($n=22$) or not performed ($n=13$).

Fifty-eight patients were evaluable for the presence of EVH. One patient met the criteria for EVH (eculizumab subgroup); this patient had an LDH level $<1.5 \times \text{ULN}$. In the sensitivity analyses, the number of patients with EVH increased to two (one patient in the eculizumab subgroup and one patient in the ravulizumab subgroup) when the requirement for transfusion was removed from the definition but did not change when the hemoglobin level cut-off was changed to <10 g/dL or when the reticulocytosis cut-off was changed to $\geq 100 \times 10^9/\text{L}$ or $\geq 150 \times 10^9/\text{L}$.

Discussion

We sought to evaluate the prevalence of anemia in a real-world, observational, medical chart review of 100 patients with PNH in the USA. Treatment with a C5 inhibitor improved anemia, transfusion dependence, and levels of LDH, platelets, and D-dimers. During follow-up (>6 months), LDH levels were $<1.5 \times \text{ULN}$ in 86.0% of patients. The percentage of patients with hemoglobin levels <10 g/dL decreased from 92.0% at baseline to 38.0% at follow-up; and the percentage of patients who were transfusion dependent decreased from 89.0% to 35.0%. Among the 35 patients who were transfusion dependent, 21 patients had a reticulocyte count $<60 \times 10^9/\text{L}$, 8 patients met Camitta criteria for bone marrow failure, and 1 patient met the criteria for clinically evident EVH. These results underscore the fact that in PNH, a range of hematologic causes needs to be considered when evaluating hemoglobin <10 g/dL and transfusion dependence in the setting of treatment with a C5 inhibitor.^{2,27}

In prior publications, small sample sizes and inconsistent definitions have resulted in the reported incidence of clinically significant EVH ranging from 0% if defined as positive direct Coombs test plus at least one transfusion plus no aplastic anemia,²⁶ to 12% if defined as C3+ red blood cells plus less than 50% reduction in transfusion dependence plus no aplastic anemia,²⁵ to 76% if defined as a positive direct antiglobulin test plus at least one transfusion.²⁴ Because there is no formal or agreed upon definition for EVH, we used the definition adopted in the danicopan (factor D inhibitor) phase 3 clinical trial that was specifically designed to evaluate treatment of the subpopulation of patients with PNH who develop EVH when treated with eculizumab or ravulizumab.³⁴ In the present study, only one patient (1.7%; 1/58 evaluable patients) met the definition for clinically evident EVH. In the sensitivity analysis, removing transfusion dependence as a criterion increased the number of patients with EVH to two, whereas varying the reticulocytosis and the hemoglobin cut-offs had no impact on results. Thus, although more research is needed, our study, which takes a broad survey approach, suggests that the incidence of clinically evident EVH is likely to be lower than originally suspected.

Extravascular hemolysis is a secondary mechanistic consequence of treatment with C5 inhibitors. Because red blood cells are no longer rapidly destroyed by the membrane attack complex, C3 fragment deposition on the cell membrane can occur and, potentially, opsonization and extravascular clearance.^{24,25,35} The clinical impact of C3 deposition on red blood cells in eculizumab-treated patients remains unclear as the percentage of C3 deposition does not correlate clearly with levels of hemoglobin or transfusion dependence.^{25,29} Indeed, measuring C3 deposition and assessing patients using a Coombs test has not been included in recent recommendations on how to monitor patients with PNH on a complement inhibitor.²⁷

Thrombosis is the leading cause of mortality in PNH.³⁶ Long-term studies have shown that effective terminal complement inhibition significantly reduces and controls the terminal complement activity that leads to IVH, thrombotic risk, and mortality.^{18,21,22,37} Interventional studies are assessing proximal inhibitors to determine whether blocking of the complement pathway upwards of C5 has positive consequences for those patients who may be experiencing clinically evident EVH. Danicopan, a factor D inhibitor, is being evaluated in a phase 3 trial as an add-on therapy for patients with PNH treated with eculizumab or ravulizumab. A combination of factor D and C5 inhibitors will target the proximal and terminal complement pathways, and may offer a strategy to reduce EVH while maintaining established clinical benefits conferred by terminal complement inhibition.³⁴ Recently, the findings of a 16-week phase 3 study (PEGASUS), which evaluated the efficacy of C3 inhibitor pegcetacoplan in a subset of eculizumab-treated patients who remained anemic and/or transfusion dependent, were published, demonstrating a significant improvement in hemoglobin level and resulting in US Food and Drug Administration approval.³⁸ It should be noted that patients enrolled in the PEGASUS study do not represent a broad population of patients with PNH and, as such, the conclusions drawn should be interpreted accordingly and cannot be extended to the full PNH population.^{39–41} Additional studies will be needed to evaluate the long-term safety and efficacy of proximal complement inhibitors.³⁸

Baseline characteristics prior to C5 inhibition suggest that the cohort of patients in this study was representative of patients encountered in real-world clinical practice. PNH disease burden was high: 96.0% of patients were anemic (hemoglobin level <12 g/dL), 76.0% of patients had an LDH level $\geq 1.5 \times \text{ULN}$, 89.0% of patients were transfusion dependent, while most patients did not have severe bone marrow failure (no patients had a platelet count below $45 \times 10^9/\text{L}$ at baseline). These data are consistent with previously published studies, which also reported high disease burden in untreated patients.⁴² Moreover, treatment response was consistent with that reported in published studies.^{15,19,37,43,44}

This real-world study was a retrospective chart review; therefore, accuracy and completeness of collected data was limited by the quality of data in the medical charts. In addition, this study also informs that prevailing standards of care and work-up in real-world settings may not always be met (eg missing absolute reticulocyte count data) which implies a need for ongoing education in the management of this disease. Absolute reticulocyte count during follow-up was not reported in 42.0% of patients. Because this variable informed the number of patients with EVH, EVH was assessed in 58 patients. In addition, our analysis focused on hematological parameters; other reasons for anemia, such as folate and relative erythropoietin deficiency, were not evaluated. As the analysis focused on laboratory results, data on thromboembolic events, treatment emergent adverse events, and cause of death were not collected. Lastly, because physician participation was self-selected and the randomness of patient selection was not monitored, there is a possibility of physician-introduced bias.

Conclusion

In this first, large, real-world study using clinical data in which eculizumab or ravulizumab improved hematologic parameters by reducing anemia, IVH, and transfusion dependence in patients with PNH, clinically evident EVH was uncommon (1.7%; 1/58). Concomitant bone marrow failure was identified as a major contributor to patients with PNH having hemoglobin <10 g/dL and transfusion dependence, and concomitant treatment may be required for these patients.⁴⁵ Altogether, these data underscore the fact that evaluating patients for all causes of anemia, including active bone marrow failure, is important for the comprehensive management of patients with PNH on a complement inhibitor therapy.

Abbreviations

BTH, breakthrough hemolysis; C, complement protein; EVH, extravascular hemolysis; GPI, glycosylphosphatidylinositol; IVH, intravascular hemolysis; IQR, interquartile range; LDH, lactate dehydrogenase; PNH, paroxysmal nocturnal hemoglobinuria; ULN, upper limit of normal.

Data Sharing Statement

Alexion will consider requests for disclosure of clinical study participant-level data provided that participant privacy is assured through methods like data de-identification, pseudonymization, or anonymization (as required by applicable law), and if such disclosure was included in the relevant study informed consent form or similar documentation. Qualified

academic investigators may request participant-level clinical data and supporting documents (statistical analysis plan and protocol) pertaining to Alexion-sponsored studies. Further details regarding data availability and instructions for requesting information are available in the Alexion Clinical Trials Disclosure and Transparency Policy at <https://alexionclinicaltrials.com/Disclosure-and-Transparency-Policy>. Link to Data Request Form (<https://alexion.com/contact-alexion/medical-information>).

Ethics Approval and Consent to Participate

The study was conducted in accordance with International Society for Pharmacoepidemiology Guidelines for Good Pharmacoepidemiology Practices and applicable regulatory requirements. A waiver from the WCG Institutional Review Board under Privacy Rule: 45 CFR 164.512 was received prior to the start of the study. Patient-protected health information was kept confidential at all times and in accordance with the Health Insurance Portability and Accountability Act of 1996; no data that could directly identify patients were extracted. As such, patient informed consent was not needed. Providers were compensated at fair market value for the time needed to complete data abstraction and quality control procedures.

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Author Contributions

All authors made a significant contribution to the work reported, have substantially revised and critically reviewed the article; have agreed on the journal to which the article has been submitted, have reviewed and agreed all versions of the article before submission and publication, agree to be accountable and take responsibility for the contents of the article.

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Disclosure

JS has received research support from Alexion, AstraZeneca Rare Disease, BMS, CTI, Novartis, Kartos Pharma and Stemline Therapeutics; consulting fees from Novartis; has been a speaker for Incyte, Alexion, BMS and Sanofi. She also owns stocks from Abbvie, chairs the Data and Safety Monitoring Board (DSMB) for NS Pharma and is a member of the DSMB for Apellis. AG and JK are employees of Cardinal Health Inc. YP, IT, AH and JRS are employees and shareholders of Alexion, AstraZeneca Rare Disease. DA provides consulting to Alexion, AstraZeneca Rare Disease. The authors report no other conflicts of interest in this work.

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Analysis of JAK2V617F Tyrosine Kinase Mutation in Blood Donors with Erythrocytosis – A Pilot Study in a Tertiary Care Teaching Hospital of South India

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Purpose: Transfusion services and blood centers provide immediate medical evaluation to blood donors by physical examination and hemoglobin (Hb) screening. Screening for Hb value before every blood donation is mainly aimed to rule out anemia. However, it is not uncommon to defer the donors for high Hb value which can be due to primary or secondary polycythemia. This study aimed to analyze the frequency of JAK2V617F mutation among blood donors with a high Hb of >18 g/dl.

Patients and Methods: A prospective study was conducted over a period of 18 months involving blood donors with a persistently high Hb value of >18 g/dl. Complete blood count (CBC), JAK2V617F gene mutation and Serum Erythropoietin (EPO) levels in study donors were analyzed. Descriptive statistical analysis was performed using SPSS, version 24 (IBM, USA).

Results: Of 13,798 screened donors, 48 donors (0.34%) had persistent erythrocytosis with a high Hb value of >18 g/dl. Their age ranged between 20–50 years with a mean of 31.2 ± 6.66 . The CBC parameters including red blood cell (RBC) count, Hb%, hematocrit (Hct), white blood cell (WBC) count and the platelet count ranged from $4.35\text{--}8.43$ million/ μL (6.2 ± 0.6), $18.6\text{--}24.4$ g/dl (19 ± 0.94), $51.9\text{--}83.3\%$ (58 ± 5.02), $3.99\text{--}10.8 \times 10^3/\mu\text{L}$ (7.8 ± 1.5), and $120\text{--}450 \times 10^3/\mu\text{L}$ (227 ± 57.2), respectively. Estimated mean EPO value was 8.29 mIU/ ± 0.04 . JAK2V617F mutation was detected in 2 donors (4.1%).

Conclusion: The prevalence of persistent erythrocytosis among blood donors was 0.34% and among them, two donors (4.1%) harbored the JAK2V617F mutation. Thus, blood centers play an important role in the primary screening of donors with high hemoglobin leading to early detection and management of polycythemia vera (PV).

Keywords: blood donors, high hemoglobin, erythropoietin, polycythemia vera

Introduction

Blood donation is an important part of healthcare all over the world and is a life-sustaining and life-saving procedure. More than one hundred million units of blood are donated each year all over the world.¹ Blood donors have to undergo their hemoglobin (Hb) values being checked before every blood donation to rule out anemia, which is the most frequent cause of a temporary deferral. However, it is not uncommon to observe Hb values above or near the upper limit of the normal reference range.²

Erythrocytosis is defined as an increase in the number of erythrocytes in the whole blood. Erythrocytosis refers to a red blood cell (RBC) count above the sex-specific normal range.³ It is suspected when the individual presents with a Hb >16.5 g/dl in men and >16.0 g/dl in women or hematocrit (Hct) >49% in men and >48% in women or an increase in red cell mass (RCM) of >25% above the mean predicted value.⁴ Erythrocytosis and polycythemia are frequently used synonymously. The term polycythemia refers to a group of disorders, typically characterized by persistent elevation in packed cell volume (PCV) due to an increase in the number of RBCs in the peripheral blood circulation.⁵

Erythrocytosis can be classified into relative or absolute. Relative erythrocytosis occurs due to a decrease in the plasma volume and absolute erythrocytosis is due to an increase in RCM.⁶ The etiology for an absolute erythrocytosis can be further subdivided into primary (low erythropoietin) and secondary causes (high erythropoietin). Primary causes are due to intrinsic problems in the bone marrow which leads to an increase in erythroid production. The primary causes can be further subdivided into congenital and acquired. Secondary causes are due to physiological or pathological processes, outside the bone marrow which leads to the accelerated process of erythropoiesis by means of increased erythropoietin secretion; these conditions can also be further subdivided into congenital and acquired.⁷

High Hb may be a sign of an underlying disease and can pose a risk for vascular accidents and investigations must be performed to rule out the primary disease.⁸ Studies suggest that some blood donors with erythrocytosis may have hidden pathologic conditions like polycythemia vera (PV). Studies have shown that the JAK2V617F mutation has been detected in about 65–97% of patients with PV.⁵ Based on these findings, the first step in the algorithm proposed for diagnosing PV in cases with erythrocytosis is the screening for the JAK2V617F mutation.² Thus, this study aimed to analyze the frequency of the JAK2V617F mutation among blood donors with high Hb >18 g/dl. The study will contribute to the screening of blood donors, to detect or diagnose the medical problems associated with high Hb values. Since there is a paucity of data available on blood donors with erythrocytosis in India, it is hoped that the results of this study can be used as one of the referral data for future research.

Materials and Methods

Study Design and Setting

A prospective exploratory study to analyze the JAK2V617F mutation among blood donors with high Hb was conducted in the department of Transfusion Medicine and Blood Center of an 1800 bedded university teaching hospital of southern India between November 2019 to May 2021 (18 months). The study was reviewed and approved by the institutional ethical committee of JSS Medical College, JSS Academy of Higher Education and Research in accordance with the Declaration of Helsinki. (Reference:- IEC-419/2019). Written informed consent was obtained from all individual participants included in the study.

Inclusion and Exclusion Criteria

Blood donors with persistent erythrocytosis of Hb >18 g/dl were included in the study. Donors with erythrocytosis who did not have Hb >18 g/dl following repeat blood count after 3 months or those who did not follow up were excluded from the study.

Study Methods

All the donors coming to the blood center during the study period were subjected to pre-donation counseling and screening tests for fitness as per the departmental standard operating procedure (SOP) for donor selection. This SOP is written with reference to the Drugs and Cosmetics Act (the rules thereunder) and supplemented by the Directorate General of Health Services and National AIDS (Acquired Immune Deficiency Syndrome) Control Organization (NACO) guidelines, including their amendments.^{7,9} During the screening process the donors were evaluated by entering details in donor registration cum consent forms which included a questionnaire, a brief physical examination by a medical officer followed by Hb estimation. Hb estimation was performed by an automated hematology analyzer (Sysmex XN-1000) and recorded.

As per the SOP of donor deferral, donors with a high Hb value (>18 g/dl) were temporarily deferred and counselled. A detailed history was taken from donors with emphasis on their history of smoking, alcohol consumption and travel to high altitude. All donors deferred due to high Hb were followed up for repeat complete blood count (CBC) after 3 months. During the follow up, donors with Hb >18 g/dl were enrolled in the study (Figure 1) and written informed consent was obtained from the donors by the investigators. Venous blood samples (3 mL in ethylenediamine tetraacetic acid (EDTA) vacutainer and 3 mL in plain vacutainer) were collected for the JAK2V617F mutation study and erythropoietin (EPO) assay from the donors meeting the inclusion criteria.

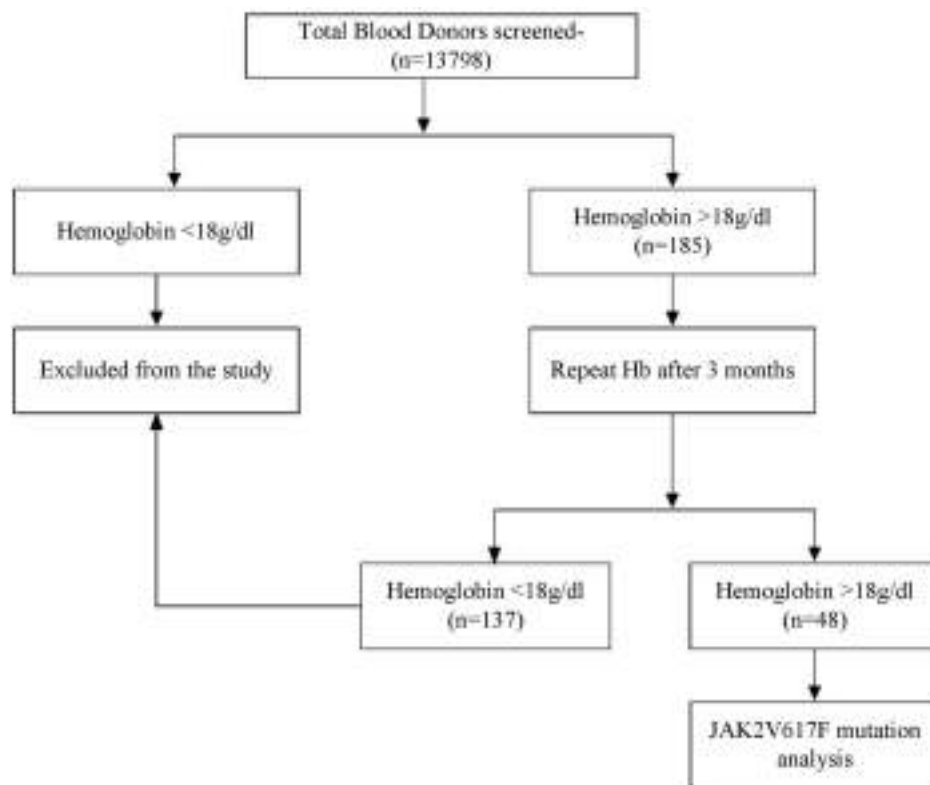


Figure 1 Algorithm showing the recruitment of study population.

The EDTA samples from the donors were centrifuged and the buffy coat was separated. Deoxyribonucleic acid (DNA) was extracted from the buffy coat sample using the DNA extraction kit manufactured by SpinStar Total DNA kit (ADT Biotech Sdn Bhd, Selangor DE, Malaysia) as per the kit instructions and stored at -80°C till further use. A blood sample from a known PV patient with JAK2V617F mutation was selected as a positive control for the RT-PCR study. Serum was separated by centrifugation of clotted blood sample and stored at -80°C for EPO estimation. The donor DNA samples were subjected to JAK2V617F mutation testing by the OncoscreenT JAK2V617F Mutation Detection Kit from the manufacturer Mylab Discovery Solutions (Maharashtra, India). EPO level was detected using the ELISA method from the manufacturer Biomerica (California, United States of America) according to the kit instructions. The EPO normal range was taken to be 3.22–31.9 mIU/mL. The CBC of randomly selected 200 donors not meeting the inclusion criteria were analyzed as controls.

Statistical Analysis

The obtained data was entered into MS Excel. SPSS computer program version 24 was used for statistical analysis. Descriptive statistical analysis was used for frequency of the CBC parameters of donors with persistent erythrocytosis. Independent sample *t*-test was used to compare the CBC parameters between patients with a sustained erythrocytosis and randomly selected normal donors. A *p*-value of less than 0.05 was considered statistically significant.

Results

A total of 13,798 blood donors were screened during the study period and among them a majority (98.8%) were men. Of the screened donors, 962 (6.9%) donors were deferred from donating blood for various reasons like low Hb, high Hb, consumption of alcohol, hypertension and medical reasons like cold, fever, dengue, asthma in decreasing order of frequency. High hemoglobin value ($\text{Hb} > 18 \text{ g/dl}$) was noted in 185 (1.3%) deferred donors and a majority of them (99.4%) were male donors. Among 185 donors, their history of smoking, alcohol intake and decreased intake of water

Table 1 Descriptive Statistics of Donors with Persistent Erythrocytosis

n = 48	Range	Mean $\pm$ SD
Age in years	20–50	31.2 $\pm$ 6.66
RBC count in millions/microliter	4.35–8.43	6.2 $\pm$ 0.6
Hb value in g/dl	18.6–24.4	19 $\pm$ 0.94
PCV in %	51.9–83.3	58 $\pm$ 5.02
MCV in fl	85.1–126	94.8 $\pm$ 7.94
MCH in pg	26–41.8	30.9 $\pm$ 2.4
MCHC in g/dl	29.2–35.5	32.8 $\pm$ 1.2
WBC count in $\times 10^3/\mu\text{L}$	3.99–10.8	7.8 $\pm$ 1.5
Platelet count in $\times 10^3/\mu\text{L}$	120–450	227 $\pm$ 57.2

throughout the day was noted in 27%, 25%, and 13% respectively. None of the donors had a history of heart or respiratory diseases, abnormal bleeding tendencies, sleep disorders or any drug intake.

Among the 185 deferred donors, 48 (25.9%) had persistent erythrocytosis on follow up and their samples were analyzed for the JAK2V617F mutation. The descriptive statistics of the 48 subjects with persistent erythrocytosis is shown in Table 1. Of the 48 cases, two cases (4.1%) harbored the JAK2V617F mutation (Figure 2). Their serum EPO levels ranged from 1.29–22.46 mIU/mL and the mean EPO was 8.75 mIU/mL with a standard deviation (SD) of 2.67. Comparison of hematological parameters between donors with persistent erythrocytosis (48 cases) and randomly selected healthy donors showed statistically significant differences in Hb, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet count values (Table 2). The details of the two donors with JAK2V617F mutation are as shown in the table (Table 3).

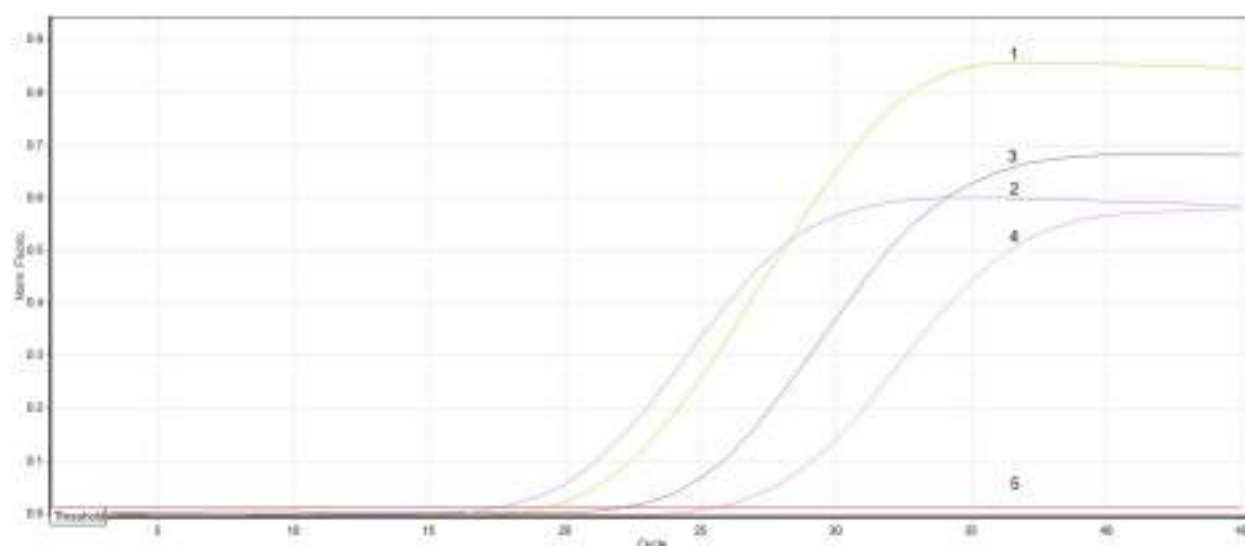


Figure 2 Detection of the JAK2V617F mutation in blood donors with persistent erythrocytosis by RT-PCR method. Amplification plot 1 corresponds to JAK2V617F mutant positive control provided in the kit. Amplification plot 2 and 3 corresponds to positive JAK2V617F mutation in the donors studied. Amplification plot 4 represents the positive JAK2V617F mutation in a known case of PV. Amplification 5 represents negative JAK2V617F mutation in the donors studied.

Table 2 Complete Blood Count Parameters of Donors with Persistent Erythrocytosis and Healthy Blood Donors

	Donors with persistent erythrocytosis	p-value	Healthy blood donors
	n = 48 (mean ± SD)		n = 200 (mean ± SD)
Age in years	31.2 ± 6.66	0.079	29.2 ± 7.3
RBC count in million/mcl	7.89 ± 1.5	0.746	7.8 ± 3.4
Hb in g/dl	19 ± 0.94	<0.001	16.1 ± 3.2
PCV in %	58 ± 5.02	<0.001	49 ± 2.9
MCV in fl	94.8 ± 7.86	<0.001	87 ± 8
MCH in pg	30.9 ± 2.4	<0.001	29.4 ± 2.3
MCHC in g/dl	32.8 ± 1.2	<0.001	33.5 ± 2.3
WBC Count × 10 ³ /μL	7.8 ± 1.5	0.477	8.2 ± 2.8
Platelet Count × 10 ³ /μL	227 ± 57.2	<0.001	275 ± 61.8

Table 3 Details of Donors with Positive JAK2V617F Mutation

	Case 1	Case 2
Age in years	36	24
RBC count in million/mcl	5.91	5.63
Hb in g/dl	18.7	20.2
PCV in %	57.9	57
WBC Count × 10 ³ /μL	6.0	9.35
Platelet Count × 10 ³ /μL	193	222
EPO level	8.76 mIU/mL	11.37 mIU/mL

Discussion

Donor screening at blood centers is conducted in accordance with the Drug and Cosmetic Act (D&C act) regulations, as well as national standards, to protect the safety of the donor, and to ensure the safety, purity and potency of the blood collection.⁹ Strict donor selection and deferral criteria are followed at the time of screening. The deferral rate of blood donors during pre-donation screening in the study period was estimated as 6.9%. Studies on donor deferrals conducted all over the world showed a deferral rate ranging from 5.19–35.6%.¹⁰ The blood donors during pre-donation screening were deferred due to various reasons and the most common cause for the donor deferral is low Hb as noted in various studies.^{11–14} A high percentage of donor deferrals are due to high Hb (0.5%) as noted in our previous study, which prompted us to study the JAK2V617F mutation among the blood donors with high Hct levels to diagnose the underlying disease.¹¹

With the change in the criteria to diagnose PV as per “The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms” the two important major criteria are high Hb value (Hb of >16.5 g/dl in men and Hb >16.0 g/dl in women) and presence of the JAK2V617F mutation.⁴ As per the guidelines bone marrow biopsy may not

be required in cases with sustained absolute erythrocytosis of Hb levels of 18.5 g/dl in men or 16.5 g/dl in women.⁴ As per the institutional SOP, the Hb cut off of more than 18 g/dl was the criteria to defer the donors. Accordingly, 1.3% of the total screened donors were deferred due to high Hb (>18 g/dl). The donor deferral rate due to high Hb in the present study was comparable to the studies done by Tagariello et al² (0.9%), Hatami et al¹⁵ (1.23%) and Al-Nuri et al¹⁶ (1.6%). In contrast to our study, studies done by Arslan et al¹⁷ (0.4%), Kandasamy et al¹² (0.39%), and by Magnussen et al¹⁸ (0.1%) had a lower rate of donor deferrals due to High Hb. Studies by Kamaruzzaman et al⁵ (7.8%) and Birjandi et al¹⁹ (2.4%) had a higher deferral rate due to a high Hb value. Although there is a precise cut-off for low Hb value (<12.5 g/dl), as per WHO and American Association of Blood Banking (AABB) for donor deferral, there is a lack of precise upper limit cut-off for Hb. Every blood center has to set its own ranges for blood donation parameters.²⁰ The wide variation in donor deferral due to high Hb can be attributed to the different methods used in the estimation of Hb value. Many blood centers in India use the copper sulfate method for Hb detection which does not have the competency to distinguish between normal and high Hb levels.¹² Hence use of the automated hematology analyzers which gives an accurate Hb value has to be encouraged in blood centers.

It is important to distinguish between PV and secondary polycythemia which is a complicated task due to various clinical profiles, evolution of the disease, and therapeutic implications.²¹ PV should be worked up by subjecting the patients to JAK2V617F mutation.²² JAK2V617F mutation is one of the major criteria as per “The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms”.⁴ In the present study, persistent erythrocytosis was noted in 48 donors and among them 2 cases (4.1%) tested positive for the JAK2V617F mutation. The prevalence of JAK2V617F mutation in donors with a Hct level above the upper limit of normal was estimated to be 1% and 2.1% in the studies performed by Tagariello et al,² and Magnussen et al,¹⁸ respectively. Kamaruzzaman et al⁵ did not find any donors positive for mutation test among the donors with erythrocytosis. Rubaie et al²³ noted a higher prevalence of 21.3% blood donors positive for JAK2V617F mutation.

The JAK2V617F negative cases also have to be evaluated extensively for causes of secondary erythrocytosis. One of the common causes of relative erythrocytosis is dehydration due to decreased intake of water or hot weather.⁵ Most of our blood donors claimed adequate intake of water, but this may not be true because India is a tropical country with a high daytime temperature, during which most of the blood donors were screened. Patients, who do not fall into the category of PV or secondary polycythemia, are grouped into idiopathic erythrocytosis, which is a diagnosis of exclusion and a substantial number of cases fall into this category.²⁴

In the present study, the 2 cases with JAK2V617F mutation had serum EPO levels in the normal range. Subnormal EPO is a minor criterion in the diagnosis of PV.⁴ Studies have documented subnormal EPO levels in patients with PV, but a low EPO level is not pathognomonic of PV.²⁵ Normal EPO levels were estimated in 20% and 32% of polycythemia patients by Vannucchi et al²⁶ and Lupak et al²⁷ respectively. Thus, the normal serum EPO level does not exclude PV and a subnormal serum erythropoietin level is not specific for the diagnosis of PV.^{26,27}

Blood donors with erythrocytosis have to be subjected to further investigations after ruling out causes of secondary polycythemia. PV has to be ruled out in donors with persistent erythrocytosis. Diagnosing PV via JAK2V617F mutation testing directs the donors in need to access proper treatment before the onset of thrombosis and other complications which can decrease their lifespan.

Limitations

The sample size was small hence results must be validated in a larger scale study. Exon 12 mutation analysis could not be performed; hence we could not rule out PV in the 46 cases. Bone marrow examination could not be performed on the JAK2V67F mutation-positive cases.

Conclusion

Blood donation is a valuable and indispensable component saving millions of lives all over the world. Hematological evaluation of all blood donors is a mandatory process in blood centers to ensure donor safety. Donors with persistent polycythemia could have hidden pathologic conditions like PV. These donors should be thoroughly evaluated and tested for JAK2V617F mutation along with serum EPO levels and bone marrow examination. The prevalence of persistent

erythrocytosis among blood donors was 0.34% and among them, two donors (4.1%) harbored JAK2V617F mutation. This study highlights the role of the blood centers in the identification of potential PV cases, refer them for further evaluation and thus play an important role in advancing preventive medicine.

Disclosure

The authors report no conflicts of interest in this work.

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Haematological Traits in Symptomatic and Asymptomatic COVID-19 Positive Patients for Predicting Severity and Hospitalization

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Haematological Traits in Symptomatic and Asymptomatic COVID-19 Positive Patients for Predicting Severity and Hospitalization

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Objective: The present investigation aims on the clinical attributes and haematological parameters between symptomatic (COVID-19 ICU) and asymptomatic (COVID-19 home isolation) patients as predisposing sign for COVID-19 related mortality.

Materials and Methods: A retrospective cohort research was conducted of admitted patients to ICU, who were suffering from severe COVID-19 in Aseer Central Hospital, Abha, Kingdom of Saudi Arabia (KSA) from July 2020 until September 2020. The study included individuals with COVID-19 and ICU admission as symptomatic group and others who are COVID-19 positives with quarantine as asymptomatic group. Epidemiological, clinical and haematological laboratory data were retrospectively collected, analysed with control subjects.

Results: Of the 38 ICU patients studied, the most common symptoms were fever and respiratory distress (100%), cough (86.8%). Majority were of Saudi origin (78.9%). Eighteen (47.4%) COVID-19 ICU patients showed leukocytosis, 6 (15.8%) had severe thrombocytopenia (with most having thrombocytopenia), 18 (47.4%) were anaemic. A significant correlation was observed between the WBC, RBC, Hb, platelets, neutrophil and lymphocyte count between ICU inmates compared with quarantine ($p < 0.001$) and RBC, Hb, neutrophil and lymphocyte count with control groups ($p < 0.001$).

Conclusion: From the observations it is evident that, the blood tests have potential clinical value in predicting COVID-19 progression. Further, patient characteristics including age, leukocyte count, RBC, platelets and differential leukocyte counts may be significant predictors for monitoring the progression of the critical illness observed in SARS-CoV-2 patients. Also, treatment procedures can be re-defined further to reduce COVID-19 mortalities in more critically ill COVID-19 individuals.

Keywords: COVID-19, blood test, ICU, quarantine, leukocyte count, erythrocyte count, platelet count

Introduction

A pandemic of coronavirus disease 2019 (COVID-19) is a viral infection which cause severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) with a variety of disease presentation and severity.¹ COVID-19 has affected almost the whole world, with death of approximately more than 6.2 million worldwide and 500 million cases, since that fateful December month of 2019, and more than 9000 deaths and more than 7.5 lakh cases have been recorded in KSA.² Epidemiological researches have depicted that 6–10% of COVID-19 affected patients progress to a more critical stage and will be in dire need of admittance to the intensive care unit (ICU) on account of acute respiratory.³ Invasive ventilation is usually required by most of these ICU admitted patients, due mostly to intensive lung injury and acute respiratory distress syndrome (ARDS).⁴ Till now, most of these patients on invasive mechanical ventilation (MV) have

depicted an increased mortality pattern.⁵ Most ICU harbouring these COVID-19 patients have shown a mortality rate of 50–65% range.^{6–8} Some ICU keeping COVID-19 patients on MV have reported a mortality rate as high as 97%.⁹

Many studies have shown a significant alteration in blood investigations associated with SARS-CoV-2 as well as the disease outcomes.^{10–13} In addition, very few studies provide correlation between peripheral blood WBC morphologic changes and disease outcomes.¹⁴ Another study demonstrated the quantitative and qualitative of the peripheral blood changes in COVID-19 cases which showed reduction in T cell count.¹⁵

In many studies, COVID-19 has shown a link with inflammation and thrombosis. It has been shown that serious cases had thrombotic complications in ICU COVID-19 patients. Another study showed the association between C-reactive protein (CRP) and infection with COVID-19.^{16,17} As platelets are critical in haemostasis, thrombosis, and inflammatory responses, one study showed that COVID-19 may induce production of autoantibodies which leads to destruction of platelets by immune responses which was approved in another study in patients infected with human immunodeficiency virus (HIV).¹⁸ It is well known that during infection, the platelets are activated and adhered to the sub-endothelium, their hyperactivity leads to thrombosis, arterial ischemia and in many cases causes pulmonary embolisms. Many viral infections such as HIV, hepatitis C virus (HCV) and influenza virus can directly lead to hyperactivity of the platelets.^{19–22} In a recent study COVID-19 has been found to induce hyperactivity of the platelets through binding of spike protein to ACE2 receptor. It has been approved that SARS-CoV-2 can induce platelet activation which may contribute in thrombus formation and inflammatory responses in COVID-19 patients.²³ One of the important inflammatory markers, which give an insight to the inflammation going on in various organs is the Platelet lymphocyte ratio (PLR).^{24,25} Some studies have also found it to be independent from either platelet count or lymphocyte count.²⁵

Fast and correct laboratory diagnosis of active COVID-19 infection is one of the keystones of pandemic control. If these COVID-19 serious cases can be identified early by haematological and other parameters, it can facilitate clinicians to take early appropriate remedial measures to control the morbidity and mortality of COVID-19 cases.²⁶ Some earlier literature on reporting on laboratory-confirmed COVID-19 cases have noticed changes in the haematological parameters, which included neutrophil count and lymphocyte count.^{27,28} With a view of the above, we undertook this particular research to further clarify the clinical attributes and haematological parameters in ICU admitted COVID-19 patients compared to COVID-19 positive patients without symptoms and healthy controls.

Materials and Methods

Study Design and Setting

A retrospective cohort study was conducted at Aseer Central Hospital, Abha, the largest government hospital in Abha, Aseer province. All critical care admissions from October to December 2020 presenting to any of the intensive care units of the hospital were included in the research. All selected ICU critically ill participants and quarantine individuals had established severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection which was earlier confirmed by positive report on polymerase chain reaction (PCR) investigation of a sample from nasopharynx or aspirate from trachea. The patients who were included in the present study had their clinical outcomes monitored until February 2021, the last date of the research follow-up.

Study Participants

Thirty-eight patients aged sixteen years to eighty-three years, admitted to the ICU with COVID-19 were included in this study. Laboratory confirmation that the patients were SARS-CoV-2 infected was based on positive reverse-transcriptase-polymerase chain-reaction (RT-PCR) assay.²⁹ They were designated as group-1. Those were compared with twenty-nine COVID-19 quarantined patients who were designated as group-2 and twenty healthy individuals who have not been infected by COVID-19 as group-3.

Exclusion and Inclusion Criteria

Exclusion criteria included previously known participants having coagulopathy, pregnancy, antiplatelet therapy, current use of systemic anticoagulants, vitamin K antagonists and patients who presented with serious cardiac problems. Quarantined patients, who fitted the criteria, were randomly selected from records.

Ethical Consent

Our study complies with the Declaration of Helsinki. Ethical approval has been provided from Regional Committee for Research Ethics [No of Registration (H-06-8-091)] vide REC-NO: REC-03-09-2020, Ministry of Health, Directorate Health Affairs - Aseer Region, KSA. Informed consent was obtained from each study participant after describing the objective and procedures of the study. Every response of the participants was kept confidential.

Detection of COVID-19 by Real Time-PCR and the Conditions of the Test

A nasopharynx samples were collected from assigned groups and proceeded for DNA and RNA extraction using magna pure DNA and viral NA kit (Roche). The extracted samples were amplified by the RealStar[®] SARS-CoV-2 RT-PCR Kit 1.0 (Altona diagnostic), to detect S gene for SARS-CoV-2 and E gene for B-βCoV. For quality control check, positive control contains both targets, B-βCoV and SARS-CoV-2 and negative controls (H₂O) were used per test. After that, samples were analysed by Light Cycler 480 II (LC480II, Roche) and performed according to the manufacturer's instructions. Samples with a CT value below 35 were considered positive amplification of the SARS-CoV-2 S gene.

Data Collection

All study data were retrieved from ICU records after proper permission. Collected clinical variables included demographics, comorbidities, and haematological parameters after ICU admission.

Retrospective Laboratory Data Analysis

All data were collected from the patient records after adequate permission and then entered in IBM SPSS version 23 [IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 22.0. Armonk, New York, USA] for further analysis. The data were subjected to general analysis like mean, standard deviation, standard error, kurtosis, skewness, etc, and then comparison was done with the help of non-parametric test, ie, Mann–Whitney test where p-value (2-tailed), Wilcoxon W, Mann–Whitney U, etc were analysed. PLR was calculated by dividing the platelet count by the lymphocytes.

Results

ICU patients had a mean±SD of $15.11 \pm 11.612 \times 10^9/L$ for WBC, with a minimum of $2.15 \times 10^9/L$ and maximum of $47.49 \times 10^9/L$ (Table 1). RBC count was 4.11 ± 1.26 million cells per mcL with a minimum of 2.38 million cells per mcL and maximum of 8.12 million cells per mcL. The Hb was 11.30 ± 3.12 g/L, with minimum being 6.00 g/L and maximum being 20.80 g/L. Platelets' mean±SD was $184.95 \pm 123.3 \times 10^9/L$, with a minimum of $18.00 \times 10^9/L$ and $553.00 \times 10^9/L$ maximum. Neutrophil count was (mean±SD) $13.34 \pm 11.38 \times 10^9/L$ with a minimum of $0.50 \times 10^9/L$ and a maximum of $43.80 \times 10^9/L$. Lymphocyte count had a mean±SD of $0.97 \pm 0.66 \times 10^9/L$, the minimum being 0 and maximum being $2.78 \times 10^9/L$. In the case of monocytes, it (mean±SD) was $0.63 \pm 0.46 \times 10^9/L$, with a minimum of $0.10 \times 10^9/L$ and a maximum of $1.97 \times 10^9/L$. The Eosinophil count had a mean±SD of $0.12 \pm 0.23 \times 10^9/L$ with minimum being 0 and maximum of $0.97 \times 10^9/L$. Basophils were in the range of $0.039 \pm 0.04 \times 10^9/L$ (mean±SD) with maximum of $0.19 \times 10^9/L$ and minimum of 0 (Table 1). PLR was 189.895.

Quarantined group had a mean±SD of $5.7 \pm 0.48 \times 10^9/L$ for WBC of with a minimum of $2.18 \times 10^9/L$ and maximum of $14.92 \times 10^9/L$ (Table 1). RBC count was 5.79 ± 0.55 million cells per mcL with a minimum of 4.87 million cells per mcL and maximum of 6.83 million cells per mcL. The Hb was 16.0 ± 1.97 g/L, with minimum being 8.20 g/L and maximum being 20.20 g/L. Platelets' mean±SD was $306.76 \pm 106.77 \times 10^9/L$, with a minimum of $156.00 \times 10^9/L$ and $567 \times 10^9/L$ maximum. Neutrophil count was (mean±SD) $2.54 \pm 1.96 \times 10^9/L$ with a minimum of $0.66 \times 10^9/L$ and a maximum of $10.94 \times 10^9/L$. Lymphocyte count had a mean±SD of $2.519 \pm 0.82 \times 10^9/L$, the minimum being 0.88 and maximum being $4.26 \times 10^9/L$. In the

Table 1 Characteristics of the Blood Parameters of ICU Patients with COVID-19, Quarantine Patients with COVID-19

Parameters	Normal Range	Mean±SD		
		ICU (n=38)	Quarantine (n=29)	Control (n=20)
WBC × 10⁹/L	4.5–11	15.11±11.612	5.7±0.48	6.53±1.96
RBC × million cells per microliter (mcL)	4.5–11	4.11±1.26	5.79±0.55	5.31±0.31
Hgb, g/L	11.5–15.0	11.30±3.12	16.0±1.97	15.5±1.18
Platelets × 10⁹/L	150–450	184.95±123.3	306.76±106.77	286.65±86.54
Neutrophil count, × 10⁹/L	2.0–8.0	13.34±11.38	2.54±1.96	3.22±1.32
Lymphocyte count, × 10⁹/L	1.5–3.5	0.97±0.66	2.519±0.82	2.54±0.785
Monocyte count, × 10⁹/L	0.2–0.8	0.63±0.46	0.49±0.21	0.53±0.126
Eosinophil count, × 10⁹/L	0.04–0.4	0.12±0.23	0.15±0.174	0.22±0.178
Basophil count, × 10⁹/L	0.01–0.1	0.039±0.04	0.04±0.022	0.039±0.0236
Platelet to lymphocyte ratio	36.63–172.68	189.895	121.746	112.766

case of monocytes, the mean±SD was $0.49\pm0.21\times10^9/L$, with a minimum of $0.14\times10^9/L$ and a maximum of $1.12\times10^9/L$. The Eosinophil count had a mean±SD of $0.15\pm0.174\times10^9/L$ with minimum being 0 and maximum of $0.79\times10^9/L$. Basophils were in the range of $0.04\pm0.022\times10^9/L$ (mean±SD) with maximum of $0.08\times10^9/L$ and minimum of 0 (Table 1). PLR was 121.746.

Considering WBC, the mean±SD for the control group was $6.53\pm1.96\times10^9/L$ with a minimum of $3.58\times10^9/L$ and maximum of $11.65\times10^9/L$ (Table 1). RBC count was 5.31 ± 0.31 million cells per mcL with a minimum of 4.62 million cells per mcL and maximum of 5.98 million cells per mcL. Hb level was $15.5\pm1.18g/L$, with minimum being 12.70g/L and maximum being 17.60g/L. The mean±SD for Platelets was $286.65\pm86.54\times10^9/L$, with a minimum of $156\times10^9/L$ and $546\times10^9/L$ maximum. Considering neutrophil count, it was (mean±SD) $3.2\pm1.32\times10^9/L$ with a minimum of $1.56\times10^9/L$ and a maximum of $6.56\times10^9/L$. The mean±SD for lymphocyte count was of $2.54\pm0.785\times10^9/L$, the minimum being 1.34 and maximum being $4.94\times10^9/L$. In the case of monocytes, the mean±SD was $0.53\pm0.126\times10^9/L$, with a minimum of $0.37\times10^9/L$ and a maximum of $0.9\times10^9/L$. The Eosinophil count had a mean±SD of $0.22\pm0.178\times10^9/L$ with minimum being 0.07 and maximum of $0.76\times10^9/L$. Basophils were in the range of $0.039\pm0.0236\times10^9/L$ (mean±SD) with maximum of $0.09\times10^9/L$ and minimum of 0.09 (Table 1). PLR was found to be 112.766.

A significant correlation was seen between the RBC (Figure 1), Hb (Figure 2), Neutrophil count (Figure 3) and Lymphocyte count (Figure 4) count ($p < 0.001$) of ICU patients with COVID-19 and healthy controls. However, no such correlation was seen between Monocyte count (Figure 5), Eosinophil count (Figure 6) and Basophil (Figure 7) count ($p = 0.294$, $p = 0.116$ and $p = 0.996$).

It was seen that there is a significant correlation between the WBC (Figure 8), RBC (Figure 1), Hgb (Figure 2), Platelets (Figure 9), Neutrophil count (Figure 3) and Lymphocyte count (Figure 4) of the ICU patients with COVID-19 and Quarantined patients with COVID-19 ($p < 0.001$). But no such relationship was seen between Monocyte (Figure 5) count ($p = 0.617$), Eosinophil (Figure 6) count ($p = 0.005$) and Basophil (Figure 7) count ($p = 0.613$) (Table 1).

It was noticed that no significant correlation existed between COVID-19 cases from quarantine with healthy participants in WBC (Figure 8) count ($p = 0.154$), Hgb (Figure 2) ($p = 0.108$), (Figure 9), (Figure 3), Lymphocyte count (Figure 4) ($p = 0.831$), Monocyte count (Figure 5) ($p = 0.476$), Eosinophil count (Figure 6) ($p = 0.041$), Basophil count (Figure 7) ($p = 0.648$). However, significant correlations occurred between platelets (Figure 9) and neutrophil count (Figure 3) ($p < 0.001$).

Discussion

The virus responsible for COVID-19 is mutating so rapidly that we are unable to understand the different speculations that its effects are having on the human body. Nonetheless, the haematological and biochemical parameters are still to be elucidated perfectly, so that future generations can control the mutated virus in the human body. Therefore, we abridged a comparative scrutiny of haematological attributes of 38 ICU COVID-19 patients, 29 quarantined COVID-19 patients and 20 controls.

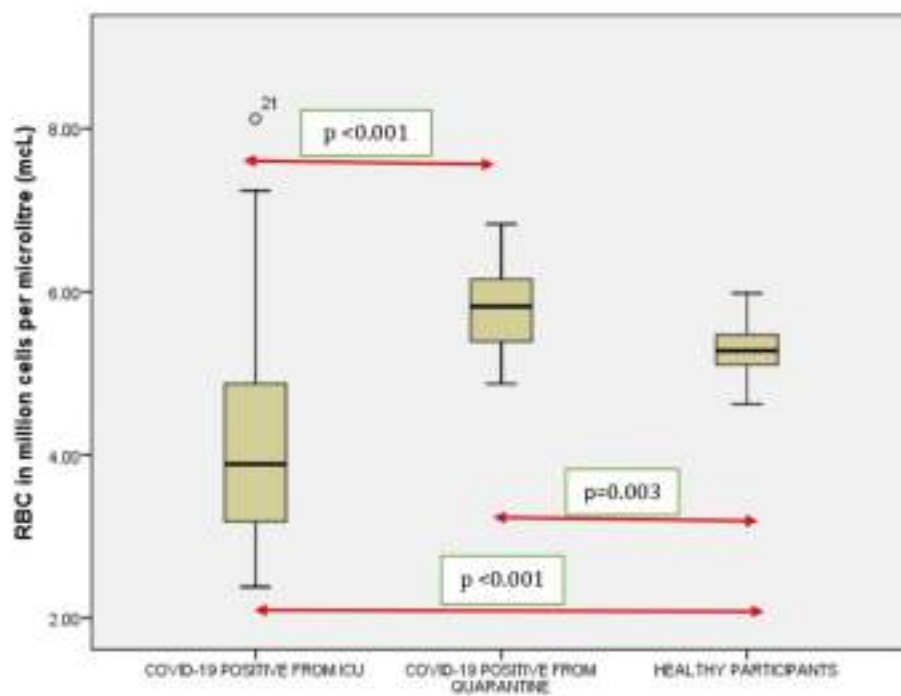


Figure 1 Comparison of RBC count in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. °Outlier (observed data points outside the boundary of the whiskers).

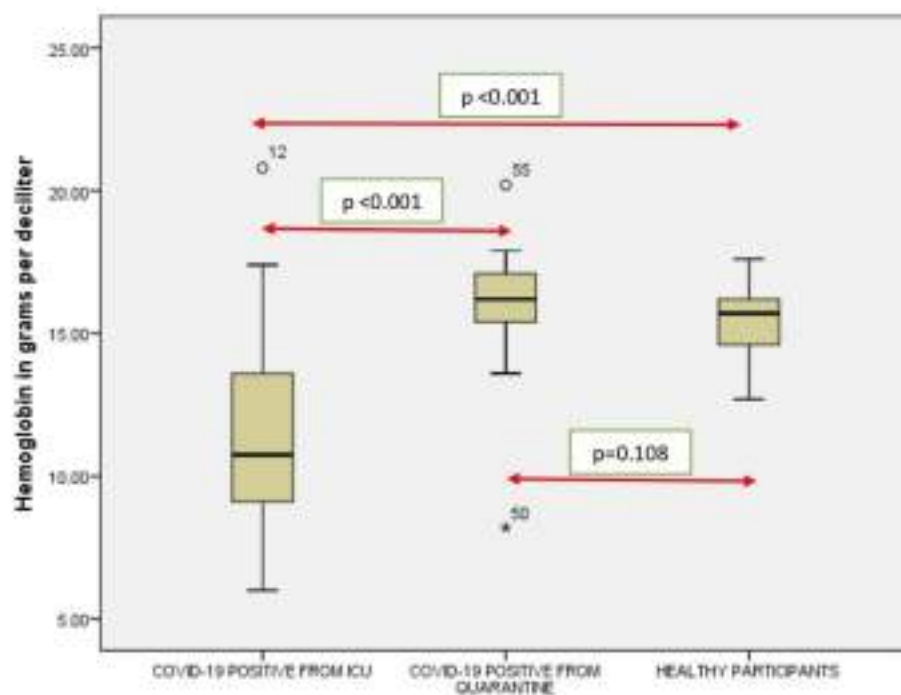


Figure 2 Comparison of hemoglobin in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. °Outlier (observed data points outside the boundary of the whiskers). *The asterisk is used for higher observed data points outside the boundary of the whiskers.

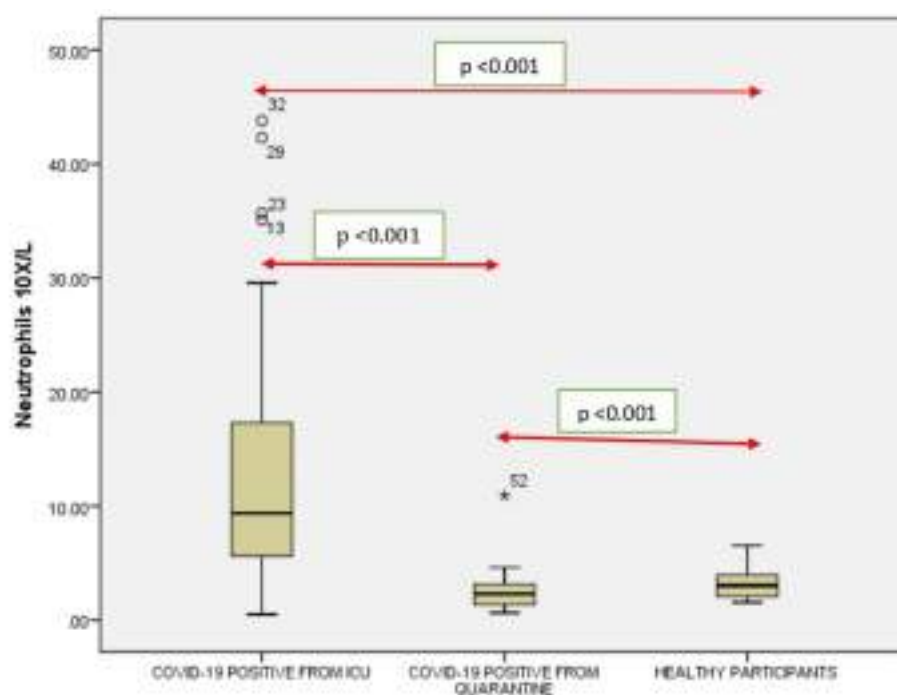


Figure 3 Comparison of neutrophils in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. *Outlier (observed data points outside the boundary of the whiskers). *The asterisk is used for higher observed data points outside the boundary of the whiskers.

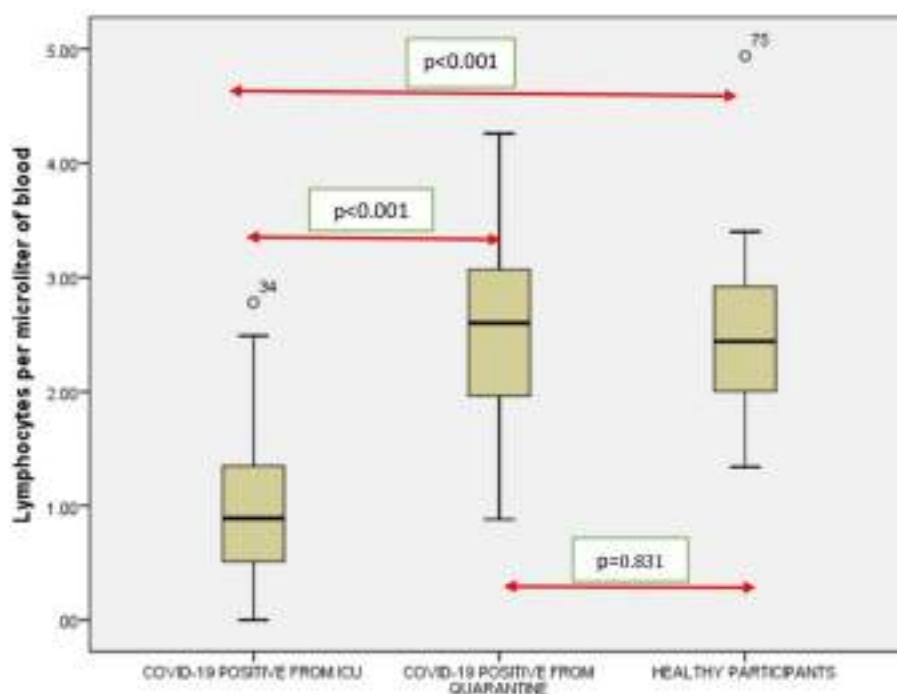


Figure 4 Comparison of lymphocytes in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. *Outlier (observed data points outside the boundary of the whiskers).

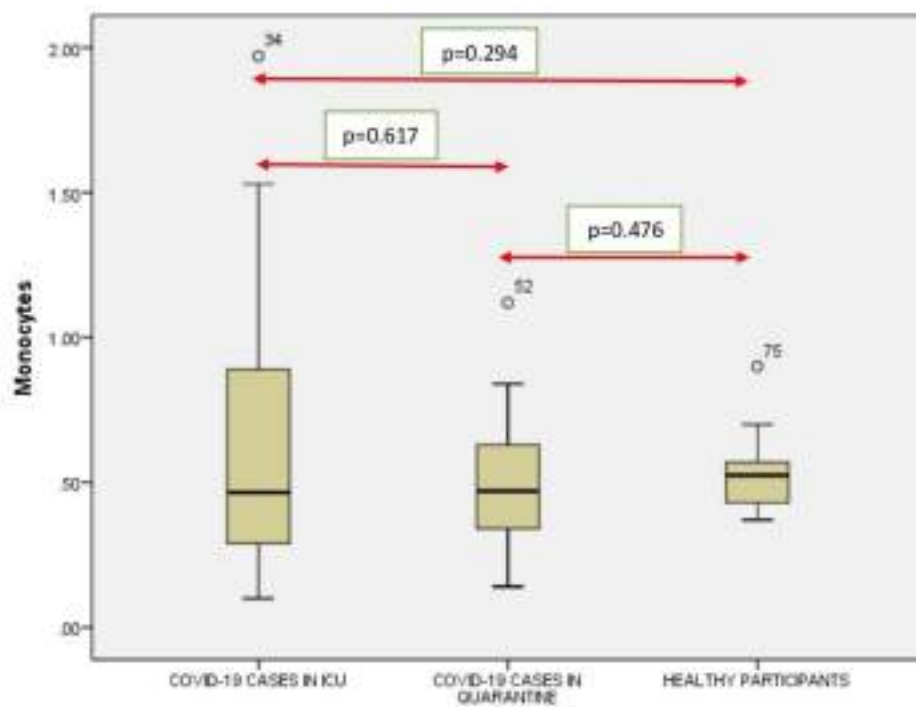


Figure 5 Comparison of monocytes in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. °Outlier (observed data points outside the boundary of the whiskers).

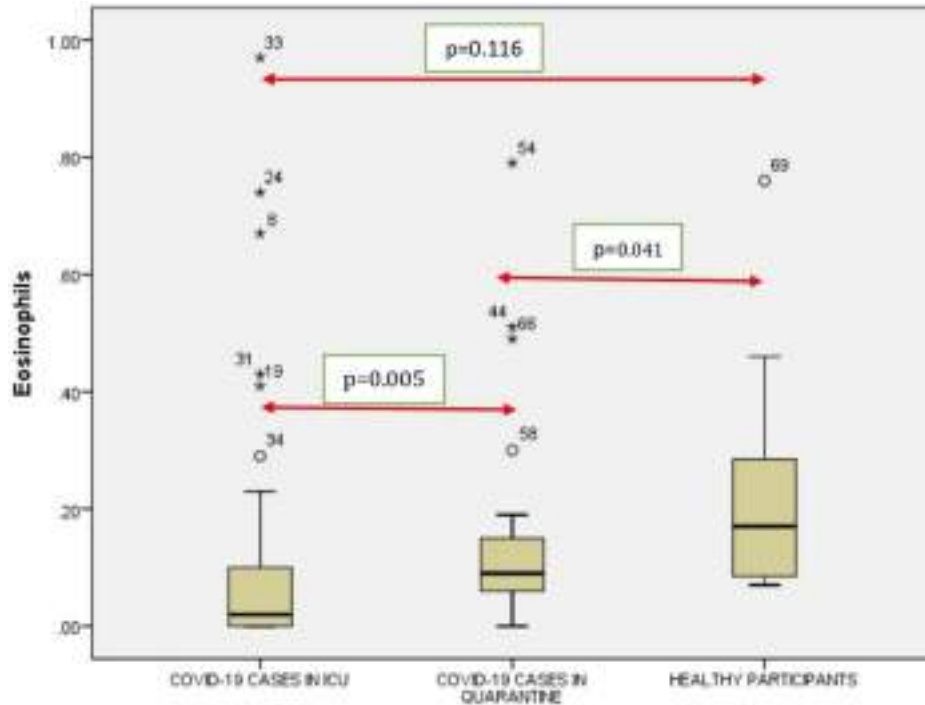


Figure 6 Comparison of eosinophils in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. °Outlier (observed data points outside the boundary of the whiskers). *The asterisk is used for higher observed data points outside the boundary of the whiskers.

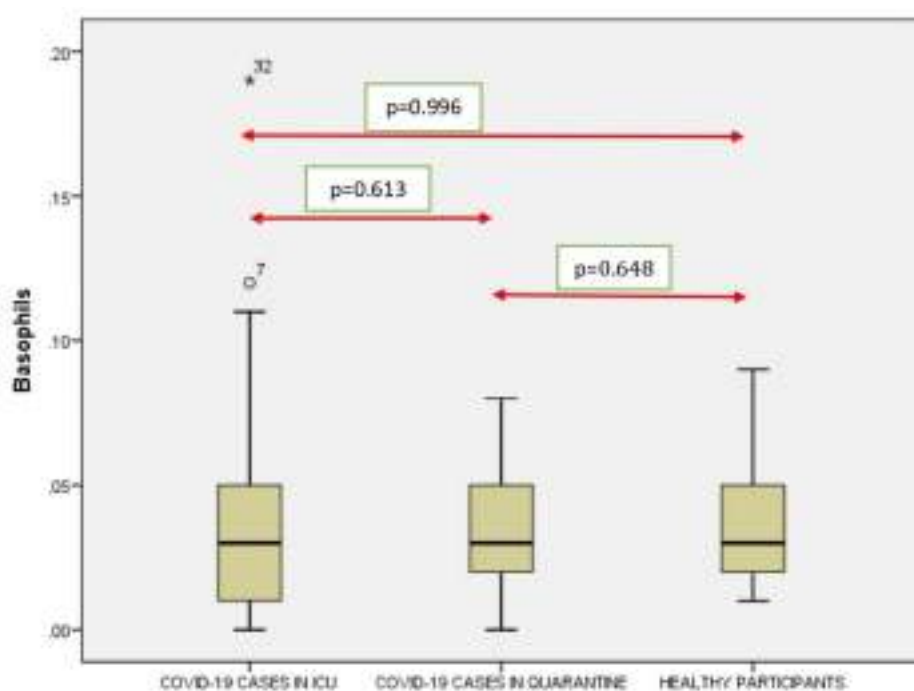


Figure 7 Comparison of basophils in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. *Outlier (observed data points outside the boundary of the whiskers). *The asterisk is used for higher observed data points outside the boundary of the whiskers.

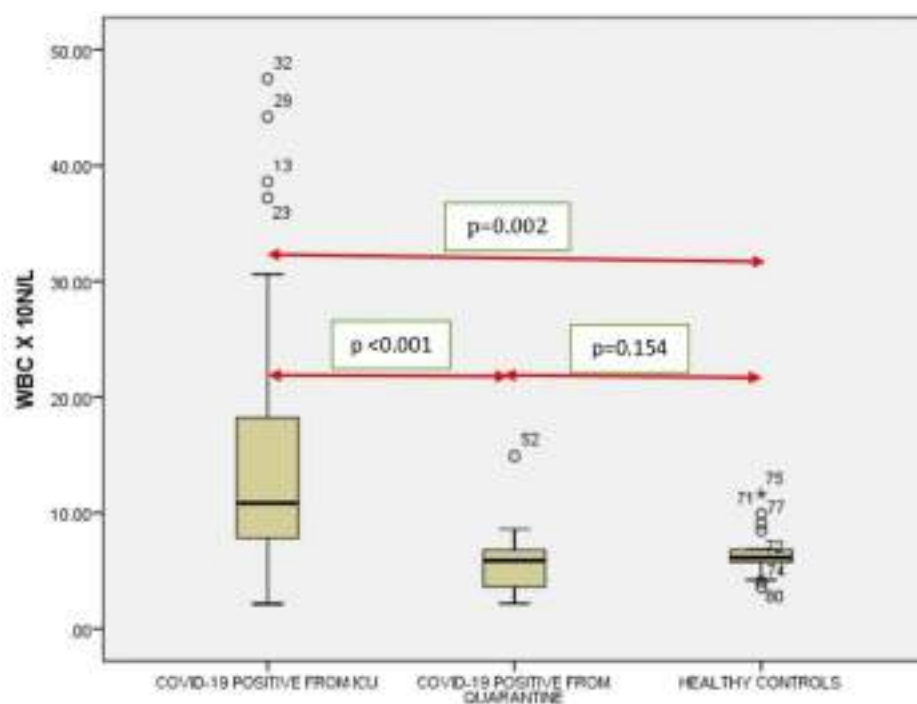


Figure 8 Comparison of WBC count in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. *Outlier (observed data points outside the boundary of the whiskers). *The asterisk is used for higher observed data points outside the boundary of the whiskers.

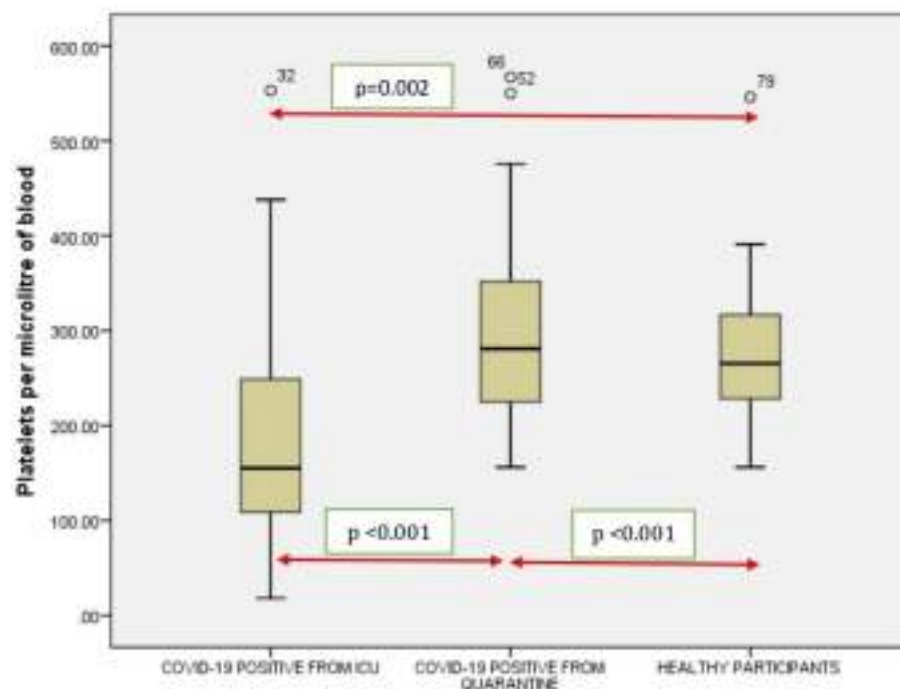


Figure 9 Comparison of Platelets in COVID-19 positive cases of ICU patients, quarantined patients with COVID-19 and healthy participants. Red double direction arrow: used to compare the p value between various groups. °Outlier (observed data points outside the boundary of the whiskers).

Leukocytosis, low RBCs (compared to quarantined subjects and controls), low haemoglobin (compared to quarantined subjects and controls), decreased platelets (compared to quarantined subjects and controls), neutrophilic leukocytosis, lymphocytopenia were some of the prominent features of our study. In Chongqing (China), the common symptoms that were observed were cough, fatigue, fever and expectoration; these symptoms were also observed in many other studies.^{30–33} But some major symptoms like myalgia, fever, malaise and chills were also reported³⁴ and common symptoms like chest pain and dyspnoea were also reported.³⁵

The haematological disorders observed in critically ill COVID-19 patients are complex and multifactorial. In this study of 38 individuals with COVID-19 hospitalized in the ICU of Aseer Hospital, it was seen that significant correlation was seen between the RBC, Hb, Neutrophil count and Lymphocyte count ($p < 0.001$) of ICU patients with COVID-19 and healthy controls. It was noticed that abnormal hematologic parameters (including lymphocytopenia, neutrophilia and eosinopenia) and declining kidney functions were correlated with a greater peril of alarming hospital itinerary. Many previous researches have depicted that approximately 5% of SARS-CoV-2 patients become grievously sick, evolving organ malfunctions and ultimately failure.³⁶ Recently, a study has depicted irreversible damage caused to the erythrocyte proteome by COVID-19.³⁷ Reactive oxygen species plays a vital role in leading to the damage of the RBCs, memorialize the neutrophil activation. SARS-CoV-2 attacking facilitates iron removal from the heme prosthetic group, which leads to the loss of functional haemoglobin.^{38,39} The authors found that oxidative stress connected with COVID-19 damages essential proteins in erythrocytes, including those that influence membrane structure and the ability to transport and deliver oxygen. Because mature erythrocytes cannot synthesize new proteins to replace damaged ones and the average lifespan of erythrocytes is 120 days, the authors hypothesize that the circulation of irreversibly damaged erythrocytes with impaired function could contribute to the long-term effects of COVID-19.²⁵ The mechanism behind the lymphopenia in ICU patients might be due to the direct attack by the virus in the lymphocytes or the mechanism might be one of immune-mediated apoptosis of lymphocytes.^{40–42} The research shows that the median age of the ICU patients was 55.5 years while the median age of the quarantined patients was

25 years; this is same as some previous studies which showed that the median age of the patients who were in quarantined with COVID-19 was lower than the ICU cohort.^{43,44} It is a renowned fact that the aged people greater number of limited organ function, comorbidities, biological aging, impaired immune system and more serious complications, which were pointed out by earlier studies on aged people afflicted with COVID-19^{45,46} hence it is of utmost importance that clinicians should treat them as a high-risk group.

Differential count (Neutrophil, eosinophil, etc) showed some remarkable differences from the normal in both ICU and COVID-19 positive cases, which also correlated with previous studies. All these add to the remarkable distinction, which can be pursued in future studies.⁴⁷⁻⁴⁹

An essential pathogenetic mechanism in COVID-19 which leads to kidney and lung injury is endothelial injury.⁵⁰⁻⁵² Comparison of haematological findings showed that the RBC, Hb, Neutrophil count and Lymphocyte count ($p < 0.001$) of ICU patients had significant differences with healthy controls. Materializing evidence hints that neutrophil, and the imbalance between neutrophil extracellular trap (NET) which plays a key role in the pathophysiology of coagulopathy, inflammation, organ damage, and also immunothrombosis, mainly is responsible for the characteristics of severe cases of COVID-19.⁵³ This also mostly matches earlier research done on patients with COVID-19.^{54,55} Damage to the endothelium leads to recruitment of perivascular T-cells and distorts the alveolar capillary barricade in the lungs.⁵⁶ Acutely damaged endothelium in COVID-19 is correlated with structurally distorted capillaries and further evidence of compensatory neovascularisation.⁵⁷ This lower than is desirable endothelial barricade provokes oedema of the lungs, and subsequent proteinuria, which are commonly seen in patients afflicted with severe kidney and lung diseases.^{58,59} As abundant NETs are found in the circulation and in kidney and lung tissues of patients afflicted with COVID-19,⁶⁰ their stockpiling delineates a key provocation to generate renal and pulmonary microvascular thrombosis, which ultimately leads to COVID-19-related organ failure.^{61,62} A nonspecific inflammatory marker, the PLR, implies concurrent interaction between platelet count and lymphocyte count, reflects aggregation, as well as inflammatory pathways. In many cases, it has been found to be elevated in response to many acute as well as chronic proinflammatory conditions. Our study had elevated PLR in ICU, compared to healthy controls^{25,63-65} and has reported a strong correlation between elevated PLR and mortality in Covid-19 patients like some other studies.

Limitations of the Study

We have to admit that the study has many limitations. First, retrospective cross-sectional research furnishes no features and details on a cause to effect relationship. Secondly, the findings have been hampered by the limited number of haematological parameters studied. Another drawback that we faced was that we were not able to perfectly match the study participants as required. Also, some more parameters were not done, and we were not able to include them in our study. Since this was a retrospective cohort study, it was impossible to follow timing of blood count. Also, another drawback was the number of patients in ICU who died; we were unable to get the data due to technical issues. Further exploration of these markers is required for utilization for risk stratification in patients afflicted with COVID-19. By doing research in a larger number of COVID-19 cases combined with COVID-19 autopsy information will surely help us to get more accurate information on the clinical characteristics and their contribution in managing the disease. This will additionally assist in formulating a vigorous scheme to dominate and attenuate the COVID-19 pandemic at large.

Conclusion

We have seen in this research that characteristics of ICU patients, including age, leukocyte count, platelet count, RBC count and differential leukocyte count may be noteworthy futuristic for developing managing plans for critically ill SARS-COV-2 afflicted patients. These alterations in the said parameters have shown that patients having severe COVID-19 disease could be treated beforehand with the said alterations, and thus these may also serve as a possible biomarker for those people who needs hospitalization and ICU care. Also, close monitoring of platelets should be done to prevent clotting in ICU patients. Also, it was seen that PLR determination was useful in COVID-19 for determination of the health peril. However, further evaluation of these parameters is needed to be utilized for peril stratification purposes. COVID-19 is very unpredictable, since it is mutating constantly in defined periods, which leads to variety in the clinical

symptoms and signs. This is the main reason that high-risk patients need constant and strict monitoring and timely supportive measures and adequate treatment.

Data Sharing Statement

The datasets used in this study are available from the corresponding author upon request.

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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.

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Disclosure

The authors report no conflicts of interest in relation to this work.

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Pyruvate Kinase Deficiency: Current Challenges and Future Prospects

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Abstract: Pyruvate kinase deficiency (PKD) is a rare autosomal recessive disease marked by chronic hemolytic anemia of various severity and frequent complications including gallstones, splenomegaly, iron overload, and others. Disease phenotype is highly heterogeneous and changes over time with children, adolescents and adult patients displaying different transfusion requirement and rates of complications. The diagnosis relies on the initial clinical suspicion in a patient with chronic hemolysis and exclusion of other more common congenital forms of hemolytic anemias; it is supported by the demonstration of reduced PK enzyme activity, and further confirmed by the detection of (homozygous or compound heterozygous) mutations of PKLR gene. Therapy is mainly supportive, with vitamin supplementation and transfusions (based on symptoms and patient growth rather than on fixed Hb thresholds). Splenectomy is widely performed, although it is less effective than in membrane defects and carries thrombotic and infectious risk. In the last decade, the allosteric PK enzyme activator mitapivat showed dramatic clinical benefit in clinical trials and gene therapy is also being studied to substitute the defective enzyme. In this review, we provide an insight in the current challenges of PKD diagnosis and management and discuss the future application of novel drugs and gene therapy, including a focus on quality of life.

Keywords: pyruvate kinase deficiency, splenectomy, mitapivat, gene therapy

Introduction

Pyruvate kinase deficiency (PKD) is a congenital hemolytic disease characterized by a variable degree of anemia from mild to transfusion dependent and heterogeneous clinical features derived from chronic red blood cell (RBC) destruction (gallstones, splenomegaly, iron overload, etc.). Disease phenotype may vary over time with children, adolescents and adult patients displaying different transfusion requirement and rates of complications (Figure 1).¹⁻³ Despite the availability of biochemical assays for enzyme activity and the identification of the several underlying genetic lesions, the *primum movens* for the diagnosis is still the clinical suspicion and the subsequent reference of patients to tertiary centers. Several confounders further challenge the diagnosis and patients may end up being treated as other congenital hemolytic anemias (CHAs). The latter may have similar phenotypes but may show different outcomes in terms of response to splenectomy and frequency of complications.⁴ Finally, the advent of novel treatments aimed at restoring PK activity, including the allosteric PK enzyme activator mitapivat and gene therapy, further advocates for a definite diagnosis in these patients.^{5,6} In this review, we will provide a clinician insight in the current diagnosis and management of PKD, including challenges in disease identification and treatment, and future prospects on novel targeted therapies and gene therapy.

Epidemiology and Physiopathology

PKD is inherited as an autosomal recessive disease and has an estimated prevalence of 3:1,000,000 to 1:20,000.^{7,8} Heterozygote frequencies range from 0.15% to 6% in the various populations and have been phylogenetically preserved due to a protective effect against malaria.⁹⁻¹¹ As a result, a high PKD frequency is reported in the Middle East and sub-Saharan Africa.^{12,13} From a physiopathology point of view, RBCs lack nucleus and organelles, and generate energy (adenosine triphosphate, ATP) via the

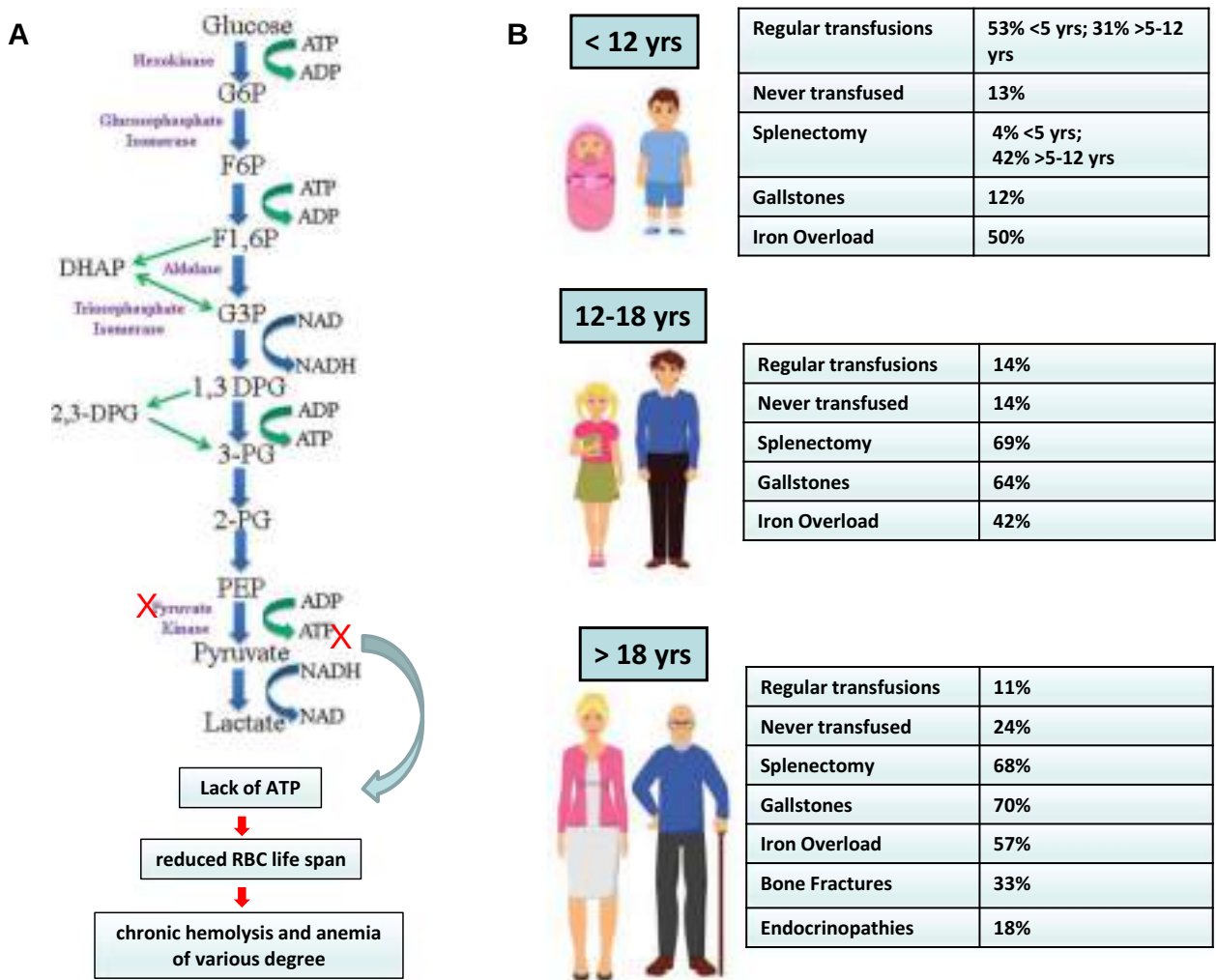


Figure 1 The physiopathology of pyruvate kinase deficiency (PKD), and its clinical features and complications. **(A)** The Embden-Meyerhof pathway alteration. **(B)** Different clinical features and complications during infancy, adolescence, and adulthood.
Abbreviations: 1,3 DPG, 1,3 biphosphoglyceric acid; 2,3 DPG, 2,3 biphosphoglyceric acid; 2-PG, 2-phosphoglyceric acid; 3-PG, 3-phosphoglyceric acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; F1,6P, fructose-1,6-biphosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose-6-phosphate; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide+hydrogene; PEP, phosphoenolpyruvate; RBC, red blood cells; yrs, years.

Embden-Meyerhof glycolytic pathway to maintain their shape, flexibility, and rheological properties over their 120-day journey. PK is a key enzyme of the glycolysis, boosting the transformation of pyruvate from phosphoenolpyruvate (PEP) with ATP generation (Figure 1A). PKD results from homozygous or compound heterozygous mutations in the *PKLR* gene (chromosome 1q22),^{14–16} including more than 350 lesions, mostly missense (encoding single aminoacid changes), and rarely disruptive mutations (stop codons, frameshifts, and large deletions). The latter are generally associated with a more severe phenotype that affects the enzyme’s structure, stability or catalytic function. Decreased PK activity results in low ATP levels and high levels of the upstream metabolite 2,3-diphosphoglycerate (2,3-DPG), that in turn favors O2 release to tissues.^{1,17,18} As a result, PKD-RBCs are rigid, less flexible, and undergo premature hemolysis in the spleen.

Current Challenges in PKD

Clinical Suspicion and Differential Diagnosis

Diagnosis of PKD is based on the presence of chronic hemolytic anemia, reduced PK enzyme activity, and molecular characterization of the pathogenic mutations. Generally, severe cases are diagnosed at an earlier age, intrauterine

complications (growth retardation, hydrops fetalis and prematurity),¹ frequent neonatal jaundice (59–90%), and possible transfusion requirement (Figure 1). On the other hand, some subjects may receive PKD diagnosis in late adulthood, possibly due to comorbidities unveiling a mild disease.^{1,19} Overall, the most frequent symptoms are anemia (90–95% of cases, mild to transfusion-dependent), splenomegaly (80–85%), jaundice (40–70%) and gallstones (30–45%). More rare manifestations include aplastic crises (2–14%), bone deformities (9%), extramedullary erythropoiesis (9%), delayed puberty (8%), hyperpigmentation (6%), leg ulcers, and pulmonary hypertension (2–3%).^{1,16,19} Hemoglobin values generally range from 6.5 to 11 g/dL and improve after splenectomy (approximately 15 g/l). Concerning hemolytic markers, reticulocytes are elevated (4%–11%) and typically markedly increase after splenectomy (20%–70%),^{19,20} unconjugated bilirubin is also elevated with a mean value of 60 mol/L (higher if coexistent with Gilbert's syndrome); and lactate dehydrogenase is usually normal.²¹ Ferritin is often increased (even >1000 ng/L), regardless transfusion dependency.^{1,22}

The great overlap with the other congenital anemias challenges the differential diagnosis (Figure 2).⁴ Generally, hemolytic features and increased mean corpuscular volume (MCV) typical of CHAs aid the distinction from hemoglobinopathies that are further investigated by searching for pathologic hemoglobin forms through high performance liquid chromatography (HPLC). The distinction from other CHAs may be more difficult and is supported by evaluation of RBC morphology (peripheral blood smear), osmotic fragility tests, and RBC deformability tests by ektacytometry that are usually normal in PKD and altered in membrane defects. Additionally, PKD is autosomal recessive whilst membrane defects are usually dominant, so that family history is generally not revealing, unless an affected sibling is present. Finally, in patients without family history and onset in adulthood, tests to exclude common causes of acquired hemolytic anemias, particularly the direct anti-globulin test for autoimmune forms, is recommended.

After the above-mentioned tests have been performed, RBC enzymatic activities are evaluated leading to PKD diagnosis.^{2,3} PK enzymatic activity is determined in RBC lysates by spectrophotometry according to Beutler et al, 1984, and is measured as international unit per Hb grams (normal reference range usually defined at each laboratory on healthy blood donors).²³ This test is fast but may give false normal results due to incomplete leukocyte/platelets depletion, reticulocytosis, and recent transfusions. Evaluating PK activity in proportion to that of other age-dependent RBC enzymes, such as hexokinase or glucose-6-phosphate dehydrogenase, may disclose relative low enzyme activity and trigger molecular confirmatory test.^{17,24} Finally, mutations in *KLF1*, a master regulator of erythropoiesis that modulates the expression of many red cell enzymes including PK, may result in PK deficiency at routine enzyme assays possibly leading to misdiagnosis.²⁵ Genotyping of PKLR is therefore recommended and more than 300 mutations have been described, mostly missense substitutions (70–80%, R510Q in Northern Europe and the United States, R486W in Southern Europe, and R479H mutation among Pennsylvania Amish).²⁶ An effort to perform a genotype–phenotype correlation has been made in the PKD Natural History Study,¹ where patients with at least one missense mutation had a milder phenotype (later diagnosis, higher Hb, reduced transfusion need, lower ferritin levels, and less frequent splenectomy) as compared with those with two non-missense mutations. Genetic testing offers several advantages, such as the need for smaller sample volumes, no interference of transfused RBCs, and suitability for prenatal diagnosis that may be performed in the more severe cases. While there are no clear indications for prenatal/pre-gestation screening programs in PKD, genetic counselling may be considered in affected individuals or in case of familiarity.

To Transfuse or Not to Transfuse

Supportive treatment is still the mainstay for PKD management, and routine folic acid supplementation is pivotal to sustain bone marrow reticulocyte compensation. B12 vitamin should also be systematically tested and supplemented if deficient. Concerning transfusions, their use is mainly dependent on the presence of clinical signs and symptoms that vary according to anemia degree and to the compensatory response. In PKD, tolerance of anemia may be higher than in other forms due to both the chronic nature of the disease and to the increased levels of RBC 2,3-DPG content, with consequent increased oxygen release to tissues. Therefore, whilst in thalassemia systematic transfusions are indicated to avoid complications of accelerated erythropoiesis, in PKD transfusion need should be individualized, based on anemia compensation, and its impact on quality of life (QoL). In any case, in a recent study, most PKD patients (84%) had required at least one transfusion in their lifetime.^{1–3} Transfusion requirement in a single patient may change over time in

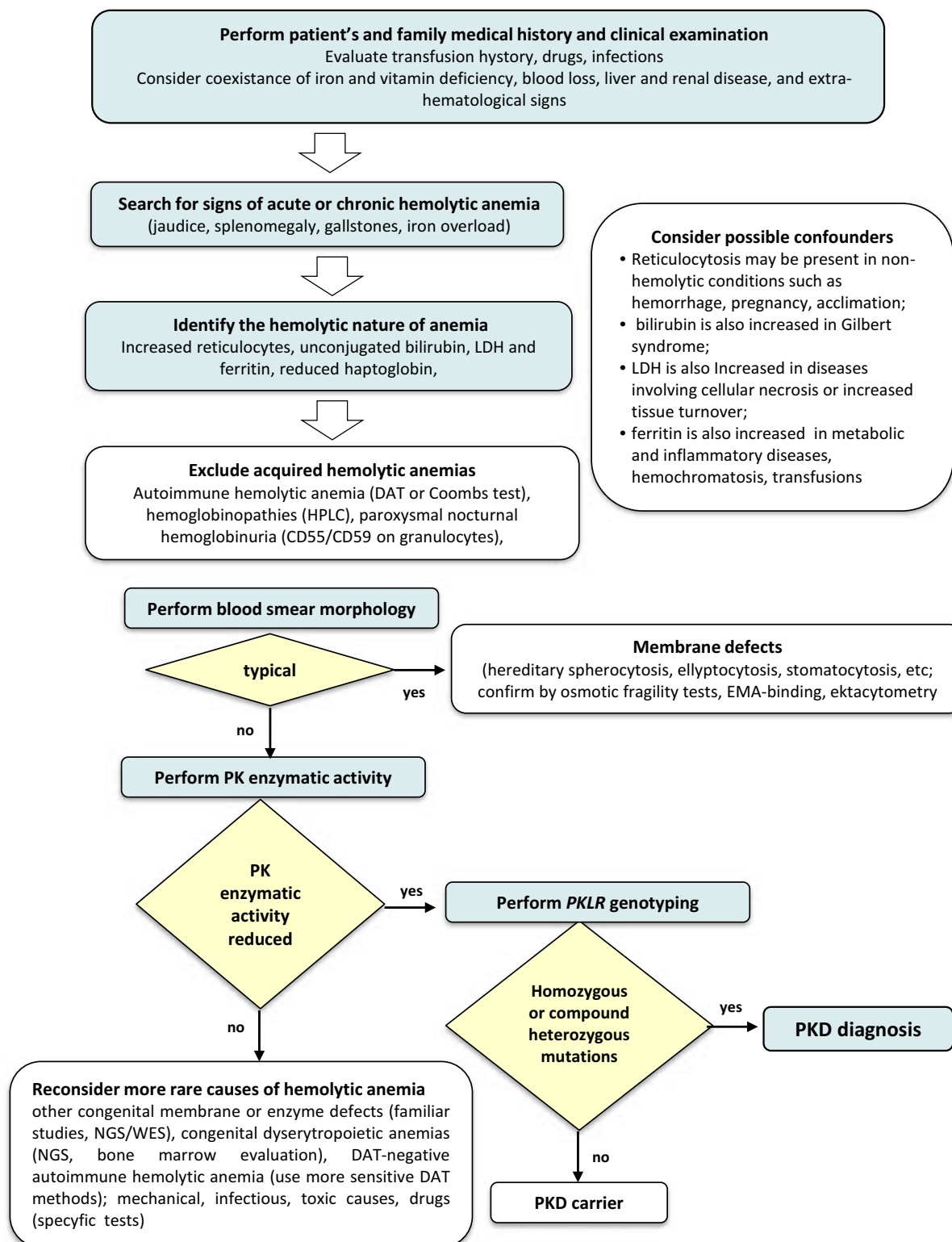


Figure 2 The differential diagnosis of pyruvate kinase deficiency (PKD).

Abbreviations: NGS, next generation sequencing; WES, whole exome sequencing; DAT, direct antiglobulin test; HPLC, high performance liquid chromatography.

parallel with anemia compensation. In newborns, transfusion requirement is frequent in the first days of life, possibly suppressing erythropoiesis. This, combined with the physiological hemoglobin nadir, may confound the assessment of anemia severity in neonatal age. It has been therefore recommended to extend the time between transfusions to allow erythropoietin and reticulocyte response in newborns. Additionally, indirect hyperbilirubinemia requires phototherapy in nearly all patients (93%) and exchange transfusions in about half of cases.¹ Subsequently, some children require regular transfusions to avoid symptoms whilst others are only intermittently transfused during acute events, particularly infections, and the Hb threshold is that allowing for normal growth/development. Infections, causing both increased hemolysis and suppression of bone marrow compensation, usually decline along with age with a reduction in transfusion need. As a result, about 50% of patients aged ≤ 5 years are regularly transfused, compared to only 25% of those >5 - <12 years and less than 10% of those aged ≥ 18 years.^{1,16,20} This is also due to the effect of splenectomy which is usually deferred after the first decade of life as discussed thereafter. Conversely, in the elderly, the accumulation of comorbidities and physiological aging may decrease anemia tolerance triggering transfusion need. However, since intra-patient variation of Hb levels is generally minimal in PKD, clearcut anemia worsening should prompt investigations for additional causes (ie, nutritional deficiencies, evolution into myelodysplasia or marrow infiltration, or presence of accessory spleen in splenectomized patients).

Splenectomy: Friend or Foe?

It has long been known that splenectomy may attenuate anemia and reduce transfusion requirement in about 90% of PKD patients,¹⁻³ with a median rise in Hb of 1.6 g/dL. However, these benefits should be weighed against the well-known infectious and thrombotic risks of splenectomy. It is generally delayed until 5 years of age, and the timing is also decided based on the frequency of RBC transfusions, the presence of allo-immunization, the presence of iron overload (including the efficacy of iron chelation), and the availability and fitness of venous access. Unluckily, assessment of red cell survival, splenic sequestration, and spleen size is not predictive for efficacy of splenectomy in PKD, likely due to liver hemocatheresis. The latter contributes to residual post-splenectomy hemolysis (ie, reticulocytosis and indirect hyperbilirubinemia) in most patients, with consequent persistent jaundice and risk of pigmented gallstones.

The main reasons to perform splenectomy are regular transfusions and severe anemia,¹ as also indicated by recent guidelines.²⁷ However, there is a high variability in splenectomy frequencies among the various centers.¹ Predictors of Hb response include less severe anemia, with lower total bilirubin, and presence of a more favorable genotype (ie, 2 missense PKLR mutations), so that most severe PKD is less likely to improve. Full splenectomy is recommended since the few reported cases of partial splenectomy had no benefit.²⁸

The risk of post-splenectomy sepsis is mainly related to encapsulated organisms, but also to malaria and babesiosis,^{29,30} and its frequency was as high as 7% in a recent study.¹ Vaccinations (anti-Meningococcal, anti-Pneumococcal, and anti-Haemophilus) may effectively reduce the risk, should be performed at least 2 weeks before splenectomy, and regularly updated based on most recent guidelines. Indications on oral antibiotics for infection prophylaxis are less clearcut and largely variable among Countries. Pediatricians generally recommend that asplenic children receive penicillin prophylaxis until 5 years of age and for at least 1 year following splenectomy. Since vaccinations do not completely abrogate infectious risk, patients' education to promptly seek for medical attention to receive broad spectrum antibiotics in case of fever (or other suggestive signs/symptoms) is mandatory.^{2,3,27}

Regarding post-splenectomy thrombosis, in PKD the risk is approximately 10%, mainly involving venous district (deep venous thrombosis and pulmonary embolism) and also including unusual sites (portal vein thrombosis and central nervous system thrombosis).^{20,31,32} Post-splenectomy thrombocytosis can be managed with low dose aspirin until the platelet count is $<500 \times 10^9/L$, particularly. Anticoagulant prophylaxis may also be considered in selected cases after careful evaluation of additional risk factors for thrombosis.

Transplant

Hematopoietic stem cell transplant (HSCT) that may virtually cure PKD, has been shown beneficial in animal models,³³ and in subsequent reports of PKD patients.³⁴ In a larger series of 16 patients undergoing HSCT in Europe and Asia³⁵ a 74% cumulative survival was reported although with a relevant rate of graft-versus-host disease (GVHD). Additionally,

pooling data is particularly difficult given the heterogeneity of donor types, conditioning, GVHD and infection prophylaxis. Altogether, in the absence of clinical trials, recent guidelines favor splenectomy and/or regular red cell transfusions rather than HSCT in PKD.^{2,3}

How to Monitor and Manage Complications

PKD patients may experience several disease-related and treatment-related complications that further challenge clinical course with variable frequency during lifetime. Gallstones may occur at all ages (median 15 years) in up to 30–45% of patients,^{1,19} are predicted by a more hemolytic pattern with hyperbilirubinemia, and favored by Gilbert syndrome.^{36,37} In a recent analysis, cholecystectomy was performed simultaneously with splenectomy in 20% of cases, whilst 50% of patients required it after splenectomy due to persistent hemolysis.¹ In case of gallstones, simultaneous surgery is therefore recommended. Whilst in their absence the indication to cholecystectomy is less clear, also due to an increase of intrahepatic cholestasis risk post-surgery.

Another frequent and underestimated complication is iron overload, both related to transfusion dependence,¹⁹ but also common in patients never/seldomly transfused. Iron overload may also be due to ineffective erythropoiesis and low hepcidin levels, to increased intestinal absorption, or to coinheritance of hemochromatosis genes.^{22,24} In the PKD Natural History Study, 38% of transfusion independent patients had ferritin >1000 lg/l,²² 82% had pathologic liver T2* MRI, and 7% cardiac one, and 10% had received chelation at some point during their life. The recommended monitoring in regularly transfused patients (>6 transfusions/year) includes ferritin levels twice a year, more frequently in those on chelation. The latter is advised after 10–14 RBC transfusions and preceded by a T2* MRI. In non-transfusion dependent patients ferritin levels should be obtained at least every 1–2 years, and MRI at least once, particularly if ferritin >500 lg/l (sensitivity 90%). Such patients may in fact require intermittent chelation.^{2,3,22} The approach to chelation in PKD is similar to other red cell and iron loading disorders and not specifically standardized for PKD. In non-transfused patients with adequate hemoglobin levels, therapeutic phlebotomy can be considered as an alternative to chelation.

During life, patients may experience sudden Hb drops due to either hemolytic or aplastic crises.^{1–3} Both are more frequent during infancy and after infections. Hemolytic crises are generally characterized by fatigue, worsening jaundice and hemoglobinuria. Aplastic crises are marked by reticulocytopenia, and possibly pancytopenia, are due to parvovirus B19, and mostly recover in about 10 days, but rarely require longer time and high transfusion need. Positive serology for anti-parvovirus IgM or DNA detection by PCR confirm the diagnosis. In both conditions, anemia may be severe and require transfusion support, and continuous folic acid supplementation.

Other rarer complications include bone deformities, due to hyperplastic bone marrow, and osteopenia, possibly related to iron toxicity, increased bone turnover, and hormone deficiencies. It is therefore advised to monitor vitamin D levels, calcium intake, and to perform bone densitometry at least once.^{1,20} Extramedullary hematopoiesis is very rare and may be recognized by CT scan showing paravertebral masses;^{1,38,39} pulmonary hypertension has been reported, should be suspected in case of dyspnea disproportionate to anemia severity, and investigated by echocardiography and cardiologist referral.^{1,40} Finally, leg ulcers have been seldomly reported,^{20,41} so that physical examination for cutaneous lesions is recommended in PKD patients.

Although it is not a complication, pregnancy in PKD deserves a mention. It is usually associated with good maternal and fetal outcomes,¹ provided close obstetrician/hematologic monitoring. However, up to 10% preterm births and 18% miscarriages have been reported. A preconception evaluation with echocardiogram is advised, as well as continuous folic acid supplementation, whilst iron supplement should be evaluated on a case-by-case basis if deficiency is present. Hemolysis typically worsens and transfusion need increase. The latter should also be tuned on fetal growth.^{2,3}

Future Prospects

Current management of PKD mainly relies on supportive measures, iron chelation, and splenectomy. In the last 10 years, a novel era of therapies directed at boosting enzyme activity, by directly activating PK or by substituting the defective gene, has begun. Published and ongoing clinical trials are summarized in [Table 1](#).

Table I Future Treatment Prospects in Pyruvate Kinase Deficiency

Drug	Study	N°Patients	Main Results	Ref
Mitapivat	NCT02476916, phase 2	52 pts (non RBC transfusion dependent)	• Increase of at least 1 g/dL of Hb in 50% pts; mean maximum increase 3.4 g/dL (range 1.1+5.8) • Median time until the first increase of more than 1 g/dL 10 days (range, 7 to 187) • Association of response with genotype (Hb responses in pts with at least one missense PKLR mutation) • Main adverse events: headache (24 pts), insomnia (22 pts), nausea (21 pts)	[5]
	ACTIVATE study; NCT03548220, phase 3	80 pts (not regularly transfused); 40 mitapivat arm, 40 placebo arm	• Sustained Hb response in 16 mitapivat pts (40%) vs 0 PBO pts ($p < 0.0001$) • Significant improvements with mitapivat vs PBO, average change from BL (difference in least squares mean): Hb concentration, hemolysis markers; PKDD, PKDIA • Main adverse events (mitapivat): nausea (17.5%), headache (15%)	[43]
	ACTIVATE Long Term Extension		• ≥ 1.5 g/dL Hb increase from BL at all time points in 13/15 M/M pts (86.7%); ≥ 1 g/dL Hb increase from BL at all time points in 2/15 M/M pts	[45]
	ACTIVATE-T study; NCT03559699, phase 3	27 pts (regularly transfused)	• 10 (37%) pts treated with mitapivat achieved a $\geq 33\%$ reduction in transfusion burden (1-sided $p = 0.0002$) • 22% transfusion-free status • Main adverse events: increased alanine aminotransferase and headache	[44]
	ACTIVATE-T Long Term Extension		• Transfusion-free status maintained in all 6 patients during LTE	[45]
Gene therapy	NCT04105166, phase I	Estimated enrollment: 6 pts	• Ongoing (two adult splenectomized patients enrolled: Hb increase from the baseline respectively from 7.4 to 13.3 g/dL and from 7 to 14.8 g/dL at 12 months; improvement in hemolysis markers)	[50]

Pyruvate Kinase Activators

Mitapivat, previously AG-348, is an oral allosteric activator of RBC PK. In biochemical assays and ex vivo experiments, this small molecule succeeded in increasing wild type and mutant PK enzyme activity.⁴² Subsequently, a Phase 2 study (NCT02476916)⁵ enrolled 52 adult patients with PKD not receiving RBC transfusions, who were randomly assigned to receive mitapivat 50 mg or 300 mg twice daily for 24 months. A Hb increase of more than 1 g/dL from the baseline was reported in 26 patients (50%, mean maximum Hb increase of 3.4 g/dL, range 1.1+5.8). Importantly, Hb response was observed only in patients with at least one missense mutation of PKLR (ie, those with residual PK activity). The drug showed a favorable safety profile, with mainly low-grade adverse events (AEs) including headache (24 patients), insomnia (22), and nausea (21), mostly transient and resolving within 7 days of treatment. Further two Phase 3 trials assessed mitapivat in PKD patients. The ACTIVATE study (NCT03548220)⁴³ was a randomized, double-blind, placebo-controlled study evaluating the efficacy of mitapivat in not regularly transfused patients. A 12-week dose escalation period was followed by a 12-week fixed dose one and 16/40 patients in the mitapivat arm (40%) achieved a sustained Hb response versus 0 patients in the placebo one. Patients receiving mitapivat had a mean Hb increase of 1.8 g/dL, an improvement in hemolytic markers, and in patients reported outcomes. Again, AEs were mainly mild and included nausea (17.5%) and headache (15%). Responders patients maintained Hb improvement across the long-term extension

(LTE, data cut off at 19.5 months); 17 patients from the placebo arm switched to mitapivat in the LTE, and 6 (35%) achieved a Hb response. The efficacy of mitapivat in regularly transfused patients was evaluated in the open label ACTIVATE-T study (NCT03559699).⁴⁴ Twenty-seven patients were enrolled and received a dose escalation period of 16 weeks, followed by a fixed dose period of 12 weeks. Ten cases (37%) met the primary endpoint (>33% reduction in transfusion burden), and 6 (22%) became transfusion independent. Mitapivat was again well tolerated, with increased alanine aminotransferase (37%) and headache (37%) as most common AEs. Responders maintained transfusion independence across the LTE study⁴⁵. Interestingly, mitapivat and etavopivat, a new allosteric PK activator (FT-4202), are currently under study in sickle cell disease (SCD) and thalassemia (NCT04610866, NCT03692052, NCT03815695, NCT04624659, NCT04987489).^{46–48} These conditions share with PKD features of chronic hemolysis with reduced RBC life span, and ineffective erythropoiesis and further highlight the potential of boosting RBC energy machinery in congenital anemias.

Gene Therapy

Being a recessive inherited disease with a single gene defect involving RBC, PKD is an excellent candidate for gene therapy. In a murine model, PKD hematopoietic stem cells were transfected with a lentiviral vector carrying PK gene and subsequently transplanted in myeloablated PKD mice.⁴⁹ This led to the restoration of normal glycolytic activity and erythropoiesis, along with reversion of secondary effects of hemolysis. Moreover, no evidence of genotoxicity was noted. Given the promising results in preclinical studies, gene therapy by lentiviral transduction of autologous stem cells and progenitor cells is currently under investigation in an open-label Phase I trial (NCT04105166).⁵⁰ At the last update, two adult splenectomized patients had been enrolled, with a substantial Hb increase from the baseline in both subjects (from 7.4 to 13.3 g/dL and from 7 to 14.8 g/dL at 12 months, respectively), associated with an improvement in hemolytic markers. Notably, no severe adverse events were reported. Gene therapy is now under evaluation in the pediatric setting.

In the present scenario of PKD therapeutic choices, the indication for gene therapy is certainly a “hot” topic. Hence, an “expanded” definition of severe PKD has recently been proposed by a panel of experts, to facilitate the identification of subjects most suitable for ongoing gene therapy.⁶

QoL Evaluation in PKD

Along with the emergence of new drugs and therapeutic strategies for PKD, health-related quality of life (HR-QoL) in PKD has been gaining more and more importance in the recent years. HR-QoL is negatively affected not only by signs and symptoms of PKD (such as fatigue, dyspnea, jaundice and splenomegaly), but also by disease complications (eg, iron overload necessitating iron chelation therapy) and transfusion requirement.²⁰ Nevertheless, due to the rarity of the disease and the lack of published HR-QoL data, it's difficult to define the burden of the disease on patients' lives. Moreover, understanding HR-QoL of PKD patients and defining specific tools to assess it is crucial to delineate the value and the utility of new treatments. To better explore the burden of disease on patients' HR-QoL, Grace et al carried out a qualitative interview study of 21 adults with PKD.²⁰ The most common reported signs and symptoms were yellow eyes (19/21), tiredness (18/21), yellow skin (17/21) and low energy (13/21). Appearance, emotional and cognitive states, work, school, sleep and the ability to perform physical, social and leisure activities were all negatively affected by the disease. Moreover, recently published data from the PKD Natural History Study (NCT02053480) described the impact of PKD on HR-QoL and fatigue.⁵¹ HR-QoL was measured at 3 different time points (enrollment, 1- and 2-year follow-up) in 254 patients (131 adults and 123 adolescents and children), using tools validated from other anemias (EuroQol 5-Dimension Questionnaire, Pediatric Quality of Life Inventory Generic Core Scale version 4.0, and Functional Assessment of Cancer Therapy-Anemia) and fatigue (Patient Reported Outcomes Measurement Information System Fatigue and Pediatric Functional Assessment of Chronic Illness Therapy-Fatigue). Significantly worse HR-QoL outcomes were reported in regularly transfused adults, children with severe anemia or with non-missense/non-missense genotype, iron overload with chelation requirement and patients with pulmonary hypertension. Jaundice was also reported as a cause of upset both in children and in adults. Salek and colleagues conducted a systematic search of the literature to identify adequate patient reported outcome (PRO)s measures for HR-QoL and fatigue suitable for PKD trials.⁵² Conditions similar to PKD, such as SCD and thalassemia,

were comprised in the research. Based on the results, the European Organisation for Research and Treatment of Cancer Quality-of-life Questionnaire Core 30 (EORTC QLQ-C30) and the Short Form 36-item Health Survey Version 2 (SF-36v2[®]) were recommended for assessing HR-QoL in PKD. However, being not disease-specific, the EORTC QLQ-C30 and the SF-36v2[®] may not cover some symptoms of PKD or may include symptoms and impacts not suitable in PKD patients. Therefore, the same Authors proposed two disease-specific PRO measures in 2020.⁵³ Twenty-one PKD patients underwent concept elicitation and then cognitive interviews to develop the PK deficiency daily diary (PKDD) and the PK deficiency-specific impact assessment (PKDIA) survey. The first is a 7-item tool that measure main PK signs and symptoms, whilst the latter is a 14-item measure of PKD impact on patients' HR-QoL. Subsequently, PKDD and PKDIA were compared with the previously recommended tools EORTC QLQ-C30 and SF-36v2[®], reporting only minimal similarities (eg, in the EORTC QLQ-C30, 43% of concepts were similar to the PKDD and 42% to the PKDIA; in the SF-36v2[®], 57% of concepts were similar to the PKDD and 17% to the PKDIA). The PKDD and PKDIA appear reliable disease-specific tools to assess PROs and HR-QoL and will likely be implemented in clinical trials of PKD.

Conclusions

The landscape of PKD management is still marked by several challenges, particularly regarding the threshold of clinical suspicion, the sensitivity and availability of diagnostic tests, the management of complications, and the choice of treatment type and timing. Regarding the first, much has been done and much more is still needed to inform clinicians about the differential diagnosis of hemolytic disorders (Figure 2). It is fundamental to differentiate the various CHAs, since treatment may have different efficacy and risks. For instance, splenectomy is generally more effective in membrane defects versus PKD while it is contraindicated in hereditary stomatocytosis for the high thrombotic risk. The clinical suspicion allows to test PK enzymatic activity, particularly in patients without familiarity and unremarkable RBC morphology. Clinicians have to take into account that falsely normal activity may be observed and that the ratio with proximal enzymes activity (ie, hexokinase) may be helpful. The diagnosis is completed by the confirmation of the genetic defect that takes advantages of the use of NGS platforms. The latter may pose several interpretation challenges since genotype/phenotype association is not clearcut and variants of unknown significance continue to emerge. It is also important to reconsider congenital forms (even in adults) after all acquired forms have been excluded and vice versa; and also to take into account the possibility of the coexistence of acquired and congenital forms in the same patient.⁵⁴ Thereafter, patient management should be individualized considering the different ages, comorbidities, and frequency of complications (gallstone, hemolytic and aplastic crises, and iron overload) (Figure 1B). The need of transfusions, iron chelation, splenectomy, and cholecystectomy will be tuned accordingly. Specifically, transfusions should be triggered by growth retard and symptomatic anemia. Splenectomy is not as effective as in other CHAs, is discouraged during the first 6 years of age, and is usually avoided in elderly comorbid patients for the infectious and thrombotic risks. A further drawback of splenectomy is the poor response observed in subjects with more severe disease and highly disruptive mutations (ie, non missense ones). The allosteric PK stimulator mitapivat is a promising new option with very good responses in the majority of patients; however, responses are generally observed only in patients with at least one missense mutation, whilst those with more severe genotype/phenotype represent an unmet clinical need. Additionally, mitapivat has only been studied in adults and cannot be therefore directly translated into pediatric care. Regarding gene therapy, it might fill the gap in severe cases by substituting the defective enzyme; however, results are very preliminary and there is still uncertainty regarding long-term safety and efficacy. Early evidence seems to favor an early use in pediatric patients, and in young adults not candidates for mitapivat due to unfavorable genotype. On the whole, the timing of patient allocation to splenectomy, mitapivat and gene therapy is another challenge for the treating physician that will require future investigation.

Data Sharing Statement

All data are available within the manuscript and further may be available upon reasonable request to the corresponding author.

Consent for Publication

All Authors approved present submission.

Author Contributions

All authors made a significant contribution to the work reported, in the conception, design, execution, acquisition of data, analysis and interpretation; took part in drafting, revising and 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.

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Anaemia, Morphological Classification and Its Associated Risk Factors Among Lactating Mothers at Mbarara City Council Health Centre IV, Southwestern Uganda

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Purpose: The objectives of this study were to determine the prevalence of anaemia, morphological classification and its associated risk factors among the lactating mothers accessing postnatal care at Mbarara City Health Centre IV, Southwestern Uganda.

Patients and Methods: A cross-sectional study was conducted at Mbarara City Health Centre IV among 264 participants. A structured questionnaire was used to capture characteristics of study participants. 4 mL of venous blood was collected from each participant for complete blood count (CBC) and peripheral blood smear (PBS). CBC was done using hematology analyzer (BC-2800 Mindray Hematology Analyzer) while PBS were taken for participants with hemoglobin (Hb) <12.0g/dl and stained using Giemsa–Maygrunwald stain. Bivariate analysis and multivariate logistic regression were used to determine the associations between factors and anaemia. A p-value of <0.05 was considered significant.

Results: The overall prevalence of anaemia was 65 (24.6%), with mean hemoglobin (Hb) concentration of 12.5g/dL. Out of the anaemic participants (24.6%), morphological classifications of anaemia were as follows: normocytic normochromic 27 (41.5%), microcytic hypochromic 20 (30.8%), normocytic hypochromic 16 (24.1%) and macrocytic normochromic 2 (3.1%). According to the severity of anaemia, majority had mild anaemia 52 (80%), moderate anaemia was 11 (16.9%), and severe anaemia was 2 (3.1%). The following risk factors were significantly associated with anaemia: duration of lactation (between 5 to 8 months, p-value 0.017, 95% CI (0.12–0.82) and alcohol consumption (p-value 0.032, 95% CI (1.12–12.16). The rest of the variables had no association with anaemia.

Conclusion: The prevalence of anaemia among lactating mothers is high and it is a moderate public health problem as defined by WHO. Nutrition promotion, and health education, may be the ideal way to reduce the prevalence of anaemia in this region.

Keywords: anaemia, prevalence, risk factors, lactating mothers

Introduction

Anaemia is a condition in which blood hemoglobin concentration is below normal for age and sex with inadequate oxygen carrying capacity of red blood cells for normal physiological processes.¹ World Health Organization (WHO) defines anaemia in non-pregnant women as blood hemoglobin concentration <120 g/L and severe anaemia as blood hemoglobin concentration <70g/L.²

Anaemia is a global public health burden affecting about 1.74 billion people,³ with 29.6% of non-pregnant women anaemic worldwide.⁴ Anaemia has impacts on health as well as social and economic development of both rich and poor countries. Regional prevalence of anaemia among non-pregnant women ranges from 16% in high income countries to 48% in central and western Africa.⁵

Several studies have reported prevalence of anaemia among lactating mothers as: 32.7% in China,⁶ 60.3% in Myanmar in 2014⁷ and 73.8% in 2016,⁸ 62.9% in India⁹ and 8–24% in Denmark.¹⁰

Lactating mothers are vulnerable to anaemia because of iron depletion during lactation and blood loss during delivery.^{8,11} Breast milk iron concentration has been known to be maintained at the expense of the mother's iron stores and mothers who had anaemia during pregnancy have higher chances to develop anaemia during lactation.¹ East African region has reported 22%¹² and 25%¹³ prevalence of anaemia among postnatal mothers in Tanzania and Kenya, respectively, and the Horn of Africa is equally affected by anaemia in lactating mothers. Studies have estimated prevalence of anaemia among lactating mothers in Ethiopia to have increased from 21.8%¹⁴ and 22.1%¹¹ to 28.3%.¹⁵

Several factors have been found to predict anaemia in lactating mothers in low income countries and these include: iron deficiency, vitamin A, C, B₁₂ and folate deficiency, parasitic infection, multi-gravidity, antepartum anaemia, low income level, young lactating age and infectious diseases.^{1,6,7}

In Uganda the prevalence of anaemia among breastfeeding mothers is 34%.¹⁶ The regional variation of anaemia among non-pregnant women ranges from 16.9% in Kigezi to 47.1% in Acholi.¹⁷ There is scarcity of data on the prevalence of anaemia among lactating mothers in Mbarara district and in Uganda in general. The aim of this study was therefore to determine the prevalence of anaemia and its associated risk factors among lactating mothers at Mbarara City Health Centre IV (MC HC IV), Southwestern Uganda.

Materials and Methods

Study Design and Site

This was a cross-sectional study carried out at Mbarara City Health Centre IV (MC-HC IV) from October to December 2021, located in Mbarara City in Southwestern Uganda. MCHC-IV is a public health facility that serves the population of Mbarara city, including referrals from other low level health centres within the city. It offers services like: immunization, family planning, ante/postnatal care, prevention of mother-to-child transmission (PMTCT), HIV/AIDS clinic, general outpatient (OPD), minor surgeries for male circumcision and laboratory services.

Sample Size Calculation

The sample size for this study was estimated using the formula for a single population proportion, with the following assumptions: anaemia prevalence of 22% in lactating mothers,¹² at 95% confidence level, and 5% marginal error. Using the formula for sample size calculation for the proportion.

$$n = Z^2 P(1-P)/d^2$$

Where: n=the desired sample size

Z=critical values of normal distribution at 95%, which corresponds to 1.96

P=the proportion of the target population estimated to have anaemia

d=estimated margin of error 5%

$$n=264$$

Sample size was then adjusted for 10% non-response rate and the minimum sample size calculated was 290 lactating mothers. There was however low turn up to postnatal clinic so this was not attained but considering the fact that ratio of each variables under study to sample size was between 5 to 10, this sample size was adequate for multivariate level of analysis.

Sampling Procedure

Two hundred and sixty four (n=264) lactating mothers were randomly selected and subjected to structured questionnaires to obtain information on sociodemographic characteristics, dietary intake, mid upper arm circumference (MUAC), iron supplementation, number of antenatal and postnatal visits, use of treated mosquito nets, tea consumption, alcohol intake, deworming status, source of drinking water, and history of post-partum hemorrhage.

Laboratory Procedure

About 4mL of venous blood was collected aseptically into an EDTA vacutainer. Complete blood count was performed using (BC 2800 Mindray Hematology Analyzer), peripheral thin blood smears were made, fixed with absolute methanol and stained using 10% Giemsa–Maygrunwald stain for the morphological classification of anaemia.

Ethical Consideration

This study was approved by the Faculty Research Committee (FRC) of Mbarara University of Science and Technology and Mbarara City health officer (approval number MUST/MLS/023). Written Informed consent was obtained from the study participants and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (1964). Privacy and confidentiality was followed all through the study process and those with anaemia were referred to physicians for management.

Results

Demographic Characteristics of the Study Participants

The mean age was 26 years, 95% CI (25.4–26.7). The participants' ages ranged from 18 years to 45 years. Majority of the lactating mothers were aged between 20 to 29 years, 192 (72.7%), and those within the first 4 months of lactation were 169 (64%), mothers who had attained secondary education 126 (47.7%), married 252 (95.4%), and employed 155 (58.7%). Those with 1–2 parities, 195 (73.9%), and those with ≥ 4 antenatal visits during pregnancy were 214 (81.1%), as shown in Table 1.

Table 1 Demographic Characteristics of Study Participants

Variable	Category	Frequency (n)	Percentage (%)
Age (years)	15–19	12	4.6
	20–29	192	72.7
	30–39	57	21.6
	40–49	3	1.1
Duration of lactation (months)	1–4	169	64.0
	5–8	53	20.1
	9–12	36	13.6
	>12	6	2.3
Education level	None	6	2.3
	Primary	63	23.9
	Secondary	126	47.7
	Tertiary	69	26.1
Marital status	Single	11	4.2
	Married	252	95.4
	Widowed	1	0.4
Occupational status of mother	Unemployed	109	41.3
	Employed	155	58.7
Occupational status of husband	Unemployed	6	2.3
	Employed	258	97.7
Parities	1–2	195	73.9
	3–4	60	22.7
	>4	9	3.4
Monthly household income (UGX)	Not aware	69	26.1
	0–99,000	53	20.1
	100,000–999,000	111	42.1
	>999,000	31	11.7
Number of antenatal care (ANC) visits	<4	50	18.9
	≥ 4	214	81.1
MUAC measurements (cm)	<23.5	11	4.2
	≥ 23.5	253	95.8

Prevalence of Anaemia

The overall prevalence of anaemia among lactating mothers was 65 (24.6%), as shown in [Figure 1](#). According to WHO's definition of severity of anaemia, anaemia is classified as mild anaemia when the Hb is (10.0–11.9g/dL), moderate (7.0–9.9g/dL) and severe (<7.0g/dL). In this study, 2 (0.7%) had severe anaemia, 11 (4.2%) had moderate anaemia and 52 (19.7%) had mild anaemia.

Morphological Classification of Anaemia

Based on the peripheral blood reports, majority 27 (41.5%), of the participants had normocytic normochromic anaemia, 20 (30.8%) had microcytic hypochromic, 16 (24.1%) had normocytic hypochromic and 2 (3.1%) had macrocytic normochromic anaemia, as shown in [Figure 2](#).

Risks Factors Associated with Anaemia

Several factors considered to be associated with anaemia during lactation were studied. These included age, duration of lactation, education level, marital status, occupation of mother, occupation of spouse, number of parities, household income, use of mosquito bed net, history of postpartum haemorrhage, use of iron supplements, alcohol consumption, tea consumption, deworming status, source of drinking water, malnutrition (MUAC <23.5 cm), number of antenatal (ANC) visits and consumption of iron-rich food (meat, fish, green vegetables, fruits and eggs).

On bivariate logistic regression, the following variables were found to be associated with anaemia: age group (40–49 years) (OR: 68.25, p-value 0.037), duration of lactation of 5 to 8 months (OR: 0.31, p-value 0.014), alcohol consumption (OR: 5.0, p-value 0.016) and having been dewormed in the last 3 months (OR: 0.41 and p-value 0.045); see [Table 2](#).

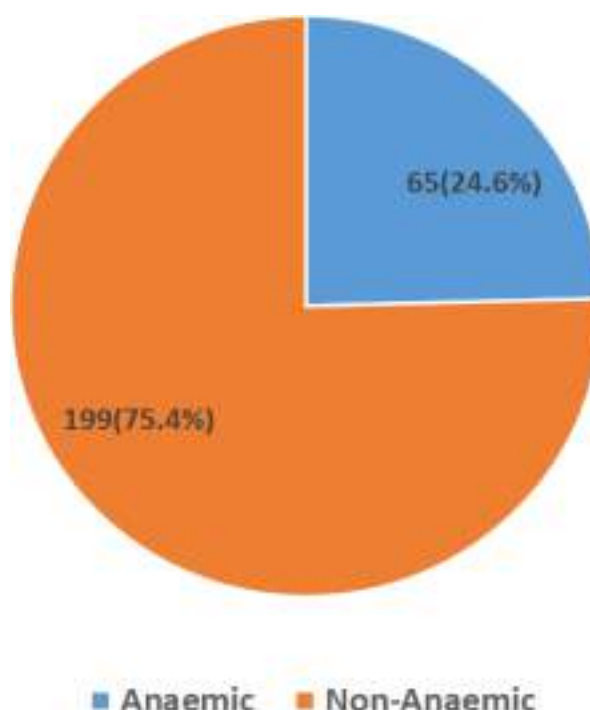


Figure 1 Pie chart showing prevalence of anaemia among lactating mothers at Mbarara City Health Centre IV.

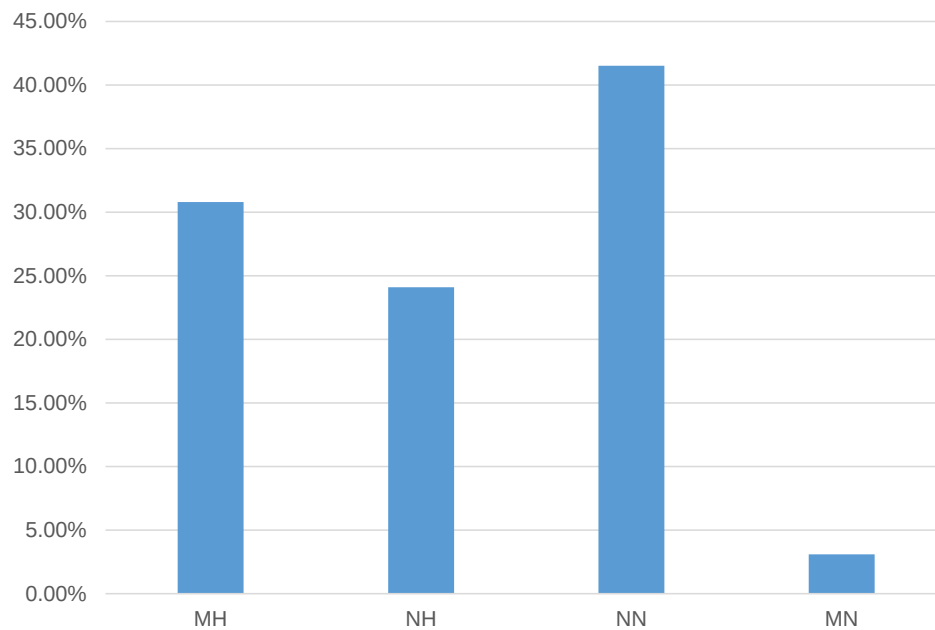


Figure 2 Morphological classification of anaemia among lactating mothers at Mbarara City Health Centre IV.

Abbreviations: MH, Microcytic hypochromic anaemia; NH, Normocytic hypochromic anaemia; NN, Normocytic normochromic anaemia; MN, Macrocytic normochromic anaemia.

The factors that were significantly associated with anaemia in the bivariate logistic regression analysis were then subjected to multivariate logistic regression. Duration of lactation of 5 to 8 months (OR: 0.32, p-value 0.017) and alcohol consumption (OR: 3.69, p-value 0.032) remained significantly associated with anaemia during lactation (Table 3).

Discussion

Prevalence of Anaemia

Anaemia in lactating mothers remains a public health burden affecting both the mother and the newborn.¹⁸ We determined the prevalence and risk factors of anaemia among lactating mothers at Mbarara City Health Centre IV (MCHC-IV). The overall prevalence of anaemia among lactating mothers was 24.6%. This prevalence is lower than the national prevalence of anaemia among lactating mothers in Uganda, which is 34%.¹⁶ The difference in prevalence in these two studies could be attributed to study design since the study across Uganda was a demographic health survey and this study was only in one region. The sample size in this study was also smaller than the health survey conducted among a very large number of study participants and differences in the techniques used in anaemia diagnosis.

This finding is comparable with studies from Kenya 25%,¹³ Tanzania with 22%¹² and several studies in Ethiopia 22.1%,¹¹ 28.3%¹⁵ and 28.9%.¹⁹ Our study was also similar to studies in Argentina and Nepal, with 25.8%²⁰ and 20%,²¹ respectively.

The prevalence of anaemia in this study is however lower than the prevalence reported in East Africa 36.5%,¹⁸ Myanmar 73.8%,⁸ India 62.9%,⁹ Binjai in Indonesia 52%,²² Ethiopia, 43%²³ and Somalia 48.7%.¹¹ The lower prevalence of anaemia could be attributed to geographical location, feeding habits, differences in sample size and socioeconomic status of these lactating mothers in these studies compared to ours.

Our study finding was however higher than the prevalence reported in Japan, 10.5%.²⁴ This higher prevalence in our study could be due to differences in socio-economic status of lactating women in Japan and Uganda. It can also be due to larger sample size, geographical location, retrospective cohort study design and larger time period employed in the Japanese study.

Table 2 Bivariate Analysis of Risk Factors Predisposing Lactating Mothers to Anaemia

Risk factor		Unadjusted Odds Ratio	95% Confidence Interval	P-value
Age (years)	15–19	1		
	20–29	3.67	0.45–29.74	0.223
	30–39	4.43	0.48–40.56	0.188
	40–49	68.25	1.28–3633.66	0.037*
Duration of lactation (months)	1–4	1		
	5–8	0.31	0.12–0.79	0.014*
	9–12	0.96	0.42–2.20	0.918
	>12	1.38	0.23–8.10	0.725
Education level	None	1		
	Primary	1.03	0.15–7.11	0.974
	Secondary	0.76	0.12–4.85	0.772
	Tertiary	0.54	0.08–3.76	0.534
Marital status	Single	1		
	Married	1.19	0.24–5.94	0.827
	Widowed	1		
Occupational status of mother	Not working	1		
	Working	0.73	0.40–1.33	0.309
Occupational status of husband	Not working	1		
	Working	0.35	0.05–2.22	0.264
Parity	1–2	1		
	3–4	0.92	0.42–1.99	0.826
	>4	0.44	0.04–4.37	0.482
Household income (Ugandan shillings)	Not aware	1		
	0–99,000	1.70	0.72–4.01	0.225
	100,000–1,000,000	0.60	0.28–1.31	0.201
	>1,000,000	0.48	0.14–1.66	0.245
Use of bed nets	No	1		
	Yes	2.72	0.37–19.81	0.322
Use of iron supplements	No	1		
	Yes	1.69	0.89–3.22	0.108
History of postpartum haemorrhage	No	1		
	Yes	1.40	0.66–2.93	0.378
Alcohol consumption	No	1		
	Yes	5.00	1.35–18.55	0.016*
Frequency of tea consumption	None	1		
	1–3	1.35	0.68–2.66	0.394
	>3	0.86	0.34–2.19	0.757
Dewormed last three months	No	1		
	Yes	0.41	0.17–0.98	0.045*
Source of drinking water	Tap	1		
	Borehole	0.30	0.03–2.63	0.276
	Well	1		
	Others	5.29	0.41–68.09	0.201
ANC visits	<4	1		
	≥4	0.69	0.31–1.56	0.376
MUAC measurement	<23.5	1		
	≥23.5	4.37	0.49–38.75	0.185
History of eating iron-rich foods	No	1		
	Yes	0.87	0.47–1.60	0.646

Note: *Significant.

Abbreviations: MUAC, Mid Upper Arm Circumference; ANC, Antenatal Care.

Table 3 Multivariate Analysis of Risk Factors Predisposing Lactating Mothers to Anaemia

Risk Factor	Adjusted Odds Ratio	95% Confidence Interval	P-value
Age (years)			
15–19	1		
20–29	3.38	0.42–27.40	0.254
30–39	3.86	0.45–33.26	0.219
40–49	18.51	0.62–554.30	0.093
Duration of lactation (months)			
1–4	1		
5–8	0.32	0.12–0.82	0.017*
9–12	1.26	0.55–2.88	0.578
>12	1.37	0.24–7.85	0.725
Alcohol consumption			
No	1		
Yes	3.69	1.12–12.16	0.032*
Dewormed last three months			
No	1		
Yes	0.45	0.20–1.02	0.055

Note: *Significant.

Morphological Classification of Anaemia

According to our study, most of the anaemic study participants had normocytic normochromic blood picture (41.5%), followed by microcytic hypochromic blood picture (30.8%), normocytic hypochromic blood picture (24.1%) and macrocytic normochromic blood picture (3.1%). This finding disagreed with a study that reported microcytic hypochromic anaemia as the most prevalent morphological type of anaemia among lactating mothers in Ethiopia.²³ Normocytic normochromic anaemia was the commonest type of anaemia in our study because lactating mothers lose blood during delivery and also are at risk of being malnourished during lactation. This blood picture could also be attributed to early stage of iron deficiency, in which majority of the red blood cells are normocytic normochromic. However, normocytic normochromic anaemia is also found in other conditions like chronic infection, inflammatory diseases, malignant diseases, renal failure, hypothyroidism, hypopituitarism, marrow failure, acute blood loss and rheumatoid arthritis.²⁵ These conditions can also happen in the lactating phase, which could have contributed to normocytic normochromic anaemia to being the major morphological type of anaemia in this study. Generally, there is a scarcity of literatures on morphological classification of anaemia in lactating mothers; most studies that studied anaemia in lactating mothers used Hb, serum iron levels and ferritin in determining anaemia.

Associated Risk Factors with Anaemia

Alcohol affects the production of red blood cells from bone marrow and promotes the production of abnormally shaped red blood cells that are destroyed earlier than their 120-day life cycle and this causes anaemia since destruction of abnormally shaped red blood cells reduces haemoglobin levels and reduces the oxygen carrying capacity of red blood cells that the body requires for normal physiology.²⁶ Alcohol consumption was found to be significantly associated with high odds of developing anaemia during lactation. This disagreed with a study done in agro-ecological zones of Ethiopia that studied drinking of Tella (local alcohol) and anaemia during lactation in rural Ethiopia and found no association.¹⁴

Lower duration of lactation that is 5 to 8 months were found to be significantly associated with lower odds of developing anaemia during lactation. This disagrees with a study that was done by Liyew et al in Ethiopia that showed that neither low nor high duration of breastfeeding was associated with anaemia during lactation.¹⁵ This could be due to health habit that since mothers lose blood during delivery, they are put on iron supplementation to

restore their haemoglobin levels when they give birth at MCC-HC IV, so this lowers their odds of developing anaemia.

Conclusion

The reported prevalence of anaemia among lactating mothers in this study is a moderate public health problem. Duration of lactation (5 to 8 months) and alcohol consumption were risk factors found to be associated with anaemia. Routine anaemia screening of all postnatal mothers and iron supplementation immediately after delivery is recommended. Lactating mothers are also advised to reduce alcohol consumption during lactation period. Further studies are recommended to explore the underlying causes of anaemia during lactation.

Limitations of the Study

This study did not collect data on factors such as malaria, tuberculosis and HIV infection that can also predispose lactating mothers to anaemia.

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Disclosure

The authors report no conflicts of interest in this work.

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Acute Malaria in Malawian Children and Adults is Characterized by Thrombocytopenia That Normalizes in Convalescence

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Acute Malaria in Malawian Children and Adults is Characterized by Thrombocytopenia That Normalizes in Convalescence

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Background: *Plasmodium falciparum* malaria has been linked with significant perturbations of the peripheral cell-mediated immune system during acute phase. Some of these changes include lower than normal platelet counts. Although the exact mechanisms that drive thrombocytopenia in *P. falciparum* malaria are not fully known, a number of hypotheses have been proposed. We conducted two sets of studies with one aimed at determining platelet counts in Malawian children, and the other in adults during acute *P. falciparum* malaria and a month post treatment.

Materials and Methods: We recruited a total of 113 HIV-uninfected children with acute malaria [n=54 with uncomplicated malaria (UCM), n=30 with severe malarial anemia (SMA), n=29 presenting with cerebral malaria (CM)]. We also recruited 42 HIV-uninfected healthy controls. Out of the 113 participants with malaria, 73 (65%) [n=34 (63%) UCM, n=21 (70%) SMA and n=18 (62%) CM] were successfully followed-up one month after treatment. A 5mL peripheral blood sample was collected for platelet count using HMX Haematological Analyzer analysis both at baseline (acute malaria) and at follow-up a month later. Platelet counts were also determined in blood samples of 106 HIV-uninfected adults, 47 of whom presented with UCM and 29 with severe malaria (SM) and these counts were compared to those of 30 healthy controls. Of the malaria cases, platelet counts for 44 UCM and 21 SM were determined again during follow-up a month after treatment.

Results: In both children and adults, platelet counts were significantly lower during acute disease compared to the levels in the healthy controls with the lowest levels observed in CM (children) or SM (adults). These lower than normal levels increased close to normal levels a month post treatment.

Conclusion: *P. falciparum* malaria in Malawian children and adults was characterized by profound thrombocytopenia which recovered during convalescence.

Keywords: acute malaria, platelet counts, children and adults, convalescence

Introduction

Although remarkable achievements have been made in reducing the devastating effect of malaria globally,^{1,2} each year *P. falciparum* malaria still causes deaths of thousands of children globally, with Sub-Saharan Africa accounting for most of these deaths.³ Clinical *P. falciparum* malaria can arbitrarily be divided into the following main categories: uncomplicated malaria (UCM), cerebral malaria (CM), severe malarial anemia (SMA), and respiratory distress (RD), with the last three considered severe forms of the disease.^{4,5} Although these four groups can be detected in isolation, some children present with malaria characterized by combined features of any two or more of these syndromes and other complications.^{4,5} Of these four groups, CM is linked with the poorest clinical outcome with mortality observed to be highest in this group, and with some CM cases who survive suffering severe complications⁴ such as severe neurological

sequelae and debilitating neurological impairments.⁶ However, the actual mechanistic basis of death in CM is yet to be fully elucidated.

Various studies have shown that *P. falciparum* malaria is characterized by profound perturbations of the cell-mediated immune system during acute stage,^{7–9} and some of the changes include significantly lower than normal peripheral platelet counts^{10–12}. Although the exact mechanisms that drive thrombocytopenia in *P. falciparum* malaria are not well characterized, several hypotheses have been proposed and these include increased platelet aggregation,¹³ microvascular sequestration,¹⁴ and endothelial activation.^{13,14}

A number of previous studies conducted in either children or adults presenting with acute malaria, have characterized either *P. falciparum* malaria or *P. vivax* malaria and a combination of these two with thrombocytopenia, with some^{15–17} even proposing that thrombocytopenia should either be used as an indicator of malaria in adults¹⁵ or be considered as one of the parameters to be used in classifying the severity of *P. falciparum* malaria disease.¹⁸ We conducted two sets of studies, one recruiting Malawian children and another adults, with the aim of determining absolute platelet counts at the time when they were presenting with acute *P. falciparum* malaria disease of different clinical types, and secondly during convalescence a month after treatment.

Materials and Methods

Study Participants

Pediatric Cohort

Study design and details of the study participants for this pediatric cohort have been reported previously.⁷ In summary, the study was conducted at Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW) and Department of Paediatrics, College of Medicine (CoM), University of Malawi (UNIMA), and Blantyre Malaria Project from November 2005 to April 2006. We recruited children who acutely presented with different clinical forms of malaria at Queen Elizabeth Central Hospital (QECH) either in the outpatient department (OPD) or short-stay ward or in children's malaria admission ward. Medically well children who were attending outpatient surgical clinics at QECH and Beit Cure International Hospital (BCIH), in Blantyre, Malawi, were also recruited. Recruitment of study participants was mainly done during the malaria season which in Malawi usually coincides with the rainy season (November/December of one year to March/April of the next year).

Firstly, for each potential study participant, informed written consent was obtained from the parent or guardian, after which they were examined by a research nurse and/or a clinical officer. Once the potential participant fulfilled the preset recruitment criteria for the study, baseline demographic data were collected and recorded and a 5mL peripheral blood sample was collected. All malaria participants, regardless of the clinical form of malaria, were then assessed for level of consciousness using the Blantyre Coma Score (BCS). The consciousness assessment was done several times for each participant starting from admission and followed by two to four hourly intervals during intensive clinical care. Although in total over forty children were prospectively enrolled into each of the four groups (UCM, SMA, CM and Healthy Controls), the data for some participants from each group were excluded from the final analysis either because they tested positive for HIV, or were asymptotically positive for malaria when included in the healthy control group, or the parents or guardians withdrew their consent after the child had already been recruited into the study.

For the purpose of this study and the other one that has already been reported⁷ we defined malaria as a clinical syndrome associated with febrile condition without any other apparent possible cause, which was confirmed by a thick blood film positive for *P. falciparum* asexual parasites on microscopy. Study participants who presented with CM had a BCS of two or less at admission and four hours later, while study participants for SMA and UCM and healthy controls had a score of five both times.⁷ SMA study participants had a blood hemoglobin concentration of 5 g/dl or less whereas study participants in UCM, CM and Healthy controls had a hemoglobin concentration above 5 g/dL.

All participants who tested positive for HIV infection during the screening stage were excluded from the study in order to eliminate HIV infection as a possible confounder and were immediately referred to the antiretroviral therapy clinic. Following exclusion of some study participants either because they were infected with HIV or due to detection of other infections that could be confounders, data from 113 children with malaria (54 with UCM, 30 with SMA, 29 with CM) and

Table 1 Demographic Details, Parasitemia Levels and Platelet Counts for the Study Participants of the Pediatric Cohort. The Presented Values are Medians with the Ranges Provided in Brackets

Clinical Group	Healthy Controls	Uncomplicated Malaria	Severe Malarial Anemia	Cerebral Malaria
Number	42	54	30	29
Died During Admission	–	0	1	4
Reviewed in Convalescence	–	34	21	18
Sex (M:F)	29:13	38:16	19:11	10:19
Age (months) (Range)	20 (5–76)	27 (6–58)	23 (5–38)	30 (5–84)
Parasites/ μ L blood) (Range)	0	52,300 (460–768,000)	3500 (20–296,000)	41,800 (900–517,000)
Blantyre Coma Score (Range)	5 –	5 –	5 –	1 (0–2)
Hemoglobin (g/dL) (Range)	11.2 (7.0–14.1)	9.3 (5.0–13.0)	3.9 (2.4–4.9)	7.7 (5.3–12.5)
Acute Platelet Counts ($\times 10^3$ cells/l) (Range)	441.0 (122.0–781.0)	199.0 (9.0–550.0)	98.0 (15.0–399.0)	85.5 (16.0–420.0)
Convalescent Platelet Counts ($\times 10^3$ cells/l) (Range)	– –	328.0 (65.0–711.0)	387.0 (56.0–537.0)	343.0 (154.0–500.0)

from 42 healthy controls were considered for analysis for this study. Out of the 113 children originally recruited with malaria, 73 (34 UCM, 21 SMA and 18 CM) were successfully followed-up a month after treatment and during the follow-up stage baseline demographic data were recorded and another 5mL peripheral blood sample was collected for analysis of platelet counts. Demographic data for all study participants whose data were used in this study are presented in Table 1.

Adult Cohort

Similarly, demographic details for the adult cohort have previously been reported.¹⁹ Malawian adults diagnosed with either uncomplicated malaria (UCM) or severe malaria (SM) were prospectively recruited to the study after obtaining consent from the patient or a legally able guardian. Recruitment was done at the Accidents, Emergency and Trauma (AET) Centre and general medical male and female wards of QECH from July 2016 to March 2017. Of the screened 116 adults who were infected with malaria, 107 consented to participate in the study. Demographic and other related data were collected and recorded using Case Report Forms (CRFs). After recruitment, a 10mL venous blood sample was collected from each participant with a 2mL aliquot collected in EDTA tubes which was then used for confirmation of malaria parasitemia by means of thick and thin films and for the analysis of platelet counts.

For this cohort, we defined malaria as a clinical syndrome in a febrile adult who had no other possible apparent cause of disease, later confirmed by a positive malaria rapid diagnostic test (MRDT) and a positive thick blood film for *P. falciparum* asexual parasites using microscopy. The confirmed positive malaria cases were then divided into two clinical groups; UCM and SM. The UCM group had confirmed clinical malaria based on positive malaria slides and MRDT but did not have any unarousable coma, or lower than normal hemoglobin (Hb) levels or liver or kidney complications associated with the malaria infection. Among the SM cases were those who presented with cerebral malaria (CM), severe malarial anemia (SMA) and those with liver or renal dysfunction. In line with the WHO guidelines,

Table 2 Demographic Details of the Adult Study Participants and the Median Platelet Counts for Each Malaria Group During Acute Malaria and in Convalescence a Month After Treatment

Total Participants = 106	Healthy Controls [HCs] (n=30)	Uncomplicated Malaria [UCM] (n=47)	Severe Malaria [SM] (n=29)
Female [number (%)]	12 (40)	22 (46.8)	13 (44.8)
Male [number (%)]	18 (60)	25 (53.2)	16 (55.2)
Median Age (Years) (Range)	25 (18–66)	27 (18–62)	26 (28–54)
Acute Platelet Counts ($\times 10^3$ cells/l) (Range)	234.0 (100.0–485.0)	113.0 (9.0–415.0)	60.5 (9.0–248.0)
Convalescent Platelet Counts ($\times 10^3$ cells/l) (Range)	– –	200.0 (130.0–330.0)	195.0 (136.0–330.0)

we defined adult CM cases as those with a Glasgow Coma Score (GCS) of <11 , with unarousable coma which could not be attributable to any other cause, and who were unable to localize stimuli and were incomprehensible to sounds. In contrast, malaria infected participants presenting with UCM or other forms of SM had a GCS score of greater than 11 at both times of consciousness assessment.

Although study participants presenting with severe SMA were included among the SM group, these had a blood Hb concentration of 7 g/dL or less or a hematocrit concentration of 20% or less together with a parasite count of more than 10,000/L, whereas the other clinical groups had an Hb concentration above this level. All malaria infected study participants were clinically and neurologically monitored during the entire period they were in the admission wards until the day of discharge or death.

Study participants were aged 18 years or more and were either out- or in-patients at QECH presenting with a temperature $>37.5^\circ\text{C}$, a positive MRDT with a positive thick and thin blood smear microscopy test and with signs consistent with malaria disease according to the WHO guidelines.²⁰ HIV-infected individuals, pregnant women, and all those who did not give consent were excluded from the study. The demographic details for the cohort are provided in Table 2. In summary, of the total 106 HIV-uninfected study participants recruited, 47 (62%) presented with UCM and 29 (38%) presented with SM, and 30 were healthy controls. Of the malaria cases, 44 UCM and 21 SM were seen again during convalescence a month after treatment.

Investigations

Primary and confirmatory HIV tests were done using two rapid test kits; Determine (Abbott Laboratories, Japan) and Unigold (Trinity Broth, Dublin). For confirmation of malaria parasites, both thick and thin blood smears were prepared by standard methods and screened on standard microscopy. Platelet counts were determined using a HMX Haematological Analyzer (Coulter, USA) using a blood sample collected in the EDTA tube. The HMX Haematological Analyzer performs a complete blood count and the analysis for platelet counts was conducted in accordance with a provided standard protocol and manufacturer's instructions. All analyses were done by an experienced Laboratory Technologist who had been certified to operate this automated Haematological Analyzer. In summary, an aliquot of the EDTA blood sample was sucked into the Haematological Analyzer and once the analysis was complete for each sample, results were provided in a print-out. On each day, a standard sample was run first before the analysis of samples from patients. The analysis for some samples was repeated in the event of platelet counts that would be regarded as outliers (either too low or too high) based on the manufacturers' standard protocol.

Ethics Review and Approval

For both the pediatric and adult studies, ethics review and approval of the study protocols were conducted by the College of Medicine Research and Ethics Committee (COMREC) of Kamuzu University of Health Sciences (KUHeS) previously known as College of Medicine. For the pediatric cohort, written informed consent was obtained from the parent or guardian of every child before the child was enrolled into the study. A 5mL venous blood sample was taken at the time of recruitment and a month after treatment. An aliquot of this blood sample was collected in EDTA tubes and it was this sample which was used for the determination of the platelet counts. For the adult study, each participant or an appropriate guardian provided informed written consent. From the 10mL venous blood sample that was collected from each participant, a 2mL EDTA aliquot was used for confirmation of malaria parasitemia using thick and thin films and for platelet count determination. Both the pediatric and adult studies were conducted in accordance with the Declaration of Helsinki.

Data Analysis

For statistical analysis, the data from the various clinical malaria groups (Healthy Controls, UCM, SMA and CM for children and healthy controls, UCM and SM for adults) were statistically analyzed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego California USA). For all statistical comparisons, a *p* value equal to 0.05 or less than 0.05 was considered statistically significant at 95% level confidence. The Kruskal–Wallis test was employed in the comparison of absolute platelet counts. Wilcoxon-matched pairs test was used to establish if the differences in platelet counts observed during acute infection and in convalescence were statistically significant for both pediatric and adult cohorts. The results in Figures 1 and 2 (and Tables 1 and 2) are medians and 10th and 90th percentiles of absolute platelet counts during acute and convalescent stages (except healthy controls) for the different study groups. For these scatter plots presented in Figures 1 and 2, the b line through the middle of the dots corresponds to the median value with the 10th percentile presented at the bottom of the scatter plot and the 90th percentile at the top).

Results

Variation of Platelet Counts in Different Malaria Types in Children

We have previously shown that the median platelet counts for children aged between 1–6 months and 3 to 7 years were 346×10^3 cells/L and 447×10^3 cells/L respectively,²¹ which compared well with the median platelet counts for healthy controls of the same age group of 441×10^3 cells/L observed in the pediatric cohort of this study. Platelet counts were markedly lower in all malaria groups during acute infection compared to the levels in controls (Figure 1A and Table 1). The counts were significantly ($p < 0.0001$) lower in UCM (199×10^3 cells/L), SMA patients (98×10^3 cells/L) and in CM patients (85.5×10^3 cells/L) compared to the controls (441×10^3 cells/L). There was no association between parasitemia levels (median values of 52,300 parasites/L of blood in UCM, 3500 parasites/L of blood in SMA and 41,800 parasites/L of blood in CM) with the platelet counts for the three groups (199×10^3 cells/L for UCM, 98×10^3 cells/L for SMA and 85.5×10^3 cells/L for CM) (Table 1).

Platelet counts were observed to be significantly ($p < 0.0001$) higher in convalescence than the values observed during acute disease for all three clinical malaria groups (328×10^3 cells/L versus 199×10^3 cells/L for UCM, 387×10^3 cells/L versus 98×10^3 cells/L for SMA and 343×10^3 cells/L versus 85.5×10^3 cells/L for CM) (Figure 1B and Table 1).

Variation of Platelet Counts in Different Malaria Types in Adults

Similar to the child cohort, we have also previously shown that in Malawian adults aged between 20 and >60 years, the median platelet counts ranged between 214×10^3 cells/L and 253×10^3 cells/L²¹ which also compared reasonably well with the median platelet counts for the healthy controls in this study (234×10^3 cells/L). Therefore, when compared to the counts in healthy children (441×10^3 cells/L), healthy adults had significantly ($p < 0.0001$) lower platelet counts (234×10^3 cells/L). Within the adult cohort, the absolute counts of these cells were significantly ($p < 0.0001$) lower in patients presenting with acute UCM (113×10^3 cells/L) and in those presenting with acute SM (60.5×10^3 cells/L) compared to those observed in healthy controls (234×10^3 cells/L) (Figure 2A and Table 2).

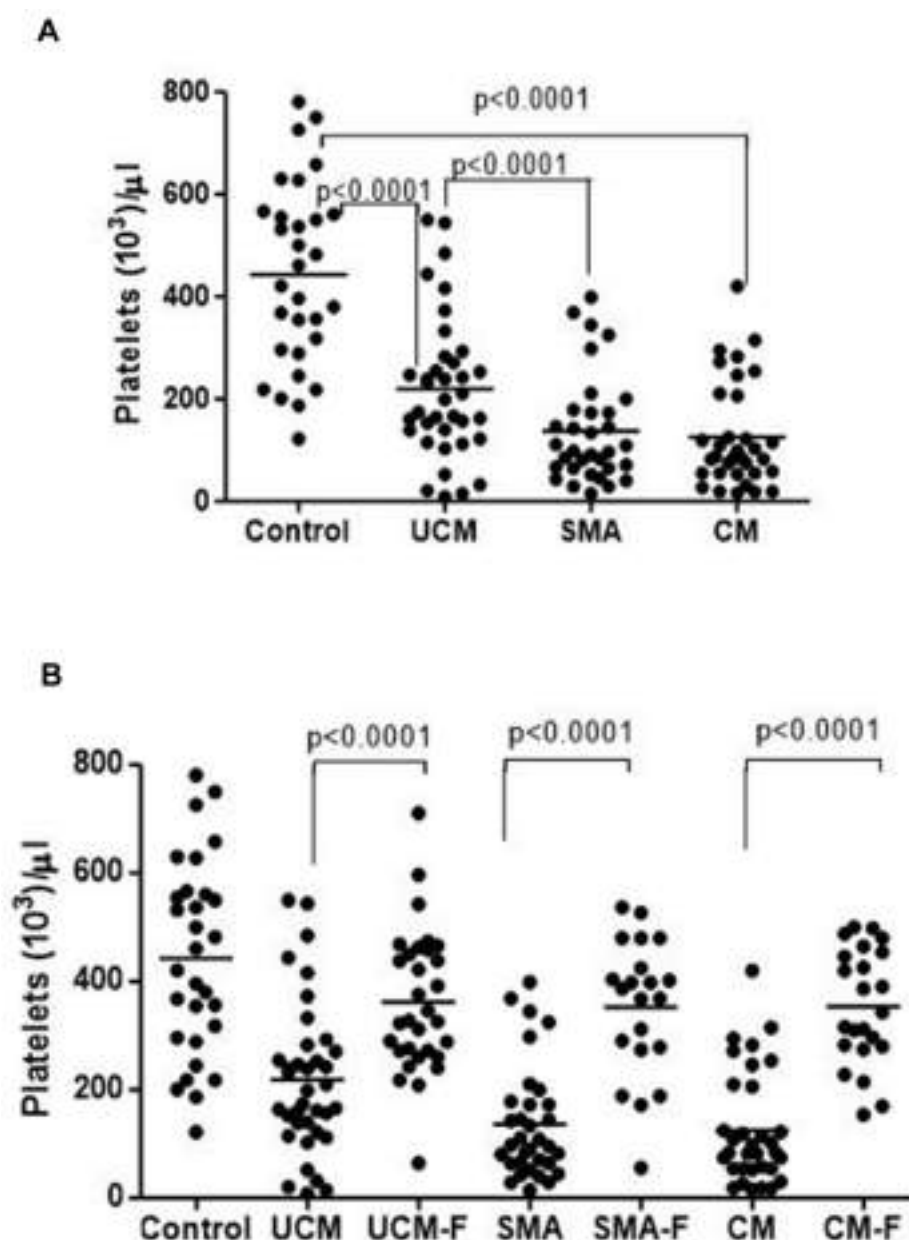


Figure 1 Medians (10th and 90th percentiles) of platelet counts in controls and different malaria groups (uncomplicated malaria (UCM), severe malarial anemia (SMA) and cerebral malaria (CM)) in acute disease (**A**) and during follow-up (UCM-F, SMA-F and CM-F) one month after treatment (**B**).

As was the case with the children, platelet counts were significantly ($p=0.0006$ for UCM versus UCM-F and $p=0.0012$ for SM versus SM-F) higher during convalescence compared to the values observed during acute disease for both UCM and SM (200×10^3 cells/L versus 113×10^3 cells/L for UCM and 195×10^3 cells/L versus 60.5×10^3 cells/L for SM) (Figure 2B and Table 2).

Discussion

Thrombocytopenia, defined as a platelet count of less than 150×10^3 cells/L, is a recognized characteristic of *P. falciparum* malaria in adults²² which, just as our results showed, has been reported to be common in malaria infected children²³ and in adults.¹⁵ Some have previously reported that platelet counts of less than 100×10^3 cells/L were linked to a higher risk of death in a pediatric Senegalese CM cohort.²⁴ Although the mechanisms that may contribute to thrombocytopenia are not

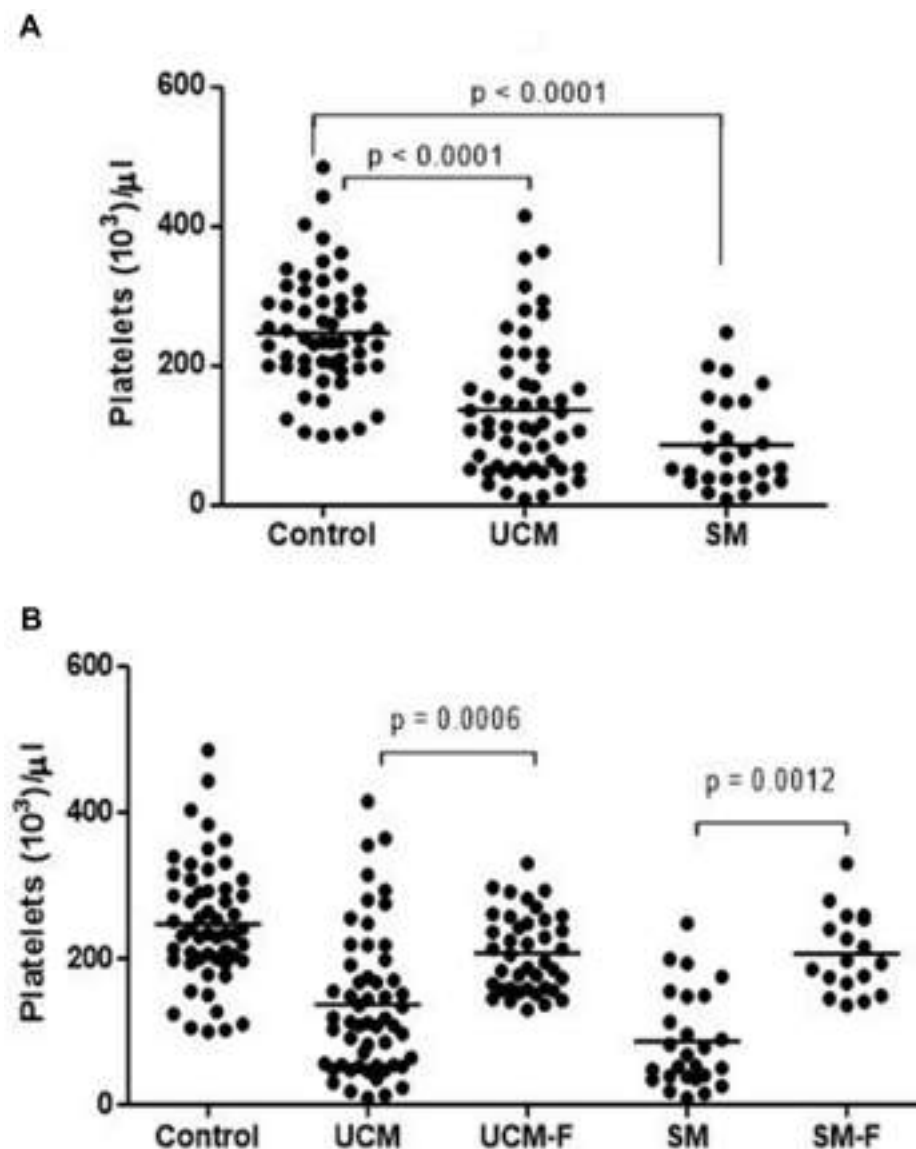


Figure 2 Medians (10th and 90th percentiles) of platelet counts in adult healthy controls (Control), in adults presenting with uncomplicated malaria (UCM) and adults presenting with severe malaria (SM) at recruitment (A), and in adults with uncomplicated malaria (UCM-F) and severe malaria (SM-F) during follow-up one month after treatment (B).

clear, sequestration of platelets and other factors in cerebral microvasculature have been linked with death in Malawian children.¹⁴

Furthermore, it is now known that platelets can adhere to endothelial cells by means of various molecules such as CD36. In addition to various endothelial cells and monocytes, human red blood cells which express CD36 are capable of binding to platelets. As such, CD36 could act as a receptor for some parasitized red blood cells (PRBCs) and could therefore indirectly play an important role in platelet and leucocyte sequestration in blood vessels in the brain.¹⁴ Platelets are also known to play a role in facilitating the clumping of PRBCs, a feature which is also known to characterize severe malaria.²⁵

This study's main finding, for both children and adults, showing that the number of platelet counts significantly increased in convalescence suggests that the malaria-associated thrombocytopenia is a transient phenomenon. Previous studies investigating *P. vivax* malaria²⁶ and *P. falciparum* malaria²⁷ have made a similar observation with the latter study showing that the lower than normal platelet counts observed during acute malaria increased significantly in both UCM

and SM, attaining normal levels within seven days post treatment. This rapid recovery therefore suggests that whether the platelets are contributing toward the pathogenesis of severe malaria¹⁴ or are involved in parasite clearance²⁸ once the malaria infection is cleared, there is a rapid re-emergence or formation of new platelets.

These two sets of studies of ours had a few limitations, the main one being the time difference between when the pediatric study was conducted (2005/2006) and when we recruited the adult cohort (2016/2017). Secondly, overall the sample sizes for both the child and adult cohorts were not as large as was the case in other previous studies. In addition, although we were able to divide the pediatric cohort (aged between 5 months and seven years) into three different clinical malaria groups (UCM, SMA and CM), for adults we only had two clinical groups (UCM and SM with the SM group combining SMA, CM and other severe manifestations of adult malaria). Furthermore, although we were able to confirm positivity for *P. falciparum* for both the pediatric and adult cohorts, we did not test for *P. vivax* malaria.

Although the overall prevalence of *P. vivax* malaria in Malawi is very low, with one recent study reporting its prevalence to be as low as 0.1% in adolescents and adults across the nation,²⁹ it is still possible that some of the adults might have been co-infected by both *P. falciparum* malaria parasites and *P. vivax* parasites at the time of recruitment. Lastly, although pseudothrombocytopenia is now recognized to be a common phenomenon in the field of blood analysis of samples collected from individuals presenting with various infectious diseases including malaria,³⁰ we did not account for this in our analyses. Nevertheless, we feel that the inclusion of analyzing samples from healthy controls adequately addressed this problem.

Conclusion

Thrombocytopenia is now a well-known and extensively studied characteristic of severe *P. falciparum* and *P. vivax* malaria that manifests during acute disease.^{31–33} In this report, we have shown that the thrombocytopenia that had previously been reported in different clinical forms of severe *P. falciparum* malaria in subjects of different age groups in other countries where malaria is endemic, also occurs in Malawians of different ages during acute malaria infection but disappears during convalescence a month post treatment.

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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.

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Disclosure

All authors for this study declare no conflicts of interest.

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Clinical Characteristics and Prognostic Risks of Philadelphia-Negative Myeloproliferative Neoplasms at Cipto Mangunkusumo General Hospital

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Background: Philadelphia-negative myeloproliferative neoplasms (Ph-MPNs) are clonal hematopoietic cell malignancy that comprises polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). They prone to develop thrombosis, bleeding, fibrotic progression, and leukemic transformation. We aimed to study clinical characteristics, thrombosis complications, and prognostic risk in Indonesians with Ph-MPNs.

Methods: This was a single-center retrospective cross-sectional study, including patients with Ph-MPNs who attended Hematology and Medical Oncology outpatient clinic at Cipto Mangunkusumo General Hospital, Jakarta, between 2016 and 2021. Demographic data, clinical characteristics, previous antiplatelet treatment, baseline laboratory data, JAK2V617F mutation, and treatment were reviewed for analysis. The prognostic risk model was assessed for PV (IPSS), ET (IPSET-thrombosis), and PMF (IPSS and DIPSS) patients.

Results: One hundred forty-six patients were classified as PV (31.5%), ET (38.4%), and PMF (30.1%) with median ages of 54, 53.5, and 55 years, respectively. PMF presented with the most diverse clinical presentations. JAK2V617 mutations were detected in 82%, 42.5%, and 76.5% of PV, ET, and PMF patients. PV had the highest thrombosis event pre/at diagnosis (26.1%), with predominantly arterial thrombosis. Bleeding occurred more in PMF (20.5%). Phlebotomy and hydroxyurea are the only treatment available for Ph-MPNs in the government hospital in Indonesia. IPSS intermediate risk in PV (47.8%), IPSET-thrombosis low risk in ET (46.4%), and IPSS and DIPSS intermediate-2 in PMF (38.6% and 50% respectively) were the most common risk groups.

Conclusion: Essential thrombocythemia was the most prevalent Ph-MPNs in Indonesia. Arterial thrombosis was the highest thrombosis event pre/at diagnosis in PV despite IPSET-thrombosis low risk being the most common risk group. In JAK2V617 mutation-positive MPN population, thrombosis event was also the highest in PV.

Keywords: Ph-MPNs, clinical characteristics, thrombosis, prognosis

Introduction

Philadelphia-negative myeloproliferative neoplasms (Ph-MPNs) are a group of hematopoietic malignancies which encompasses polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF).¹ All entities of Ph-MPNs shared a feature of mutations that is associated with physiologic pathways for hematopoiesis, as the disease arises from single hematopoietic stem cell that underwent somatic mutation.²

A study of Ph-MPNs in 17 countries unveiled the prevalence of Ph-MPNs in 2016 of 64.14 per 100,000 and the incidence cases of 12.05 per 100,000.³ Another study reported the incidence rates of Ph-MPNs per 100,000 population were between 1.0 and 1.5 for PV, 2.0 and 3.0 for ET, and 0.3 and 0.5 for PMF.⁴

In the initial stage, Ph-MPNs generally appear with no symptoms, thus usually diagnosed accidentally based on routine laboratory findings or when symptoms or complications occurred.^{5,6} These complications influence the survival and prognosis of MPN patients.

Patients with Ph-MPNs experienced poor quality of life due to symptoms that affect general activity, anxiety, mood disorder, and cancelation of life plan.^{7,8} On top of that, those with Ph-MPNs have an increased risk of morbidity and mortality compared to the general population. The relative survival ratios (RSR) of 10 years was 0.64, 0.68, and 0.21 for patients with PV, ET, and PMF, respectively.⁹ Previous prospective study showed that the median life expectancy among patients with Ph-MPNs was 91.7 months (7.5 years).¹⁰

Thrombosis and major cardiovascular adverse events are important causes of mortality in Ph-MPNs patients.^{11,12} Moreover, patients with Ph-MPNs are at risk for developing leukemic and fibrotic progression which contribute further to the morbidity and mortality of the patients.^{13,14}

Given the insufficient data on Ph-MPNs in Indonesia, we conducted this study to find out the clinical characteristics, thrombosis complications, and prognosis in Indonesian with Ph-negative MPN.

Methods

This cross-sectional study was performed at Cipto Mangunkusumo General Hospital, Jakarta. The information of all Ph-negative patients (PV, ET, and PMF) who have attended the Hematology and Medical Oncology outpatient clinic from 2016 until 2021 were retrospectively retrieved from medical records, including demographic data (age, sex), clinical characteristics (comorbidities, signs and symptoms, thrombosis and bleeding events pre/at diagnosis, previous antiplatelet treatment), baseline laboratory data at diagnosis (complete blood count), JAK2V617F mutation status, and treatment administered.

The status of JAK2V617D mutation was determined qualitatively using RNA extraction kit which employs silica-based membrane followed by cDNA synthesis, endpoint/conventional PCR and capillary/Sanger sequencing. This test has up to 20% sensitivity to detect mutation within the samples. It has 100% specificity to detect V617F mutation.

For PV patients, the International Study of PV¹⁵ was applied for estimated survival ([Supplementary Table 1](#)). For ET patients, the International Prognostic Score for ET-thrombosis (IPSET-thrombosis)¹⁶ was evaluated for thrombosis risk ([Supplementary Table 2](#)). For PMF patients, the International Prognosis Scoring System (IPSS)¹⁷ and Dynamic IPSS (DIPSS)¹⁸ were calculated for estimated survival ([Supplementary Table 3](#)).

This study was conducted in accordance with Declaration of Helsinki, and the study protocol was approved by the Local Ethics Committee of Faculty of Medicine Universitas Indonesia (No. KET-558/UN.2.F1/ETIK/PPM.00.02/2021). Verbal informed consent was obtained from all subjects before blood sampling. Since our study did not apply any intervention, The Ethical Committee waived the requirement for written informed consent. The data were analyzed by descriptive statistics. The data were expressed in frequency (percentage, %) for categorical variables and median (range) for continuous variables. All analysis was performed using SPSS version 24 for Mac.

Results

We identified 146 patients with Ph-negative Ph-MPNs, including 46 patients with PV (31.5%), 56 patients with ET (38.4%), and 44 patients with PMF (30.1%). Baseline clinical and laboratory characteristics of the subjects are represented in [Table 1](#). The median age of PV, ET, and PMF patients were 54, 53.5, and 55 years, respectively. PV and ET occurred more in males.

Asymptomatic cases were found in 10.9% of PV, 16.1% of ET, and 4.5% the of PMF patients. The most common signs and symptoms in PV patients were dizziness/headache (43.5%), splenomegaly (34.8%), and abdominal symptoms (17.4%). Similarly, most ET patients had dizziness/headache (41.1%), abdominal symptoms (23.2%), and splenomegaly (21.4%). PMF patients presented with splenomegaly (68.2%), abdominal symptoms (52.3%), fatigue (40.9%), and fever (15.9%).

Regarding complete blood count parameters, the median hemoglobin and hematocrit of PV patients were 18.05 g/dL and 57%. Median platelet counts in ET and PV patients were $1120 \times 10^9/L$ and $638 \times 10^9/L$. Patients with PMF had a wide range of WBC and platelet counts with median WBC of $16.2 \times 10^9/L$ (range $0.79\text{--}65.12 \times 10^9/L$) and median platelet count of $220 \times 10^9/L$ (range $24\text{--}1851 \times 10^9/L$).

Due to lack of testing, JAK2V617F mutation analyses could not be reported in a total of 43 patients. The majority of PV were positive for JAK2V617 mutation (82%), and only 42.5% of ET and 76.5% of PMF harbored this mutation. About 84.8% of PV,

Table 1 Baseline Clinical and Laboratory Characteristics of 146 Subjects

	PV, n (%) 46 (31.5%)	ET, n (%) 56 (38.4%)	PMF, n (%) 44 (30.1%)
Age (years), median (range)	54 (22–72)	53.5 (23–79)	55 (22–72)
Female sex	21 (45.7%)	27 (48.2%)	22 (50%)
Symptoms			
Fever	4 (8.7%)	4 (7.1%)	7 (15.9%)
Night sweat	0 (0)	0 (0)	0 (0)
Weight loss	2 (4.3%)	3 (5.4%)	8 (18.2%)
Fatigue	0 (0)	11 (19.6%)	18 (40.9%)
Dizziness/ headache	20 (43.5%)	23 (41.1%)	3 (6.8%)
Abdominal symptoms	8 (17.4%)	13 (23.2%)	23 (52.3%)
Others ^a	56 (54.3%)	22 (39.3%)	11 (25%)
Asymptomatic	5 (10.9%)	9 (16.1%)	2 (4.5%)
Splenomegaly	16 (34.8%)	12 (21.4%)	30 (68.2%)
Schuffner 1–4	15 (93.75%)	12 (100%)	25 (83.3%)
Schuffner >4	1 (6.25%)	0 (0)	5 (16.7%)
Laboratory (median, range)			
Hemoglobin (g/dL)	18.05 (15.4–24.5)	13.55 (7.1–17.2)	8.9 (3.8–14.8)
Hematocrit (%)	57 (47.7–75.5)	40.5 (12.6–50.8)	28.7 (11.1–46.3)
WBC count ($\times 10^9/L$)	18.63 (3.24–46.91)	16.12 (6.78–52.54)	16.2 (0.79–65.12)
Platelet count ($\times 10^9/L$)	638 (110–2146)	1120 (536–2168)	220 (24–1851)
JAK2V617F mutation status			
Positive	32 (82%)	20 (42.5%)	13 (76.5%)
Negative	7 (18%)	27 (57.5%)	4 (23.5%)
Comorbidity			
Yes	39 (84.8%)	44 (80.4%)	27 (61.4%)
Cardiovascular disease	15 (32.6%)	9 (16.1%)	8 (18.2%)
Hypertension	25 (54.3%)	27 (48.2%)	11 (25%)
Diabetes mellitus	2 (4.3%)	3 (5.4%)	3 (6.8%)
Smoking	6 (13%)	10 (17.9%)	3 (6.8%)
Others ^b	14 (30.4%)	23 (41.1%)	17 (38.6%)
No	7 (15.2%)	11 (19.6%)	17 (38.6%)
Treatment			
Observation	5 (10.9%)	2 (3.6%)	29 (65.9%)
Phlebotomy	8 (17.4%)	1 (1.8%)	0
Hydroxyurea	10 (21.7%)	49 (87.5%)	15 (34.1%)
Phlebotomy + hydroxyurea	23 (50%)	4 (7.1%)	0

Notes: ^aErythromelalgia, edema, ecchymosis, epistaxis, dyspnea, hemiparesis; ^bLiver failure, hepatitis, chronic renal failure, asthma, thalassemia.

Abbreviations: WBC, white blood count; NA, not applicable.

80.4% of ET, and 61.4% of PMF patients had at least one comorbidity; hypertension and cardiovascular disease were the most prevalent.

Table 2 shows the thrombosis and bleeding events in our patients. A 26.1% of PV patients experienced thrombosis pre/at diagnosis, which was more common than in ET and PMF patients (21.4% and 4.5%, respectively). Arterial thrombosis was more prevalent than venous thrombosis, primarily manifesting with ischemic stroke. Bleeding events pre/at diagnosis occurred more frequently in PMF (20.5%) and ET (10.7%) patients, manifesting commonly as gastrointestinal hemorrhage and skin and/or mucosal bleeding. About 30% of PV and ET patients had been prescribed antiplatelet therapy, either aspirin and/or clopidogrel. Table 3 shows thrombosis and bleeding events on the JAK2-positive MPN subjects. Similarly, thrombosis in patients with positive JAK2 V617F mutation was more common in PV (21.8%), compared to ET (15%) and PMF (7.7%).

Table 2 Thrombotic and Bleeding Events of 146 Subjects

	PV, n (%) 46 (31.5%)	ET, n (%) 56 (38.4%)	PMF, n (%) 44 (30.1%)
Thrombotic events pre/at diagnosis	12 (26.1%)	12 (21.4%)	2 (4.5%)
Ischemic stroke	11 (23.9%)	7 (12.5%)	2 (4.5%) ^b
Acute myocardial infarction	1 (2.2%)	3 (5.4%)	1 (2.3%) ^b
Peripheral arterial disease	1 (2.44%)	0 (0)	0 (0)
Acute limb ischemia	0 (0)	0 (0)	1 (2.3%) ^b
Deep vein thrombosis	0 (0)	1 (1.8%)	0 (0)
Recurrent pregnancy loss	0 (0)	1 (1.8%)	0 (0)
Bleeding events pre/at diagnosis	2 (4.3%)	6 (10.7%)	9 (20.5%)
Cerebral hemorrhage	1 (2.2%)	2 (3.6%)	0 (0)
Gastrointestinal hemorrhage	1 (2.2%)	0 (0)	6 (13.6%)
Skin and/or mucosal bleeding ^a	0 (0)	4 (7.1%)	3 (6.8%)
Previous antiplatelet treatment, n (%)	14 (30.4%)	21 (37.5%)	3 (6.8%)

Notes: ^aEcchymosis, epistaxis, gum bleeding, ^bOne patient developed three thrombosis events.

Table 3 Thrombotic and Bleeding Events of 65 JAK2V617F Mutation-Positive Subjects

	PV, n (%) 32 (49.2%)	ET, n (%) 20 (30.8%)	PMF, n (%) 13 (20%)
Thrombotic events pre/at diagnosis	7 (21.8%)	3 (15%)	1 (7.7%)
Ischemic stroke	7 (21.8%)	2 (10%)	1 (7.7%) ^b
Acute myocardial infarction	1 (3.1%)	1 (5%)	1 (7.7%) ^b
Peripheral arterial disease	1 (3.1%)	0 (0)	0 (0)
Acute limb ischemia	0 (0)	0 (0)	1 (7.7%) ^b
Deep vein thrombosis	0 (0)	1 (5%)	0 (0)
Recurrent pregnancy loss	0 (0)	0 (0)	0 (0)
Bleeding events pre/at diagnosis	2 (6.2%)	2 (10%)	3 (23.7%)
Cerebral hemorrhage	1 (3.1%)	1 (5%)	0 (0)
Gastrointestinal hemorrhage	1 (3.1%)	0 (0)	3 (23.7%)
Skin and/or mucosal bleeding ^a	0 (0)	1 (5%)	0 (0)
Previous antiplatelet treatment, n (%)	9 (28.1%)	8 (40%)	2 (15.4%)

Notes: ^aEcchymosis, epistaxis, gum bleeding, ^bOne patient developed three thrombosis events.

Table 4 IPSS in 46 PV Subjects

	n (%)
Age >66 years	4 (8.7)
Age 57–66 years	14 (30.4)
WBC $\geq 15 \times 10^9/L$	34 (73.9)
Previous venous thrombosis	0 (0)
IPSS score	
0 (low risk)	8 (17.4)
1–2 (intermediate risk)	22 (47.8)
≥ 3 (high risk)	16 (34.8)

Tables 4–6 display the distribution of patients in the prognostic model risk category in PV, ET, and PMF subjects, respectively. By applying the IPSS stratification in PV subjects, most of the patients were distributed between intermediate- and high-risk groups (47.8% and 34.8% respectively). When patients with ET were classified using IPSET-thrombosis model, most of the patients were distributed between low- and high-risk groups (46.4% and 32.1%

Table 5 IPSET-Thrombosis Model in 56 ET Subjects

	n (%)
Age ≥60 years	8 (14.3)
Previous thrombosis event	12 (21.4)
Cardiovascular risk ^a ≥1	33 (58.9%)
JAK2V617 mutation	20 (35.7)
IPSET-thrombosis score	
0–1 (low risk)	26 (46.4)
2 (intermediate risk)	12 (21.4)
≥3 (high risk)	18 (32.1)

Note: ^aHypertension, diabetes mellitus, and/or tobacco smoking.

Table 6 IPSS and DIPSS Model in 44 PMF Subjects

	n (%)
Age >65 years	5 (11.4)
Constitutional symptoms ^a	20 (45.5)
Hemoglobin <10 g/ dL	32 (72.7)
WBC >25×10 ⁹ /L	10 (22.7)
Circulating blast ≥1%	9 (20.5)
IPSS score	
0 (low risk)	5 (11.4)
1 (intermediate-1 risk)	13 (29.5)
2 (intermediate-2 risk)	17 (38.6)
≥3 (high risk)	9 (20.5)
DIPSS score	
0 (low risk)	5 (11.4)
1–2 (intermediate-1 risk)	15 (34.1)
3–4 (intermediate-2 risk)	22 (50)
5–6 (high risk)	2 (4.5)

Note: ^aFever, fatigue, and/or weight loss.

respectively). Using IPSS and DIPSS model in PMF, the majority of the patients were classified into the intermediate-2 risk group.

Figure 1 shows the thrombotic and bleeding events of all PV subjects according to IPSS score. The thrombotic risk appears to be similar between low-risk, intermediate-risk and high-risk groups. Figure 2 shows that in all patients with ET, both the risk for thrombosis and bleeding rise gradually as the risk stratification increases.

Discussion

This study presents the clinical manifestation of Indonesian with Ph-negative Ph-MPNs. ET was the most frequent Ph-MPNs found in our study (38.4%). A study by Valladares et al also revealed similar results.¹⁹ There were 46 PV patients included in this study with the median age at diagnosis of 54 years, which was slightly lower than the median age from the International Study of PV (61 years).¹⁵ In addition, male-to-female ratio was close to 1:1, which was similar to the previous studies.^{15,20} For ET patients, the median age in our study was similar to the median age in IPSET-thrombosis study (53.5 vs 54.4 years).¹⁶

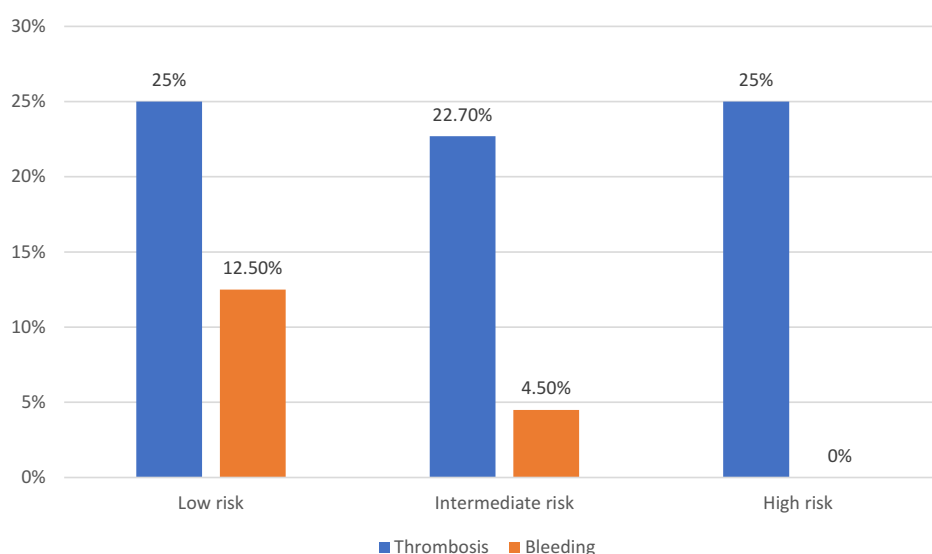


Figure 1 Thrombotic and bleeding events of 46 PV subjects according to IPSS score.

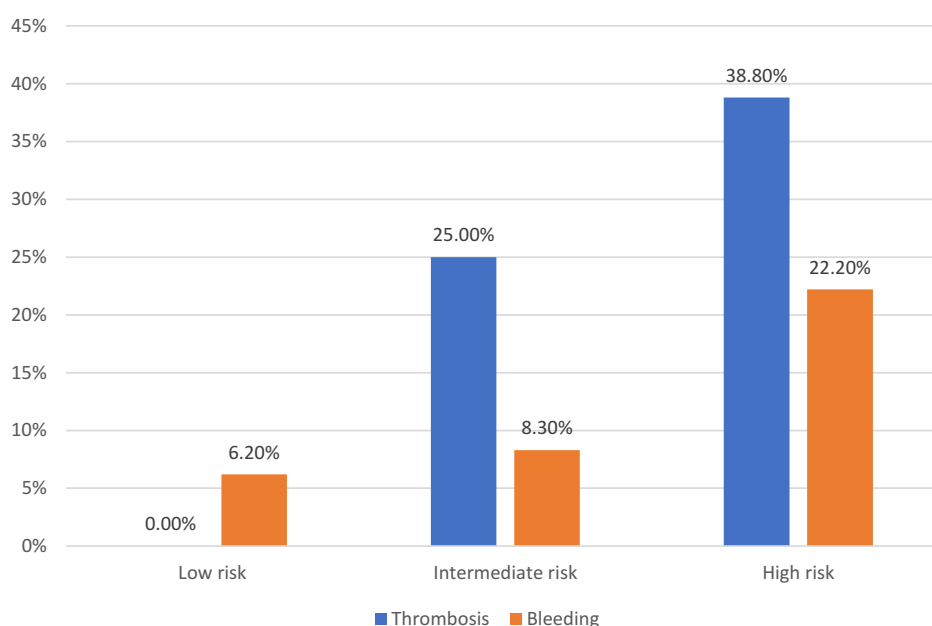


Figure 2 Thrombotic and bleeding events of 56 ET subjects according to IPSET-thrombosis score.

The median age of PMF in our study was 55 years, which was lower than the result of a study using the Mayo Clinic database for PMF and Porto-Soares et al (mean age of 65 and 64.7 years, respectively).^{21,22} The predominant symptoms of PMF were abdominal symptoms, fatigue, and splenomegaly. In the present study, patients with PMF, on average, had the greatest decrease in hemoglobin (anemia) and a wider range of WBC and platelet counts. Gangat et al and Duangnapasatit et al reported similar results.^{21,23} Cytopenias are essentially a difficult clinical problem in PMF. Anemia is typically presented in approximately 30% of the patients with PMF at diagnosis, and eventually all the patients develop anemia.²⁴ The pathogenesis of anemia in PMF is incompletely understood, the contributing factors

include ineffective erythropoiesis due to bone marrow scarring, aberrant inflammatory cytokine expression, and marked splenomegaly.²⁵

The JAK2V617F mutation is an acquired somatic mutation that is found in the vast majority of PV patients and in half of ET and PMF patients.²⁶ We found a lower percentage of JAK2 positive for PV and a higher percentage of JAK2 positive for PMF compared to multiple previous studies by Porto-Suares et al (93.5% of PV, 48% of ET, 60.8% of PMF), Valladares et al (86.6% of PV), and Gangat et al (66% of PMF).^{19,21,22} JAK2 is involved in cellular signaling processes, such as cell proliferation, differentiation, survival, and self-renewal. Mutated JAK2 cells confer hypersensitivity to several cytokines, including erythropoietin, thrombopoietin, granulocyte colony-stimulating factor. This may explain why patients with JAK2 mutation had a predominant rise in several blood parameters, including hemoglobin, hematocrit, and/or platelets, thus associated with thrombosis.^{27–29} In this study, we also evaluate thrombotic and bleeding events among 65 JAK2V617F mutation-positive subjects (Table 3) and found similar risk of thrombosis and bleeding compared to all patients regardless of their mutation status. Mutational analysis of JAK2V617F is not reimbursable by National Health Insurance, therefore not every patient was tested. Additionally, ischemic stroke is the most common thrombotic event in all population and JAK2V617F-positive population.

High hematocrit and WBC counts affect blood viscosity and blood flow, and further induce platelet activation and thrombosis.^{30,31} In addition, clinical factors, such as older age, obesity, history of thrombosis event, hypertension, dyslipidemia, contribute to the increased risk of thrombosis.¹¹ In the present study, thrombotic events pre/at diagnosis were more pronounced in PV (26.1%) and followed by ET (21.4%) as PV patients had higher levels of hemoglobin, hematocrit, and WBC counts. Arterial thrombosis occurred at higher rates than venous thrombosis. This finding is in line with studies from Malaysia and the IPSET-thrombosis study, despite most studies in the Western countries revealed a higher rate of venous thrombosis.^{32,33} This is most probably because of a higher rate of arterial risk factors in older patients, such as hypertension, diabetes, or dyslipidemia. Hypertension dominated as the preexisting risk factor of thrombosis in our patients.

A prognostic model for PV with three-tiered categories was developed by the International Study of PV, and the median survivals were 26 years (in low risk), 15 years (in intermediate-risk), and 8.3 years (in high-risk).¹⁵ In our study, 47.8% of PV patients fell into intermediate-risk categories. The survival risk score was significantly associated with thrombosis.²³ However, in our study, thrombotic risk did not differ significantly between IPSS risk groups (Figure 1). This is because the study population had very high prevalence of comorbidities (84.8%), including those with low risk. Therefore, thrombosis occurs even in low-risk individuals. Thrombotic complications are one of the most common causes of death in patients with PV. The International Study of PV reported that 32 out of 183 patients (20%) with a known cause of death deceased due to thrombotic complications.¹⁵ Therefore, cytoreductive therapy is recommended for high-risk PV.³⁴

Advanced age (≥ 60 years), previous thrombotic event, cardiovascular risk factors (hypertension, diabetes mellitus, tobacco use), and JAK2V617F mutation are well-characterized predictors for thrombotic complications in patients with ET.^{34,35} The IPSET-thrombosis study incorporates those risk factors in the international prognostic scoring system for thrombosis. In the present study, when ET patients were stratified using IPSET-thrombosis model, 46.4%, 21.4%, and 32.1% of them were part of low-risk, intermediate-risk, and high-risk groups, respectively. Moreover, the risk for thrombosis and bleeding increased incrementally along the higher risk scores (Figure 2). Sevindik et al revealed that 33%, 26.8%, and 40.2% of the patients were categorized into low-, intermediate-, and high-risk groups. Furthermore, one or more thrombotic episodes occurred in 5.4% of low-risk, 26.7% of intermediate-risk, and 66.2% of high-risk groups.³⁶

The estimated median survival of PMF is 6 years, but it can be a few months to years. In clinical practice, IPSS is used at diagnosis to predict survival in patients with PMF. Based on the four risk categories, the median survivals were 135 months (in low risk), 95 months (in intermediate-1 risk), 48 months (in intermediate-2 risk), and 27 months (in high risk). During the disease course, patients may acquire additional risk factors that may interfere patient's prognosis. In the present study, the proportion of patients in four prognostic groups using IPSS was similar to the previous study.¹⁷

In current practice, the therapy in PV and ET is intended to alleviate symptoms and prevent thrombosis. Aspirin, cytoreductive drug (in high-risk ET and high-risk PV), and phlebotomy (in PV with intended hematocrit target $<45\%$) have been shown to have an antithrombotic effect.³⁷ In our study, previous antiplatelet therapy was recorded in 21

(37.5%) patients with ET and 14 (30.4%) patients with PV. While there is a clear benefit of antiplatelet therapy in patients with PV, studies revealed inconsistent results regarding the benefit of antiplatelet therapy in patients with ET.³⁸ Another study reported that antiplatelet therapy did not lower the thrombosis risk but increased the risk of bleeding in patients with ET.³⁹ In the present study, bleeding events pre/at diagnosis occurred in 9 (20.5%) of PMF patients and 6 (10.7%) of ET patients, despite the low usage of antiplatelets in these groups compared with PV. Moreover, there was 5 (8.9%) patients with ET who were initially treated with phlebotomy due to increased hematocrit. After hematocrit reached its intended target, standard therapy for ET was administered.

This study provides a better understanding of the clinical characteristics and thrombotic events in Indonesian patients with Ph-negative MPN, who have been underreported in Indonesia. We used IPSS, DIPSS, IPSET-thrombosis in the analyses of prognosis of patients with MPN. However, some limitations of this study are needed to be acknowledged. First, this was a single-center study which only had a small number of subjects. Second, this was a retrospective study that resulted in incomplete several data. Third, JAK2V617F mutation status could not be evaluated for all the patients.

In conclusion, essential thrombocythemia was the most prevalent Ph-MPNs in Indonesia. Arterial thrombosis was the highest thrombosis event pre/at diagnosis in PV despite IPSET-thrombosis low risk being the most common risk group. In JAK2V617 mutation-positive MPN population, thrombosis event was also the highest in PV group.

Disclosure

The author reports no conflict of interest in this work.

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The Impact of Pharmacokinetic-Guided Prophylaxis on Clinical Outcomes and Healthcare Resource Utilization in Hemophilia A Patients: Real-World Evidence from the CHES II Study

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Background: Using a pharmacokinetic (PK)-guided approach to personalize the dose and frequency of prophylactic treatment can help achieve and maintain targeted factor VIII (FVIII) trough levels in patients with hemophilia A.

Objective: Investigate clinical and healthcare resource use outcomes in patients with hemophilia A treated with or without PK-guided prophylaxis using data from the Cost of Haemophilia in Europe: A Socioeconomic Survey (CHES) II database.

Methods: CHES II was a cross-sectional, retrospective, burden-of-illness study incorporating data from eight European countries. Patients were eligible for this analysis if they were male, ≥ 18 years of age, and diagnosed with congenital hemophilia A of any severity. The clinical endpoints included annualized bleeding rate (ABR), presence and number of problem/target joints, and occurrence of joint surgeries. Healthcare resource utilization endpoints included the number of hematologist consultations and bleed-related hospitalizations or emergency department admissions. Data from November 2018 to October 2020 were included and were stratified according to treatment regimen and use of PK-guided dosing.

Results: Altogether, 281 patients on prophylaxis had available FVIII trough level data. Mean (SD) age was 35.7 (13.8) years. A specific FVIII trough level was targeted in 120 (42.7%) patients and 47 (39.2%) received PK-guided dosing. Patients receiving PK-guided dosing had a mean (SD) ABR of 2.8 (2.1) and target joint number of 0.5 (0.7), compared with 3.9 (2.7) and 0.9 (1.4), respectively, for patients receiving non-PK-guided treatment. The mean (SD) number of hematologist consultations was 7.1 (5.3) for patients receiving PK-guided dosing versus 10.7 (5.7) for those who were not. A higher proportion of patients in the non-PK-guided group required hospitalization during their lifetime compared with the PK-guided group.

Conclusion: This analysis of real-world data suggests that PK-guided dosing for prophylaxis has a beneficial impact on clinical and healthcare resource utilization outcomes in patients with hemophilia A.

Keywords: hemophilia A, recombinant factor VIII, prophylaxis, PK-guided dosing, personalized treatment, CHES II

Introduction

Hemophilia A is a congenital, recessive, X-linked disorder characterized by a deficiency or absence of clotting factor VIII (FVIII), leading to frequent, acute, and prolonged spontaneous or traumatic bleeding events.¹ The severity of hemophilia A is classified on the basis of FVIII levels as severe (<0.01 IU/mL), moderate (0.01–0.05 IU/mL), or mild (0.05–0.4 IU/mL).^{1,2} Individuals with severe hemophilia A often experience hemophilic arthropathy due to joint bleeding, characterized by chronic inflammation and progressive joint deformity, leading to chronic pain, disability, and impaired quality of life.^{2,3} Treatment for hemophilia includes replacement of FVIII through intravenous injections to achieve adequate hemostasis, or the use of agents to enhance hemostasis independently of factor replacement, such as with the bispecific antibody emicizumab, which mimics FVIII activity.^{2,4–6} Patients can be treated with on-demand FVIII

replacement, to stop a bleed that has already occurred or prophylactically.⁵ The current standard of care for severe hemophilia A includes prophylaxis, involving 2–3 or more weekly intravenous injections with FVIII replacement therapy to keep FVIII levels above 1% in order to prevent bleeding episodes, target joint development, and resultant hemophilic arthropathy.²

The standard prophylactic treatment strategy was initially based on data demonstrating a significant reduction in bleeding frequency when FVIII levels of $\geq 1\%$ were maintained.^{7,8} However, although achieving a target FVIII trough level of 1% significantly reduces bleeding, some patients with hemophilia A still experience bleeding events, including into joints and soft tissue, leading to high morbidity.^{1,8,9} In addition, the shorter half-life of standard half-life FVIII replacement therapies necessitates frequent infusions to maintain FVIII levels, which can be challenging for patients, resulting in suboptimal treatment adherence. Extended half-life (EHL) recombinant FVIII concentrates have been developed to address this need. Many approaches to FVIII prophylaxis exist, and there is no consensus on the optimal strategy for dosing and treatment frequency to reduce the number and intensity of bleeds in individual patients. The optimal FVIII target trough level depends on a patient's individual pharmacokinetic (PK) profile, lifestyle, hemorrhagic history, and joint health.

In order to further improve the current standard of care, the World Federation of Hemophilia guidelines now recommend using a more personalized and tailored prophylactic treatment approach in patients who do not have satisfactory outcomes with standard prophylaxis. This approach targets FVIII trough levels $>3\text{--}5\%$ on the basis of an individual's PK profile, bleeding pattern, and lifestyle.^{2,10}

A number of studies have addressed the potential benefits of using PK profiling to personalize FVIII prophylaxis to help achieve and maintain a target trough level.^{10–16} PK profiling has also been shown to have economic benefits compared with standard prophylaxis.^{17,18} However, data from controlled clinical trials are limited, and information on the benefit of targeting higher FVIII trough levels has therefore been derived from a modeling approach.^{19–23} The recent phase 3, prospective, randomized, open-label, multicenter PROPEL study (NCT02585960) evaluated the safety and efficacy of the EHL recombinant FVIII rurioctocog alfa pegol at two PK-guided target FVIII trough level ranges (1–3% and 8–12%). This study demonstrated that a higher proportion of patients achieved zero bleeds when an elevated FVIII trough level was targeted.²⁴

However, despite more patients achieving a goal of zero bleeds, specifically in the group targeting a higher trough level, avoidable bleeds still occurred in both treatment groups.⁷ This raises the question of what the ideal target trough level for optimized bleed protection is and suggests that there is a need to match the target trough to the individual patient's needs. Given the variability associated with the care of individual patients and their responses to FVIII treatment, clinicians must evaluate the various risk factors and estimate the personalized target trough level a patient requires for the prevention of bleeding. Personalizing FVIII prophylaxis to a patient's PK profile can serve as a useful tool to aid clinicians in decision-making.

There remains, however, a lack of real-world evidence to suggest optimal prophylactic treatment strategies and, specifically, the benefits of PK-guided dosing in comparison with standard prophylaxis on clinical outcomes and healthcare resource utilization. The aim of the current analysis was to describe and compare clinical outcomes and healthcare resource utilization between patients with hemophilia A treated with or without PK-guided dosing using data from the Cost of Haemophilia in Europe: A Socioeconomic Survey (CHESS) II database.

Methods

Study Design and Patient Population

CHESS II is a cross-sectional, 12-month, retrospective, prevalence-based, burden-of-illness study conducted across eight European countries (France, Germany, Italy, Spain, the UK, Denmark, the Netherlands, and Romania). The CHESS II study used the same methodology that was used for the previously published CHESS study.²⁵ Patients were eligible to participate in CHESS II if they were male, ≥ 18 years of age, had been diagnosed with non-acquired hemophilia A or B of any severity and were able to read, understand, and sign the informed consent form. Patient data were collected from two questionnaires: a physician-completed, web-based clinical record form and a corresponding paper-based patient self-completion questionnaire

(Patient and Public Involvement and Engagement [PPIE]). The clinical record form contained information about the patient's medical history and consultations, and the PPIE covered non-medical costs, health-related quality of life (via the EuroQol EQ-5D-5L), and work impairment (via the Work Productivity and Activity Impairment instrument).

The present retrospective analysis was conducted on data from patients with hemophilia A of any severity without inhibitors, with complete responses for the variables of interest from the interim CHES II dataset. Data collected between November 2018 and October 2020 were analyzed.

All patient participants provided informed consent and the study protocol was approved by the Research Ethics Sub Committee of the Faculty of Health and Social care within the University of Chester. The approval stipulated that the study was to be carried out in correspondence with regional and relevant guidelines.

Analysis Endpoints

This retrospective analysis of the CHES II dataset included primary clinical and healthcare resource utilization outcomes associated with the care of patients with hemophilia A. The primary clinical endpoints included annualized bleeding rate (ABR) for the 12-month observation period immediately preceding data collection, joint outcomes, and whether the patient had undergone joint surgery in the previous 12 months or during their lifetime. Joint outcomes were characterized as the presence and number of target joints and/or problem joints. A target joint is defined as a joint in which ≥ 3 spontaneous bleeds have occurred within a consecutive 6-month period. Where there have been ≤ 2 bleeds into the joint within a consecutive 12-month period, the joint is no longer considered a target joint.²⁶ A problem joint is defined as a joint affected by chronic pain and/or limited range of motion due to compromised joint integrity (ie, suffering from chronic synovitis, and/or hemophilic arthropathy), with or without recurrent bleeding.²⁷ Physician-reported adherence to the prophylaxis treatment regimen was also assessed and is reported as the proportion of patients who are fully adherent to their regimen (defined as missing $<15\%$ of infusions).

Healthcare resource utilization was also assessed across subgroups by analyzing the proportion of patients requiring specialist hematologist or nurse consultations and the number of specialist consultations needed in the 12 months prior to data collection. Bleed-related hospitalizations and emergency department admissions were also assessed, including the type of hospitalization (ward, intensive care unit [ICU], day case), number of hospitalizations, and duration of hospital stay.

Statistical Analysis

Demographic data and outcomes of interest were reported descriptively and stratified according to subgroups defined by the treatment characteristics. Specifically, strata were defined by whether the patients received primary prophylaxis (ie, treatment has always been prophylactic with regular factor infusions to prevent bleeding commencing before significant bleeding has occurred) or secondary prophylaxis (treatment was previously on demand but, after experiencing several bleeds into a target joint, the patient is now receiving regular prophylactic infusions). Among those for whom a FVIII trough level was targeted, data were also stratified by how the specific treatment dose was determined (ie, whether the dose was PK guided or not).

Univariate and multivariate regression analyses for bleeding outcomes were performed with continuous and categorical variables using ordinary least-squares regression (OLS). Models were specified for both subcohorts with information on dose-determination method ($n = 120$). Basic demographics and covariates that were clinically relevant or significantly associated with ABR and resource utilization outcomes ($p < 0.10$) were selected for multivariate analysis of the relationship of clinical attributes, treatment strategy and use of PK-guided dosing with bleeding and resource utilization outcomes (hospitalizations). Multicollinearity between covariates was assessed using Pearson's correlation coefficient (PCC) before inclusion in the regression model. The assumptions of normality of OLS were assessed via visual inspection of quantile–quantile plots and distribution of residuals. Robust standard errors were employed to account for heteroscedasticity.

Data analysis was undertaken using Stata Statistical Software: Release 16, 2019 (StataCorp, College Station, TX, USA). Imputation of missing data was not conducted. Patients with missing responses for the outcomes of interest were

excluded from the analysis. Continuous variables and study outcomes were summarized using non-missing sample size (n) and percentage of non-missing sample size and means and standard deviations (SD).

Results

Sample Characteristics

The dataset used in this analysis included 1337 patients treated by 185 hematologists and hemophilia care providers based in hospitals and clinics, and captured 366 PPIEs. Of these, 281 patients with hemophilia A receiving prophylaxis met the inclusion criteria and were included in this analysis. Patient demographics and characteristics are presented in Table 1. The

Table 1 Patient Demographics and Baseline Characteristics

Parameter	Secondary Prophylaxis	Primary Prophylaxis	PK-Guided	Non-PK-Guided	Full Sample
Total patients, N	128	153	47	73	281
Age, years					
Mean $\pm$ SD	38.3 $\pm$ 14.4	33.5 $\pm$ 12.9	38.4 $\pm$ 15.9	35.3 $\pm$ 14.0	35.7 $\pm$ 13.8
Median (range)	38 (19–79)	29 (18–77)	32 (18–79)	29 (19–75)	32 (18–79)
Country, n (%)					
Italy	54 (42.2)	33 (21.6)	15 (31.9)	20 (27.4)	87 (31.0)
Spain	36 (28.1)	24 (15.7)	8 (17.0)	28 (38.4)	60 (21.4)
UK	12 (9.4)	43 (28.1)	7 (14.9)	2 (2.7)	55 (19.6)
Germany	7 (5.5)	42 (27.5)	10 (21.3)	10 (13.7)	49 (17.4)
France	19 (14.8)	11 (7.2)	7 (14.9)	13 (17.8)	30 (10.7)
Ethnicity, n (%)					
White	118 (92.2)	144 (94.1)	46 (97.9)	67 (91.8)	262 (93.2)
Asian-other	2 (1.6)	3 (2.0)	0	0	5 (1.8)
Mixed	4 (3.1)	1 (0.7)	0	2 (2.7)	5 (1.8)
Black/Afro-Caribbean	2 (1.6)	2 (1.3)	0	0	4 (1.4)
Middle Eastern	1 (0.8)	2 (1.3)	0	2 (2.7)	3 (1.1)
Asian-Indian subcontinent	0	1 (0.8)	1 (2.1)	2 (2.7)	1 (0.4)
Prefer not to answer	1 (0.8)	0	0	0	1 (0.4)
Body mass index, kg/m ² , mean $\pm$ SD	24.9 $\pm$ 2.7	24.5 $\pm$ 2.9	24.8 $\pm$ 2.3	24.1 $\pm$ 2.6	24.7 $\pm$ 2.8
Weight, kg, mean $\pm$ SD	74.2 $\pm$ 9.3	75.9 $\pm$ 10.1	75.6 $\pm$ 9.2	73.3 $\pm$ 9.7	75.1 $\pm$ 9.8
Physician targeting a specific FVIII trough level for the patient^a, n (%)					
Yes	61 (47.7)	59 (38.6)	47 (100)	0	120 (42.7)
No	67 (52.3)	94 (61.4)	0	73 (100)	161 (57.3)
Dosage guided by PK profiling, n (%)					
Yes	17 (27.9)	30 (50.8)	47 (100)	0	47 (39.2)
No	44 (72.1)	29 (49.1)	0	73 (100)	73 (60.8)
Treatment type, n (%)					
Extended half-life	21 (16.4)	36 (23.5)	13 (27.7)	13 (17.8)	57 (20.3)
Standard half-life	107 (83.6)	117 (76.5)	34 (72.3)	60 (82.2)	224 (79.7)

Notes: ^aThe specific FVIII trough levels targeted were recorded as part of this study; however, owing to the high levels of variation, these data were not included as part of this analysis.

Abbreviations: FVIII, factor VIII; PK, pharmacokinetic; SD, standard deviation.

mean (SD) patient age was 35.7 (13.8) years, and the mean (SD) patient body mass index was 24.7 (2.8) kg/m². All patients had severe hemophilia A (FVIII < 1%). Most patients were from Italy (87 [31.0%]) and Spain (60 [21.4%]), and >90% of patients were of White ethnicity (262 [93.2%]). No patients from Denmark, Romania, or the Netherlands were included owing to small sample sizes and failure to meet the inclusion criteria. Of the 281 included patients, 153 (54.4%) were receiving primary prophylaxis and 128 (45.6%) were receiving secondary prophylaxis. A specific FVIII trough level was targeted by the physician for 120 patients (42.7%) and, of those, treatment dosage was guided by PK profiling for 47 (39.2%). Although the specific FVIII trough levels targeted were recorded as part of the CHES II study, because of the small sample size, these have not been included in the reported analysis. Use of EHL therapy was numerically more common in the primary prophylaxis group compared with the secondary prophylaxis group (23.5% vs 16.4%, respectively), and in patients receiving PK-guided therapy compared with non-PK-guided therapy (27.7% vs 17.8%, respectively).

Descriptive Analysis of Clinical Attributes and Treatment Characteristics

ABRs and Joint Deterioration

ABRs and joint health outcomes stratified by treatment groups are shown in Table 2. Patients receiving primary prophylaxis exhibited a numerically lower mean (SD) ABR than patients receiving secondary prophylaxis (2.9 [2.6] vs 4.0 [3.0], respectively; $p = 0.001$). In addition, only 27.5% of patients receiving primary prophylaxis had target joints, compared with 46.9% of the patients receiving secondary prophylaxis ($p = 0.001$). The proportions of patients with problem joints and the overall number of target joints and problem joints were similar between the two treatment strategy groups (Table 2). A numerically higher proportion of patients receiving secondary prophylaxis were found to be fully adherent compared with patients receiving primary prophylaxis (84.4% vs 77.1%, respectively) (Table 2).

Patients receiving PK-guided prophylaxis exhibited a lower ABR than patients in the non-PK-guided group (2.8 [2.1] vs 3.9 [2.7], respectively). Patients receiving PK-guided dosing also reported a slightly lower mean (SD) number of target joints compared with the non-PK-guided group (0.5 [0.7] vs 0.9 [1.4], respectively). However, a higher proportion of patients in the PK-guided group (51.1%) experienced problem joints compared with the non-PK-guided group (42.5%) (Table 2). The proportion of fully adherent patients was very similar between patients receiving PK-guided (78.1%) and non-PK-guided prophylaxis (78.7%) (Table 2).

Table 2 Bleeding Rates, Joint Deterioration, and Treatment Adherence Stratified by Treatment Group

	Treatment Strategy (N = 281)			Targeting FVIII Trough Level (N = 120)		
	Secondary Prophylaxis ^d (n = 128)	Primary Prophylaxis ^e (n = 153)	p-value	PK-Guided (n = 47)	Non-PK-Guided (n = 73)	p-value
ABR, mean $\pm$ SD	4.0 $\pm$ 3.0	2.9 $\pm$ 2.6	0.001	2.8 $\pm$ 2.1	3.9 $\pm$ 2.7	0.022
Presence of target joints ^a , n (%)	60 (46.9)	42 (27.5)	0.001	18 (38.3)	30 (41.1)	0.760
Number of target joints ^a , mean $\pm$ SD	0.8 $\pm$ 1.1	0.5 $\pm$ 1.0	0.058	0.5 $\pm$ 0.7	0.9 $\pm$ 1.4	0.036
Presence of problem joints ^b , n (%)	59 (46.1)	64 (41.8)	0.473	24 (51.1)	31 (42.5)	0.356
Number of problem joints ^b , mean $\pm$ SD	0.8 $\pm$ 1.0	0.7 $\pm$ 1.1	0.995	0.8 $\pm$ 0.9	0.8 $\pm$ 1.1	0.834
Fully adherent ^c , n (%)	108 (84.4)	118 (77.1)	0.116	37 (78.7)	57 (78.1)	0.759

Notes: ^aA target joint was defined as a joint in which ≥ 3 spontaneous bleeds occurred within a consecutive 6-month period. The joint was no longer considered a target joint if the patient experienced ≤ 2 bleeds into the joint within a consecutive 12-month period. ^bA problem joint was defined as a joint exhibiting any symptoms among chronic joint pain, chronic synovitis, hemophilic arthropathy, limited motion, or recurrent bleeding. ^cFull adherence was reported by the physician and defined as "Missing <15% of infusions." ^dSecondary prophylaxis was defined as treatment that was previously on-demand but is currently prophylactic. ^ePrimary prophylaxis was defined as treatment that has always been prophylactic.

Abbreviations: ABR, annualized bleeding rate; FVIII, factor VIII; PK, pharmacokinetic; SD, standard deviation.

Joint Surgeries

The number of patients who underwent joint surgery and the number of joint surgeries received, stratified by treatment group, are presented in Table 3. A higher proportion of patients receiving primary prophylaxis underwent joint surgery during the 12 months prior to data collection compared with those receiving secondary prophylaxis (60.5% vs 45.2%, respectively). However, the mean (SD) number of surgeries performed per patient was 1.7 (1.1) for patients receiving primary prophylaxis, compared with 2.4 (1.6) for the secondary prophylaxis group (Table 3).

A higher proportion of patients receiving PK-guided prophylaxis also underwent joint surgeries in their lifetime compared with those receiving non-PK-guided prophylaxis (29.8% vs 24.7%). However, a lower proportion of patients in the PK-guided group underwent a joint surgery in the 12 months immediately prior to data collection (50.0% vs 83.3%), with only two patients (4.3%) undergoing ≥ 3 joint surgeries in the prior 12 months. The mean (SD) number of surgical procedures in the 12 months prior to data collection was also slightly lower in patients receiving PK-guided prophylaxis (2.1 [1.3] vs 2.7 [1.5]) (Table 3).

Healthcare Resource Utilization

Specialist consultations, including the number of scheduled and unscheduled visits, stratified by treatment group are presented in Table 4. Patients receiving primary prophylaxis reported a similar mean (SD) number of specialist hematologist visits (7.8 [5.6]) to those who received secondary prophylaxis (8.0 [5.3]). However, patients receiving primary prophylaxis reported a lower mean (SD) number of specialist nurse visits (6.5 [6.1] vs 7.9 [9.5]).

Patients whose dosing was PK guided reported a lower mean (SD) number of hematologist and nurse consultations (7.1 [5.3] and 5.2 [4.2], respectively) than patients receiving non-PK-guided dosing (10.7 [5.7] and 10.3 [11.7], respectively). Additionally, patients receiving PK-guided dosing reported fewer unscheduled specialist consultations (hematologist consultations = 1.9 [4.4] vs 2.3 [2.5]; nurse consultations = 1.2 [1.7] vs 2.3 [3.2]) (Table 4).

The proportions of patients requiring bleed-related hospitalization in their lifetime and during the 12 months immediately prior to data collection, as well as the number and type of hospitalizations stratified by treatment group,

Table 3 Joint Surgeries Stratified by Treatment Group

	Treatment Strategy (N = 281)		Targeting FVIII Trough Level (N = 120)	
	Secondary Prophylaxis ^c (n = 128)	Primary Prophylaxis ^d (n = 153)	PK-Guided (n = 47)	Non-PK-Guided (n = 73)
Patients who underwent joint surgery in their lifetime, n (%)	32 (25.0)	38 (24.8)	14 (29.8)	18 (24.7)
Patients who underwent joint surgery in the 12 months prior to data collection, n (%)	16 (50.0)	22 (57.9)	7 (50.0)	15 (83.3)
Number of joint surgeries in the 12 months prior to data collection, n (%)				
0	112 (87.5)	131 (85.6)	40 (85.1)	58 (79.5)
1	6 (4.7)	11 (7.2)	3 (6.4)	4 (5.5)
2	4 (3.1)	7 (4.6)	2 (4.3)	3 (4.1)
≥ 3	6 (4.7)	4 (2.6)	2 (4.3)	8 (11.0)
Total number of surgical procedures on target^a and problem^b joints in the 12 months prior to data collection, mean $\pm$ SD (n = 37)	2.4 $\pm$ 1.6 (n = 14)	1.7 $\pm$ 1.1 (n = 23)	2.1 $\pm$ 1.3 (n = 7)	2.7 $\pm$ 1.5 (n = 15)

Notes: ^aA target joint was defined as a joint in which ≥ 3 spontaneous bleeds occurred within a consecutive 6-month period. The joint was no longer considered a target joint if the patient experienced ≤ 2 bleeds into the joint within a consecutive 12-month period. ^bA problem joint was defined as a joint exhibiting any symptoms among chronic joint pain, chronic synovitis, hemophilic arthropathy, limited motion, or recurrent bleeding. ^cSecondary prophylaxis was defined as treatment that was previously on demand but is currently prophylactic. ^dPrimary prophylaxis was defined as treatment that has always been prophylactic.

Abbreviations: FVIII, factor VIII; PK, pharmacokinetic; SD, standard deviation.

Table 4 Healthcare Resource Utilization Stratified by Treatment Group

	Treatment Strategy (N = 281)		Targeting FVIII Trough Level (N = 120)	
	Secondary Prophylaxis ^a (n = 128)	Primary Prophylaxis ^b (n = 153)	PK-Guided (n = 47)	Non-PK-Guided (n = 73)
Number of hematologist consultations, mean ± SD	8.0 ± 5.3	7.8 ± 5.6	7.1 ± 5.3	10.7 ± 5.7
Number of scheduled hematologist consultations	6.2 ± 4.0	6.1 ± 3.9	5.2 ± 3.0	8.4 ± 4.2
Number of unscheduled hematologist consultations	1.8 ± 2.1	1.8 ± 3.2	1.9 ± 4.4	2.3 ± 2.5
Number of nurse consultations, mean ± SD	7.9 ± 9.5	6.5 ± 6.1	5.2 ± 4.2	10.3 ± 11.7
Number of scheduled nurse consultations	5.9 ± 7.6	5.1 ± 5.0	4.0 ± 3.3	8.0 ± 9.4
Number of unscheduled nurse consultations	2.0 ± 2.8	1.5 ± 2.3	1.2 ± 1.7	2.3 ± 3.2

Notes: ^aSecondary prophylaxis was defined as treatment that was previously on demand but is currently prophylactic. ^bPrimary prophylaxis was defined as treatment that has always been prophylactic.

Abbreviations: FVIII, factor VIII; PK, pharmacokinetic; SD, standard deviation.

are shown in Table 5. A higher proportion of patients receiving secondary prophylaxis were hospitalized within the 12 months prior to data collection (62.6%) compared with patients receiving primary prophylaxis (86.4%).

The proportion of patients requiring hospitalization during their lifetime was also lower for those receiving PK-guided dosing (80.9%) than for those receiving non-PK-guided dosing (90.4%). Patients receiving PK-guided dosing were also observed to have experienced fewer ward hospitalizations during the 12 months prior to data collection than those receiving non-PK-guided dosing, with mean (SD) numbers of hospitalizations of 0.4 (0.8) and 0.6 (1.0), respectively.

Regression Analysis

Table 6 summarizes the univariate regression analysis. Variables of clinical relevance and those demonstrating a significant association with the outcome of interest were included in the multivariate analysis.

Table 5 Bleed-Related Hospitalizations Stratified by Treatment Group

	Treatment Strategy (N = 281)		Targeting FVIII Trough Level (N = 120)	
	Secondary Prophylaxis ^b (n = 128)	Primary Prophylaxis ^c (n = 153)	PK-Guided (n = 47)	Non-PK-Guided (n = 73)
Patients requiring hospitalization during their lifetime, n (%)	107 (83.6)	132 (86.3)	38 (80.9)	66 (90.4)
Patients requiring hospitalization in the 12 months prior to data collection, n (%)	67 (62.6)	114 (86.4)	33 (86.8)	44 (66.7)
Hospitalizations occurring in the 12 months prior to data collection, mean ± SD				
Number of ward hospitalizations	0.5 ± 0.9	0.5 ± 0.9	0.4 ± 0.8	0.6 ± 1.0
Number of days on ward	5.7 ± 4.2 (n = 51)	6.9 ± 5.1 (n = 44)	8.4 ± 6.9 (n = 14)	5.7 ± 4.1 (n = 27)
Number of day cases ^a	0.1 ± 0.3	0.1 ± 0.6	0.1 ± 0.4	0.1 ± 0.3
Number of day-case days	1.0 ± 0.0 (n = 9)	2.3 ± 1.5 (n = 8)	1.5 ± 0.6 (n = 4)	1.3 ± 0.6 (n = 3)
Number of ICU hospitalizations	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.2	0.6 ± 1.0
Number of days in ICU	2.7 ± 1.3 (n = 12)	2.3 ± 1.1 (n = 7)	1.7 ± 1.2 (n = 3)	1.5 ± 0.6 (n = 4)

Notes: ^aA day case was defined as a patient who visited hospital but did not stay overnight. ^bSecondary prophylaxis was defined as treatment that was previously on demand but is currently prophylactic. ^cPrimary prophylaxis was defined as treatment that has always been prophylactic.

Abbreviations: FVIII, factor VIII; ICU, intensive care unit; PK, pharmacokinetic; SD, standard deviation.

Table 6 Multivariate Regression Results – Covariate Relationships with ABR and Hospitalizations

Variable	Relationship with Bleeding Rate		Relationship with Hospitalization Number	
	B-coefficient	95% Confidence Interval	B-coefficient	95% Confidence Interval
BMI	−0.007	−0.214, 0.199	0.017	−0.053, 0.088
Age group				
18–25	Reference	–	Reference	–
26–35	0.029	−1.022, 1.080	−0.159	−0.530, 0.211
36–50	−0.097	−1.486, 1.292	−0.107	−0.601, 0.386
51+	0.465	−1.212, 2.142	−0.436	−1.000, 0.127
Country^a				
Germany	Reference	–	–	–
Spain	1.627	0.101–3.153	0.244	−0.431, 0.920
France	0.504	−0.652, 1.660	−0.314	−0.823, 0.195
Italy	1.218	−0.178, 2.614	−0.085	−0.781, 0.612
UK	1.496	0.265–2.727	−0.006	−1.055, 1.042
PK-guided dosing use	−0.903**	−1.790, −0.016	0.114	−0.169, 0.397
ABR	NA	NA	0.198***	0.125–0.272
Problem joint number	0.563**	0.037–1.090	0.240***	0.062–0.418
Full adherence^b	–	–	−0.574**	−1.062, −0.085
Treatment type (EHL)	1.407**	0.161–2.653	−0.623**	−1.122, −0.124
Treatment strategy				
Primary prophylaxis	Reference	–	Reference	–
Secondary prophylaxis	−0.718	−1.829, 0.393	0.068	−0.266, 0.403

Notes: ^aNo patients from Denmark, Romania, or the Netherlands were included owing to small sample sizes and failure to meet the inclusion criteria. ^bThere was no significant relationship with ABR in the univariate analysis ($p=0.43$). ** $p<0.05$; *** $p<0.01$.

Abbreviations: ABR, annualized bleeding rate; BMI, body mass index; EHL, extended half-life; NA, not applicable; PK, pharmacokinetics.

Bleeding Outcomes

The multivariate regression of data from the PK subsample results suggest that ABR is higher in patients from Spain and the UK compared with those from Germany ($p < 0.05$ for both; Table 6). Presence and increasing number of problem joints were found to be a significant driver of bleeding outcomes (both $p < 0.05$; Table 6). However, use of EHL FVIII concentrates had a positive and significant relationship with ABR ($p < 0.05$), despite demonstrating a weak correlation with the bleeding outcome itself (PCC = -0.03 ; $p = 0.74$). After controlling for treatment history and body mass index, use of PK-guided dosing was negatively associated with bleeding outcomes ($p < 0.05$; Table 6).

Hospitalizations

In this multivariate model, the aggregate number of hospitalizations (ie, the sum of all types of hospitalizations) was used as the dependent variable. ABR and number of problem joints were both identified as positively and significantly associated with number of hospitalizations ($p < 0.001$ and $p < 0.01$, respectively; Table 6). Fewer hospitalizations were experienced by fully adherent patients compared with non-fully adherent patients ($p < 0.05$; Table 6). The use of PK-guided dosing showed a positive association with number of hospitalizations, although this was not statistically significant ($p = 0.426$).

Discussion

The objective of this analysis of the CHESS II dataset was the investigation of relevant patterns in clinical or resource utilization outcomes across different treatment strategies and dosing approaches in the real world. This will generate relevant examples and assist stakeholders in the determination of the best-suited approach for managing patients with hemophilia A. The impact of a prophylactic treatment regimen guided by PK profiling was compared with a regimen without PK-guided dosing. Patients who were treated with primary prophylaxis appeared to have lower ABRs and a lower likelihood of experiencing target joints than patients treated with secondary prophylaxis. Importantly, ABR is the main outcome in hemophilia as bleeding is the source of all other complications. A reduction in ABR is therefore important in achieving better joint health in the longer term. Patients receiving primary prophylaxis did, however, have a slightly higher incidence of joint surgery in the 12 months before data collection compared with patients receiving secondary prophylaxis (58% vs 50%). In addition, patients whose prophylactic dose and regimen was guided by PK profiling had a lower ABR (2.8) than patients whose regimen was not PK guided (3.9). These ABRs are consistent with values presented for patients using PK-guided prophylaxis to target FVIII trough levels in the PROPEL study.²⁵ Short-term joint health outcomes were also more favorable for patients in the PK-guided group, with these patients experiencing fewer target joints and being less likely to have required joint surgery in the previous 12 months. This is despite the worse long-term joint health issues characterizing the patients belonging to the PK-guided group, inferred from the larger proportion of patients with existing problem joints and who had undergone joint surgeries in their lifetime when compared with the patients in the non-PK-guided group. Additionally, a slightly larger proportion of the non-PK-guided group of patients underwent >3 surgeries in the 12 months prior to data collection.

Specialist consultations were common across the groups, with patients receiving secondary prophylaxis requiring a higher number of nurse consultations than those treated with primary prophylaxis. Interestingly, of the patients receiving targeted treatment, those whose dosage was PK guided required fewer hematologist consultations (7.1 vs 10.7) and fewer nurse consultations (5.2 vs 10.3) than patients whose dosage was not PK guided. In addition, patients in the non-PK-guided group were more likely to require an unscheduled consultation than patients receiving PK-guided dosage.

In the regression analysis of data from the PK subsample, ABRs were higher in patients from Spain and the UK than in those from Germany (the reference population). In addition, the presence and number of problem joints were a significant driver of bleeding outcomes. In the analysis of hospitalizations, higher ABR and number of problem joints were significantly associated with increased number of hospitalizations, while full adherence to therapy was associated with fewer hospitalizations.

EHL FVIII use was positively and significantly related to increased bleeding, which was unexpected given the theoretical improved protection provided by these agents compared with standard half-life products. This can be explained if, as seems probable, patients with more extensive joint damage and pain are more likely to receive EHL products than those with less severe disease. In the more severe patients, the existing joint damage may exacerbate bleeding outcomes despite EHL FVIII treatment. It should also be noted, however, that few patients in the analysis received EHL products, which may have skewed the results.

These data suggest that tailoring treatment using a PK-guided approach to target a specific FVIII trough level is associated with reduced bleeding and improved joint outcomes. This improvement in joint health occurred even though the patients receiving PK-guided prophylaxis had evidence of long-standing chronic joint damage, as can be inferred from the larger proportion of patients with existing problem joints and joint surgeries in their lifetime.

Additionally, the data presented here showing an association between ABR and PK-guided prophylaxis suggest that PK-guided prophylaxis may be connected with a reduced need for specialist consultations and fewer unscheduled consultations as a result of its proposed relationship with reduced bleeding. Due to the debate currently surrounding the most appropriate FVIII trough level to target in patients with hemophilia A, further real-world data are needed to investigate the use of PK profiling and how it can be used to provide a more accurate determination of the optimal target

FVIII trough range. This will aid our understanding of the benefit that personalized care can bring to patients and the entire healthcare system.

This analysis should be considered in the context of some limitations. This study was a secondary analysis of data from the existing CHES II dataset. Therefore, the data were not collected for the specific purpose of this analysis. Because of the voluntary and retrospective nature of the participation of patients and physicians in the original CHES II study, neither a degree of selection or recall bias nor errors in the transfer of data from the medical charts can be ruled out. Only a small sample of the hemophilia A population is represented, and information on the endpoints of interest was available for a limited number of patients. Given the small sample sizes, the outputs from this analysis should be interpreted with caution.

Conclusions

This analysis is an initial contribution to the investigation around determining the real-world impact that PK-guided prophylaxis may have on clinical and healthcare resource utilization outcomes in patients with hemophilia A. Primary prophylaxis and PK-guided prophylaxis seem to be associated with a reduced need for specialist consultations. In addition, reduced bleeding, improved joint health outcomes, and a reduced need for joint surgeries were observed in patients receiving PK-guided prophylaxis. In this cohort, PK-guided therapy was started when considerable damage was already present, suggesting that some patients receive less than optimal treatment until symptoms become substantial. The relationship of PK-guided therapy with reduced bleeding suggests that earlier initiation of PK-guided therapy may help preserve joint health. Further research is warranted to explore the specific FVIII trough levels targeted, as well as other potential drivers in the differences in clinical and healthcare resource utilization outcomes among patients who receive PK-guided prophylaxis.

Abbreviations

ABR, annualized bleeding rate; CHES, Cost of Haemophilia in Europe: A Socioeconomic Survey; EHL, extended half-life; FVIII, factor VIII; ICU, intensive care unit; OLS, ordinary least-squares regression; PCC, Pearson's correlation coefficient; PK, pharmacokinetic; PPIE, Patient and Public Involvement and Engagement; SD, standard deviation.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are held under license by the University of Chester and are not publicly available. Upon reasonable request, and subject to review, the corresponding author will provide the analyses that support the findings of this research. Subject to certain criteria, conditions, and exceptions, access to the related data for researchers who provide a methodologically sound proposal may be considered by data owners HCD Economics and the University of Chester. The data will be provided after its de-identification, in compliance with applicable privacy laws, data protection, and requirements for consent and anonymization.

Ethics Approval and Informed Consent

All patient participants provided informed consent and the study protocol was approved by the Research Ethics Sub Committee of the Faculty of Health and Social care within the University of Chester. The approval stipulated that the study was to be carried out in correspondence with regional and relevant guidelines.

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Author Contributions

All authors contributed 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.

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Disclosure

EFG, TB, and JOH are employees of HCD Economics. SXS is an employee of Takeda Development Center Americas, Inc. The authors report no other conflicts of interest in this work.

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Comparison of Real-World Dose and Consumption for Two Extended Half-Life Recombinant Factor VIII Products for the Treatment of Hemophilia A in the United States

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Background: US patients with hemophilia A can receive prophylaxis with extended half-life recombinant factor VIII (rFVIII) products, including efmoctocog alfa (fragment crystallizable fusion protein) and rurioctocog alfa pegol (antihemophilic factor [recombinant], PEGylated).

Objective: To evaluate dosing patterns and weekly consumption of extended half-life rFVIII products in the United States.

Methods: We performed a retrospective analysis using the US Specialty Pharmacy Database (2015–2018). Included patients had a diagnosis of hemophilia A, ≥ 2 consecutive monthly claims for efmoctocog alfa or rurioctocog alfa pegol for prophylaxis, and weight data. Outcome measures included weekly dosing frequency and dispensed weekly dose.

Results: The analysis included 774 patients (efmoctocog alfa, 506; rurioctocog alfa pegol, 268). Mean (SD) age was 24.2 (15.8) and 26.3 (14.9) years for patients receiving efmoctocog alfa and rurioctocog alfa pegol, respectively; mean (SD) weight was 68.4 (36.8) and 79.8 (37.7) kg, respectively. The most frequent efmoctocog alfa regimen was twice weekly (45.7%), followed by every 4 days (20.6%), every 3 days (9.1%), and 3 times per week (7.5%). The most frequent rurioctocog alfa pegol regimen was twice weekly (72.4%), followed by 3 times per week (8.7%), every 4 days (7.6%), and every 3 days (5.5%). The proportion of efmoctocog alfa twice-weekly dispensing records increased from 31.5% to 50.9%, and every 4 days dispensing records decreased from 31.3% to 14.5% (2015–2018). The proportion of rurioctocog alfa pegol dispensing records remained broadly stable (2016–2018). Overall, mean (SD; median) weekly prophylactic dose was 105.4 (77.9; 92.6) IU/kg with efmoctocog alfa, and 96.8 (41.9; 90.9) IU/kg with rurioctocog alfa pegol.

Conclusion: In this database study, the most frequently observed dosing frequency was twice weekly for patients receiving efmoctocog alfa or rurioctocog alfa pegol. The observed mean weekly consumption was slightly higher, and variation was greater, in patients receiving efmoctocog alfa versus rurioctocog alfa pegol.

Keywords: hemophilia A, fragment crystallizable fusion protein, efmoctocog alfa, antihemophilic factor (recombinant), PEGylated, rurioctocog alfa pegol, dosing patterns

Introduction

Hemophilia A is a recessive congenital bleeding disorder caused by a deficiency of factor VIII (FVIII).¹ The estimated worldwide prevalence is 17.1 cases per 100,000 males.² The recommended care for patients with severe hemophilia A is prophylactic replacement therapy with recombinant FVIII (rFVIII) to prevent bleeding and maintain joint health.^{3,4} However, conventional factor concentrates have a short half-life of 8–12 hours,⁵ which necessitates frequent intravenous infusions. Therefore, standard half-life (SHL) rFVIII concentrates can result in a high treatment burden for patients, which can have a negative impact on treatment adherence,⁶ potentially leading to poorer bleeding outcomes. Extended

half-life (EHL) rFVIII products with improved pharmacokinetic profiles have been developed, allowing patients to extend the interval between treatments.⁷

The plasma half-life of FVIII has been extended through recombinant technology, either by fusion with the fragment crystallizable (Fc) region of the immunoglobulin G1 molecule or by conjugation with the polymer polyethylene glycol (PEG).⁸ Efmoroctocog alfa (Eloctate; Bioverativ Therapeutics Inc., Waltham, MA, USA) is a rFVIII-Fc fusion protein.⁹ A phase 3 trial analyzed the pharmacokinetics of efmoroctocog alfa and reported that the terminal half-life was extended 1.5-fold versus a commercially available rFVIII product, octocog alfa (antihemophilic factor [recombinant], plasma/albumin-free method [Advate [Europe]; Takeda Manufacturing Austria AG] geometric mean = 19.0 vs 12.4 hours, respectively).¹⁰ Rurioctocog alfa pegol, antihemophilic factor (recombinant), PEGylated (Adynovate [United States]/Adynovi [Europe]; Baxalta Inc., a Takeda company, Lexington, MA, USA) is a recombinant full-length human coagulation FVIII covalently conjugated with at least one molecule of PEG.¹¹ A pivotal study showed that the half-life for rurioctocog alfa pegol increased 1.4-fold versus octocog alfa (14.3 vs 10.4 hours, respectively) in patients aged ≥ 12 years.¹²

Efmoroctocog alfa was approved in the United States in 2014 for use in adults and children with hemophilia A.⁹ According to the product label, the recommended starting regimen for prophylaxis (at the time of the study) in patients aged ≥ 6 years is 50 IU/kg administered every 4 days. The dose can be adjusted, based on the patient response, within a range of 25–65 IU/kg every 3–5 days. For patients aged < 6 years, the recommended starting regimen is 50 IU/kg administered twice weekly. The regimen can be adjusted based on patient response, with dosing in the range of 25–65 IU/kg at 3–5-day intervals, with more frequent or higher doses up to 80 IU/kg if required. A pivotal phase 3 study of males aged ≥ 12 years with severe hemophilia A found that prophylaxis with efmoroctocog alfa was well tolerated and effective in the prevention and treatment of bleeding and did not result in increased immunogenicity.¹⁰

Rurioctocog alfa pegol was first approved for use in November 2015.¹¹ The prescribing information recommends a dose of 40–50 IU/kg twice weekly for patients aged ≥ 12 years. For patients aged < 12 years, the recommended starting dose is 55 IU/kg twice weekly. The dose can be adjusted based on the patient's clinical response, with a maximum of 70 IU/kg.¹¹ A phase 3b study among patients with severe hemophilia A found that rurioctocog alfa pegol as long-term prophylaxis provided good hemostatic efficacy and had a similar safety and immunogenicity profile to previously published data.¹³

There is a lack of published studies of real-world treatment patterns for EHL rFVIII among patients with hemophilia A. In 2020, an observational study of US patients with hemophilia A reported a reduction in the frequency of treatment administration (mean days/week) after patients switched from an SHL rFVIII to rurioctocog alfa pegol (from 2.8 to 2.2 days).¹⁴ The authors also reported lower consumption by patients who switched from SHL rFVIII to rurioctocog alfa pegol (from 109.8 to 100.6 IU/kg). A Canadian report of efmoroctocog alfa utilization for the first 8 months following availability in 2014 showed a 19% reduction in factor utilization (based on weekly IU/kg) among patients with severe hemophilia A who switched from SHL rFVIII to efmoroctocog alfa.¹⁵ Another Canadian study reported a retrospective analysis of pharmacokinetic data from 23 patients with hemophilia A who were switched from efmoroctocog alfa to rurioctocog alfa pegol.¹⁶ The results showed minor differences in the pharmacokinetic profile of the two agents, which were statistically significant ($p < 0.05$ for all) for in vivo recovery (mean 2.73 vs 2.41 IU/dL per IU/kg), clearance (mean 0.163 vs 0.194 mL/h), and volume of distribution at steady state (mean 42.5 vs 49.8 mL/kg), respectively. The authors commented, however, that the clinical implications of these differences are uncertain.¹⁶ Indeed, an earlier study from the same centers showed that the pharmacokinetic profiles of efmoroctocog alfa to rurioctocog alfa pegol in adolescents with hemophilia A were almost identical (mean [range] terminal half-life 16.1 (10.4–23.4) hours vs 16.7 (11.0–23.6) hours, respectively [$p = \text{NS}$]).¹⁷

There is a need for further real-world evidence on EHL products to improve the understanding of prophylaxis dosing patterns associated with each EHL rFVIII to inform health care decision-making and identify the most appropriate treatment regimens for different patient groups. We therefore undertook a retrospective analysis of US specialty pharmacy data to evaluate real-world dosing patterns (weekly dose, infusion frequency) of efmoroctocog alfa and rurioctocog alfa pegol for the prophylactic treatment of patients with hemophilia A without inhibitors.

Methods

Study Design and Data Source

This study was a retrospective analysis of prescription dispensing data from the US Specialty Pharmacy Database and includes >2400 patients with hemophilia A from all 50 states. The database comprises de-identified data including patient demographics, detailed prescription information, and clinical data such as diagnosis, disease severity, and inhibitor status, and is fully compliant with regulations from the Health Insurance Portability and Accountability Act (HIPAA) of 1996. De-identified prescription dispensing data were collected for the period between January 1, 2015 and December 31, 2018.

Patient Population

Patients were included in the analysis if they had a diagnosis of hemophilia A using International Classification of Diseases (ICD)-9 code 286.0 or ICD-10 code D66. For registration in the database, patients were required to have received medication for hemophilia A from one of the Specialty Pharmacy Providers included in the database. This study included patients from the database that had at least two consecutive monthly pharmacy dispensing records for efmoctocog alfa or ruriotocog alfa pegol administered for prophylaxis during the study period. Body weight data and valid dispensing units in the patient record were also required. Patients were excluded if they received immune tolerance induction therapy or more than one dose per day, because this is given to patients receiving immune tolerance induction therapy or could be a coding error. Patients receiving perioperative management were not included in the database.

Outcome Measures

We evaluated the weekly consumption (IU/kg) of efmoctocog alfa or ruriotocog alfa pegol. The frequency of dosing was evaluated according to the number and percentage of patients with a prophylactic dose recorded (once weekly, twice weekly, 3 times per week, every 3 days, every 4 days, every 5 days). Infusion frequency data for efmoctocog alfa were available from 2015 to 2018; data for ruriotocog alfa pegol were available from 2016 to 2018. We evaluated the number and percentage of pharmacy dispensing records with doses ≤ 50 and > 50 IU/kg. For the purposes of comparison between the two drugs, this threshold was chosen because 50 IU/kg is the starting dose for efmoctocog alfa, per the label. The above outcomes (consumption, frequency of dosing) were analyzed for patients in each treatment group; consumption was also analyzed by age category (< 12 and ≥ 12 years).

Statistical Analyses

Descriptive analyses were performed for patient demographic characteristics and clinical conditions. Patient characteristics were summarized using mean (SD) for continuous outcomes and counts, while proportions and counts described categorical variables. Consumption was described by the mean (SD) and median (interquartile range). Between-group comparisons were made using *t*-test for the weekly consumption (continuous variable) and chi-square test for dosing frequency (categorical variable).

Mean weekly prophylaxis was calculated based on the following:

$$\frac{\text{dispensed units}}{\text{number of days between pharmacy dispensing records} \times \text{patient weight}} \times 7$$

The dose from the last observed pharmacy dispensing claim was excluded because the number of days covered by that record could not be calculated. Only consecutive claims were included; claims with more than a 1-month gap to the next claim were excluded.

The proportion of patients receiving efmoctocog alfa and ruriotocog alfa pegol doses that were above, below, and equal to the prophylactic dose of 50 IU/kg was calculated. Subgroup analyses according to age groups (< 12 and ≥ 12 years) were performed for patients who received efmoctocog alfa or ruriotocog alfa pegol using pharmacy dispensing records from 2017, when both EHL products had been approved for use in the United States for ≥ 1 year.

Results

Patient Characteristics

A total of 774 patients were included in the study (efmoroctocog alfa, n=506; rurioctocog alfa pegol, n=268) (Table 1). The mean (SD) ages of patients who received efmorocotocog alfa and rurioctocog alfa pegol were 24.2 (15.8) and 26.3 (14.9) years, respectively.

A higher proportion of patients aged 0 to <12 years was observed in the efmorocotocog alfa group versus the rurioctocog alfa pegol group (24.3% vs 14.6%, respectively). Among patients aged ≥ 12 years, the mean age was 31.2 years in the efmorocotocog alfa group and 29.7 years in the rurioctocog alfa pegol group, with a similar age distribution between groups. The mean (SD) weight was 68.4 (36.8) and 79.8 (37.7) kg for patients receiving efmorocotocog alfa and rurioctocog alfa pegol, respectively. A higher proportion of patients receiving rurioctocog alfa pegol had severe hemophilia A versus those receiving efmorocotocog alfa (79.9% vs 57.5%). The states with the most patients in the database were California, Florida, Massachusetts, and Ohio. Florida had the highest proportion of patients receiving efmorocotocog alfa (20.2%), while Ohio had the highest proportion of patients receiving rurioctocog alfa pegol (16.0%).

Dosing Frequency

From 2015 to 2018, the most frequent dosing regimens overall were twice weekly for both efmorocotocog alfa (45.7%) and rurioctocog alfa pegol (72.4%) (Supplementary Figure 1). For efmorocotocog alfa, this was followed by every 4 days (20.6%), every 3 days (9.1%), and 3 times per week (7.5%). For rurioctocog alfa pegol, it was followed by 3 times per week (8.7%), every 4 days (7.6%), and every 3 days (5.5%) ($p < 0.001$ for distribution of dose frequencies between efmorocotocog alfa and rurioctocog alfa pegol). During 2015–2018 (after US approval in June 2014), the proportion of efmorocotocog alfa twice-weekly dispensing records increased from 31.5% in 2015 to 50.9% in 2018, and every 4 days dispensing records decreased from 31.3% in 2015 to 14.5% in 2018. During 2016–2018 (after US approval in November 2015), the proportion of rurioctocog alfa pegol dispensing records remained broadly stable.

Table 1 Patient Characteristics by Treatment Group

Characteristic	Efmorocotocog Alfa n=506	Rurioctocog Alfa Pegol n=268
Age group (as of 2018), n (%)		
0 to <6 years	38 (7.5)	10 (3.7)
6 to <12 years	85 (16.8)	29 (10.8)
12 to <18 years	75 (14.8)	45 (16.8)
≥ 18 years	267 (52.8)	182 (67.9)
Missing	41 (8.1)	2 (0.8)
Hemophilia A severity, n (%) ^a		
Mild (>5 –30%) ^b	10 (2.0)	4 (1.5)
Moderate (1–5%) ^b	26 (5.1)	15 (5.6)
Severe (<1 %) ^b	291 (57.5)	214 (79.9)
Unknown	179 (35.4)	35 (13.1)
Top four health care provider states, n (%) ^c		
California	48 (9.5)	26 (9.7)
Florida	102 (20.2)	23 (8.6)
Massachusetts	23 (4.6)	27 (10.1)
Ohio	41 (8.1)	43 (16.0)

Notes: ^aDisease severity as recorded in the database records. ^bValues in parentheses indicate FVIII levels, percentage of normal. ^cBased on data from all 50 states.

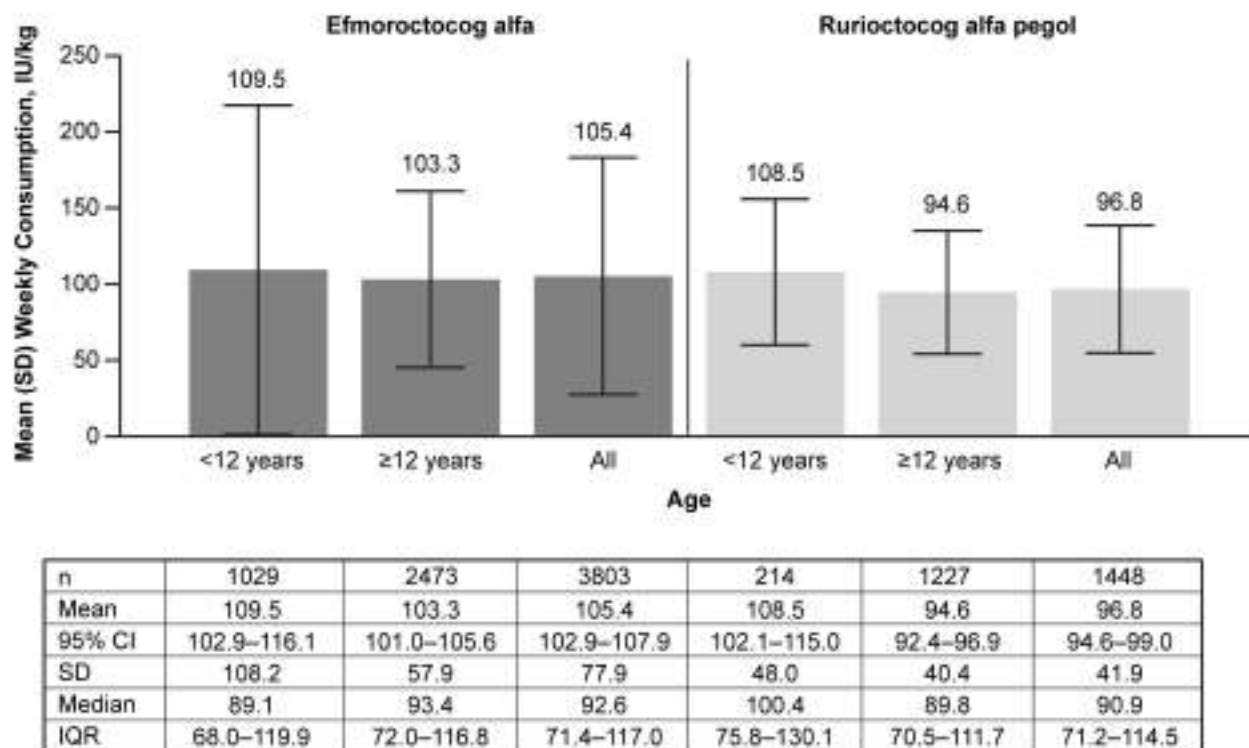


Figure 1 Mean (SD) Weekly Consumption (2015–2018), by age category.

Notes: The n values for both of the “All” columns include records with age missing: 301 missing records for efmoroctocog alfa and seven missing records for rurioctocog alfa pegol.

Abbreviation: n, number of pharmacy dispensing records.

Weekly Consumption

Overall, for patients receiving efmoroctocog alfa, the mean (SD) weekly consumption was 105.4 (77.9) IU/kg; the weekly consumption for patients receiving rurioctocog alfa pegol was 96.8 (41.9) IU/kg ($p < 0.001$ vs efmoroctocog alfa). Mean weekly consumption was slightly higher in patients aged <12 versus ≥ 12 years for both efmoroctocog alfa and rurioctocog alfa pegol from 2015 to 2018. By age category, mean weekly consumption was 109.5 (108.2) IU/kg for patients aged <12 years and 103.3 (57.9) IU/kg for patients aged ≥ 12 years receiving efmoroctocog alfa. For patients receiving rurioctocog alfa pegol, mean weekly consumption was 108.5 (48) IU/kg for patients aged <12 years and 94.6 (40.4) IU/kg for patients aged ≥ 12 years (Figure 1).

Subgroup Analysis

The subgroup analysis using 2017 data showed higher mean weekly consumption for patients who received efmoroctocog alfa versus those who received rurioctocog alfa pegol. By age category, the mean consumption for patients aged ≥ 12 years or <12 years was higher for patients receiving efmoroctocog alfa versus rurioctocog alfa pegol (Supplementary Table 1).

Discussion

This analysis of the US Specialty Pharmacy Database showed that the most frequently observed dispensed treatment regimen between 2015 and 2018 was twice weekly for both efmoroctocog alfa and rurioctocog alfa pegol. This aligns with previously published clinical trial efficacy data, where twice-weekly prophylaxis regimens of efmoroctocog alfa and rurioctocog alfa pegol resulted in low annualized bleeding rates.^{7,10,12} Further, in separate trials prophylaxis with rurioctocog alfa pegol twice-weekly or efmoroctocog alfa every 3–5 days resulted in 40% and 45% of patients, respectively, having zero bleeds.^{10,12} It seems likely that the choice of twice-weekly dosing was made for convenience

reasons, as it is less challenging for patients than dosing every 4 days. The advantage of dosing every 4 days, however, is the reduction in the number of total infusions by 1–2 per month, particularly as reduction in dosing frequency is seen as highly desirable by patients and caregivers.¹⁸ In patients who are highly active or have other clinical needs, more frequent dosing than every 4 days may be necessary even with EHL products, and studies have demonstrated the importance of individualized dosing.^{19,20}

In the present study, overall weekly consumption was slightly higher for efmoctocog alfa versus rurioctocog alfa pegol. Twice-weekly dosing for efmoctocog alfa increased from 31.5% in 2015 to 50.9% in 2018 but remained broadly stable for rurioctocog alfa pegol (67.0% to 72.2%). A possible explanation for this is that efmoctocog alfa was approved in June 2014 and clinicians may have become familiar with its use before rurioctocog alfa was approved ~1.5 years later. It has been estimated that the use of EHL rFVIII products results in a 30% reduction in the numbers of annual infusions versus SHL products.²¹ The results of the current study provide real-world evidence to support the finding that EHL rFVIII allows patients with hemophilia A to extend the time periods between intravenous injections.

Notably, most patients receiving efmoctocog alfa and rurioctocog alfa pegol resided in Florida and Ohio, respectively, likely due to different drug formularies between regions/states. A higher mean weekly consumption was observed for patients receiving efmoctocog alfa (105.4 IU/kg) versus rurioctocog alfa pegol (96.8 IU/kg), a finding that is meaningful economically. These results are broadly consistent with a published real-world exploratory analysis of SHL products (moctocog alfa, octocog alfa, simoctocog alfa, turoctocog alfa) versus EHL products (efmoctocog alfa and rurioctocog alfa pegol).²²

Our consumption results for efmoctocog alfa are very similar to those presented previously, where the authors reported a mean weekly consumption (all ages) of 104 (79.4) IU/kg.²³ Our observed consumption findings for rurioctocog alfa pegol are also similar to the findings of a real-world study published in 2020 of patients with hemophilia A who received rurioctocog alfa pegol after switching from an SHL rFVIII (overall mean weekly dose, 100.6 IU/kg).¹⁴ The slight increase in consumption for patients receiving efmoctocog alfa versus rurioctocog alfa pegol in the current study is likely explained by the higher dose and infusion frequency recommended in the prescribing information for efmoctocog alfa (according to the label, the dosing frequency for adults is every 4 days).⁹ In contrast to the prescribing information, our real-world data show that twice weekly is the most frequently used infusion frequency, which is likely due to convenience as it is less challenging for patients to follow than dosing every 4 days.

The proportion of children aged <12 years was substantially higher in the efmoctocog alfa group than the rurioctocog alfa pegol group. We observed that mean weekly consumption was slightly higher in patients aged <12 versus ≥12 years for both efmoctocog alfa and rurioctocog alfa pegol, which could have affected our observed results, given the possibility of age-dependent pharmacokinetic variation and the shorter half-life of FVIII in young children than in adults.^{3,24} Our finding of greater consumption variability among patients receiving efmoctocog alfa suggests less predictable utilization and indicates differences in the prescribing information for efmoctocog alfa versus rurioctocog alfa pegol (eg, different dosing frequencies for patients aged ≥6 vs <6 years). It may also reflect greater use of personalized treatment regimens with efmoctocog alfa, based on its longer period of availability and data obtained from clinical trials and presented in the prescribing information.

Limitations

We acknowledge some limitations that are inherent to database analyses. The US Specialty Pharmacy Database does not include all specialty pharmacy providers and therefore may not be representative of the total US population with hemophilia A. The recording of treatment regimen, infusion frequency, and disease severity may be inconsistent in a pharmacy dispensing records database. For example, no information on disease severity was recorded for 35% of patients in the efmoctocog alfa group and 13% in the rurioctocog alfa pegol group. In addition, the treatment selection and clinical response may affect dosing patterns. These factors were not investigated owing to lack of information in the pharmacy dispensing records data. The current analysis focused on FVIII utilization (frequency of infusions, consumption), but did not link the data to clinical outcomes. Finally, it should be noted that there were important differences between the two treatment groups, potentially affecting comparisons between the two agents. For example, there were

almost twice as many patients in the efamoroctocog alfa group than the ruriotocog alfa pegol group, reflecting the longer availability, and therefore potential study period, of efamoroctocog alfa. There was also a higher percentage of children aged <12 years in the efamoroctocog alfa group (24%) compared with the ruriotocog alfa pegol group (15%), and a higher proportion of patients receiving ruriotocog alfa pegol had severe hemophilia A (79.9%) versus those receiving efamoroctocog alfa (57.5%).

Conclusions

This retrospective database analysis provides additional evidence to increase the understanding of real-world dosing and utilization patterns of EHL rFVIII in patients with hemophilia A without inhibitors, which may help providers to understand clinical effectiveness implications for these therapies. Together, the results of this real-world study and those of clinical trial data suggest that the use of EHL rFVIII may reduce the treatment burden for patients. Additional research is needed to evaluate the reasons for changes in dosing patterns over time and the associated clinical outcomes.

Abbreviations

EHL, extended half-life; FVIII, factor VIII; HIPAA, Health Insurance Portability and Accountability Act; ICD, International Classification of Diseases; IQR, interquartile range; PEG, polyethylene glycol; rFVIII, recombinant factor VIII; SHL, standard half-life.

Data Sharing Statement

The data that support the findings of this study are available from Specialty Pharmacy Database records from individual states, but restrictions apply to the availability of these data due to applicable privacy laws and data use agreements. The data were used pursuant to data use agreements between Shire US Inc., a Takeda company, and the individual states.

Ethics Approval and Informed Consent

No institutional review board approval was required for this retrospective database analysis 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.

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Author Contributions

All authors made a significant contribution to the conception, study design, execution, acquisition of data, analysis and/or interpretation of the manuscript. In addition, all authors took part in drafting, revising, and 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.

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Disclosure

Y. Wu and S.X. Sun are employees of Takeda Development Center Americas, Inc., Cambridge, MA, USA, and Takeda stock owners. T. Fan is an employee of Takeda Pharmaceuticals USA, Inc., Lexington, MA, USA, and Takeda stock owner. The authors report no other conflicts of interest in this work.

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Hematological Profile Changes Among Oral Contraceptive Users: A Narrative Review

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Abstract: Hematological profiles are measurable indices of blood that can be used to identify and monitor some pathological and physiological abnormalities. Oral contraceptives are one of the factors which may exert an influence on hematological laboratory test results and have a critical importance. Therefore, the main goal of this review was to evaluate the hematologic profile alterations brought on by the use of oral contraceptives. Accordingly, even though there were studies that showed no significant difference, most of the oral contraceptive users had higher hemoglobin, platelet, and erythrocyte counts than non-users. Other erythrocyte metrics, such as mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and red cell dispersion width, did not significantly change. Moreover, leukocyte count and leukocyte differential count had no significant change among the oral contraceptive users in comparison to non-users. Oral contraceptive users typically have shorter prothrombin times, activated partial thromboplastin times, thrombin times, and greater levels of fibrinogen and D-dimer compared to non-users. However, this review is a narrative review and did not show the pooled effect of oral contraceptives on these hematological parameters. Therefore, it is recommended to do systematic and meta-analysis to determine the pooled effect of oral contraceptives.

Keywords: contraceptives, hematological parameters, hematopoiesis, hemostasis, oral contraceptives, thromboembolism

Introduction

Background

Hematological profiles are measurable indices of blood that can be used to identify and monitor some pathological and physiological abnormalities. Different illness situations that influence hematopoietic physiology and immunological responses can have an impact on these profiles,^{1,2} and they are frequently utilized in clinical settings in the medical field.^{3,4} These include parameters routinely given by complete blood count that gives a clue about erythrocyte count (EC), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin concentration (MCHC), Mean corpuscular hemoglobin (MCH), Erythrocyte volume fraction (EVF), Hemoglobin (Hb) concentration and Red cell dispersion width (RDW).⁵

Hematological markers that have levels outside of normal ranges can be used to diagnose disorders like cancer, immunological diseases, and cardiovascular disease.⁶ For example, RDW, RBC parameter that measures the variability in the size of erythrocytes circulating in the blood circulation, is usually used for the differential diagnosis of hematological disorders⁷ including iron deficiency anemia (anemia that results when there is deficiency of iron for the production of erythrocytes), megaloblastic anemia, and thalassemia^{8,9} and a variety of other diseases, such as heart failure,⁵ atrial fibrillation,¹⁰ lung cancer¹¹ or disorders characterized by inflammation.¹² Hematocrit, more commonly known as the EVF, is a determinant of whole blood viscosity, the viscosity of which will affect peripheral resistance to blood flow, which will affect blood pressure.¹³ Red cell destruction due to RBCs' reduction in deformability and an increase in cell size, high and low Hb levels are associated with cardiovascular risk.¹⁴

The second most frequent cell type, platelets are small anucleated cell fragments that are essential for maintaining vascular integrity and controlling hemostasis. In addition to their primary role in hemostasis, platelets are crucial in the defense against viral infections.¹⁵ The most important parameters related to platelet count include plateletcrit, Mean platelet volume (MPV)

which is the measure of average size of platelets, and Platelet distribution width (PDW).⁴ The morphology of platelets changes as a result of their activation, and an increase in platelet swelling results in a rise in MPV and PDW.¹⁶ A more precise index of activation platelet is the distribution width of platelets, which represents the reactivity of the thrombocytes.¹⁷ In circumstances like hypertension, activation of thrombocytes is a risk factor for thrombotic disorders.¹⁸

White blood cells (WBC) are immune system cells that protect the body against external substances and infectious diseases. Similar to how RBCs and thrombocytes are created, they are all created in the bone marrow microenvironment from hematopoietic stem cells (HSC). These cells, called WBCs, can be seen all over the body, especially in the lymphatic and blood systems.¹⁸ Changes in WBC may indicate different disorders, including infection, inflammation, and hematological cancer.⁶

Coagulation tests encompassing Prothrombin time, Activated partial thromboplastin time (APTT), and fibrinogen are frequently used to evaluate coagulopathy and to guide hemostatic interventions.^{19,20} Additional tests can be performed to quantify the levels of particular coagulation factors, screen for inhibitors of particular coagulation factors, and evaluate the thrombin clotting time.²⁰ Thrombin, activated factor XIII and plasmin work in sequence to create a D-dimer antigen, which is another coagulation parameter and a distinctive hallmark of fibrin breakdown. Its testing has primarily been used in clinical settings to rule out venous thromboembolism (VTE).²¹

The rise in fibrinolytic activity balances out the heightened coagulation activity, maintaining hemostatic equilibrium. However, thrombus formation can happen when there is local vascular endothelium damage or when other thromboembolism risk factors, such smoking and advanced age, combine. The little variations in coagulation factor levels among women using different oral contraceptive (OC) formulations may be significant in these circumstances.²²

The laboratory results of hematological tests may be impacted by various conditions. Age, activity, bed rest, food ingestion, alcohol ingestion, menstrual cycle, obesity, posture, pregnancy, race, gender, smoking, and OC utilization are among factors that may influence laboratory results.²³ The present review was designed to give a brief account of the studies carried out to elucidate the changes in hematological profiles linked to the usage of OCs.

Mechanism of Action of Oral Contraceptives on Hematopoiesis

Estrogen administration significantly increases the division and proliferation of HSCs and thus explains the higher blood counts in women during the reproductive years.^{24,25} High amounts of the estrogen receptor (ER) are expressed by HSCs, and estrogen/ER α signaling promotes self-renewal of HSC, HSC expansion and erythropoiesis.²⁵

The use of hormonal contraceptives, which is generally associated with lighter menstrual periods, is associated with better indicators of iron status indicators.^{26–29} Poorer iron status indicators (low levels of serum ferritin and Hb) have been linked to menstrual blood loss.^{27,30,31} Serum iron levels have been shown to be higher among OC users.^{29,32} The endometrium shrinks as a result of progestogenic tablets' endometrial atrophy, and their usage results in scanty withdrawal bleeding that is less than in a typical cycle. This is the first factor that is assumed to be the cause of elevated blood iron content.³³

Additionally, OCs improve gut absorption of iron³⁴ and were known to increase biomarkers of tissue iron stores (serum ferritin) thus lowering the risk of developing anemia.^{26,35,36} The OC pill increases serum ferritin, therefore improves the body's iron status.^{37,38} Oral contraceptives enhanced inflammatory-driven increases in ferritin while decreasing the frequency and volume of menstrual bleeding.³⁹ As a result, the usage of OCs may be linked to an improvement in Hb status, enhancing the health of low-socioeconomic-class women.⁴⁰

Oral contraceptive decreases intestinal re-absorption of folate and vitamin B-12 which resulted in significantly decreased serum levels of these vitamins in women taking OC pills, even when controlling for intake through diet, and may end up in megaloblastic anemia.^{41–44}

Mechanism of Action of Oral Contraceptives on Hemostasis System

With enhanced procoagulant effects, decreased anticoagulant effects, and equivocal effects on fibrinolysis, oral contraceptives have a net prothrombotic impact (with increases in the activity of the antifibrinolytic system).⁴⁵ The biologic, unifying mechanism underlying the thrombotic effects of OC pills is still unclear, despite the fact that individual alterations in procoagulants and anticoagulants with OC pill usage are widely characterized.⁴⁶

Procoagulant Effects

Oral contraceptives increase adenosine diphosphate, which causes fibrinogen binding and platelet aggregation.⁴⁷ They also cause a noticeable and long-lasting increase in the synthesis of cell membrane lipids.⁴⁸ An increased synthesis can have a significant influence on hemostatic physiology since platelet membrane lipids are crucial pro-coagulant factors in hemostasis.⁴⁹ There have also been reports of increased endothelial replication of endothelial cells and increased endothelial synthesis of the Von Willebrand factor (VWF).⁵⁰ This may also explain why platelets stick to vascular endothelium more readily and why thrombosis is more likely to occur.

Estrogen is linked to a variety of prothrombotic pathways that modify coagulation proteins and tip the hemostatic scales in favor of a prothrombotic state. Increased levels of factors II, VII, VIII, X, and fibrinogen, reduced levels of protein S and Antithrombin (AT), and activated protein C resistance are some of these changes. The PT and APTT may become shorter as a result of these modifications.⁵¹

Prothrombin, factor VII, factor VIII, factor X, fibrinogen, and prothrombin fragment 1+2 levels rise while factor V levels fall during the use of OCs, according to a recent randomized, crossover research. It is now understood that prothrombin⁵² and factor VIII⁵³ concentrations which are somewhat elevated are linked to a more risk of venous thrombosis. Since factor V may exhibit anticoagulant activity because of its ability to act as a cofactor in the inactivation of activated factor VIII that is mediated by activated protein C; a reduction in the level of factor V may contribute to the thrombotic side effects of OCs.⁵⁴

Fibrinolytic Effects

The usage of OCs is indicated to increase fibrinolytic activity through changes in fibrinolytic variables such as plasminogen, tissue plasminogen activator (T-PA), Plasminogen activator inhibitor type 1 (PAI-1), and plasmin-anti-plasmin complexes.^{55,56} Tissue plasminogen activator levels are raised and PAI-1 levels are decreased by COCs, which has an impact on fibrinolysis. Higher levels of Thrombin activatable fibrinolysis inhibitor (TAFI) in women taking COCs appear to counterbalance these effects, though.⁵⁶

Because of the lack of evidence linking alterations in the fibrinolytic system to an elevated risk of venous thrombosis, it is unknown if alterations in fibrinolytic activity during OC usage has therapeutic consequences. The TAFI, which when activated prevents fibrinolysis by removing from fibrin the lysine residues necessary for the binding and activation of plasminogen, is one antifibrinolytic mechanism.⁵⁷ Venous thrombosis is at increased risk due to elevated TAFI levels.⁵⁸

The total clot-lysis time was shown to be unaffected by OC usage in an assay for clot lyses that measured the activity of both the fibrinolytic system and the TAFI dependent antifibrinolytic pathway. This data implies that the increased fibrinolytic potential caused by the use of OCs is offset by the TAFI system's enhanced down-regulation of fibrinolysis. Increased TAFI levels and improved thrombin (the activator of TAFI) synthesis are most likely responsible for the increased TAFI-dependent inhibition of fibrinolysis.³⁹

Change in Blood Cells and Blood Cell Parameters Among Oral Contraceptive Users

Red Blood Cell Parameters

To make sure the tissues receive a proper amount of oxygen, the red cell volume must be maintained. Erythropoietin primarily regulates the process that links red cell synthesis with tissue oxygen supply. The creation of Hb, a specialized protein necessary for delivering oxygen within the red cell, is a prerequisite for the formation of functioning RBCs. As the erythroid precursor cells develop, this protein is produced more and more. Iron atoms from heme molecules are introduced into globin to form it.⁵⁹ Recycled iron from the Hb of dying erythrocytes is used to maintain the Hb level, and when necessary, iron from the reticuloendothelial storage is also used. The main protein that stores iron is called ferritin, and the concentration of ferritin in the blood is a reliable predictor of the quantity of stored iron. In plasma, the carrier molecule transferrin is linked to iron.⁵⁹

RBC count⁶⁰ and Hb concentration rise as a result of OCs.^{30,61} The decrease in menstrual blood loss, which is known to be frequent among OC users, may be to blame for the rise in RBC count.^{26,30,34,36,62–64} Menstrual blood loss has been

linked to worse iron status markers.^{27,30,31} Likewise, the use of hormonal contraceptives is linked to better markers of iron status and is frequently related with lighter menstrual cycles.^{26–29} According to reports, OC users had higher serum iron levels.^{29,32} Progestogenic medications promote endometrial shrinkage and generate scanty withdrawal bleeding that is less than in a typical cycle, which is the first reason hypothesized to be the cause of elevated serum iron content.³³

Additionally, OCs have a tendency to boost gastrointestinal absorption of iron³⁴ and they are known to raise tissue iron reserves' biomarkers, which decreases the likelihood of developing anemia.^{26,35,36} Higher ferritin levels in OC users are a positive biomarker impact. Reduced menstrual bleeding frequency and volume, as well as increases in ferritin brought on by inflammation, are two reasons why there may be increased levels of ferritin.³⁹

However, several investigations revealed no discernible alteration in RBC count, EVF,^{60,65,66} and Hb values.^{46,60,66} Ended, there was also a contradictive study that showed a decrement in RBC count among OC users compared to non-users.⁶³ However, the change in other RBC characteristics such MCV, MCH, MCHC, and RDW was not significantly impacted by the change in RBC count.⁶⁰ Also a study by⁶⁶ showed no difference in MCV, MCH, and MCHC between the groups who were using OCs and those who were not using (Table 1).

Table 1 Changes in Red Blood Cell Parameters Among Oral Contraceptive Users

Parameter	OC Used	Result		Conclusion	Author
		Users	Non-Users		
RBC × 10 ¹² /L	COC	4.35 ± 0.48	4.14 ± 0.42	Increase	[61]
	Progestin only	–	–	Decrease	[63]
	COC	4.4 ± 0.51	4.6 ± 0.39	No difference	[69]
EVF (%)	COC	37.64 ± 2.46	36.52 ± 3.56	No difference	[60]
	COC	34.42	36.40	No difference	[65]
	COC	35.8 ± 3.27	37.3 ± 3.18	No difference	[69]
Hb (g/dl)	Not indicated	–	–	Increase	[30]
	COC	–	–	Increase	[61]
	COC	12.45	12.67	No difference	[46]
	COC	12.32 ± 0.81	11.93 ± 1.20	No difference	[60]
	COC	11.9 ± 1.14	12.1 ± 1.24	No difference	[69]
MCV	COC	87.04 ± 5.92	88.03 ± 4.56	No difference	[60]
	COC	81.4 ± 8.67	81.0 ± 7.73	No difference	[69]
MCH (pg)	COC	28.73 ± 2.16	28.47 ± 1.76	No difference	[60]
	COC	27.2 ± 3.32	26.7 ± 2.96	No difference	[69]
MCHC (g/dl)	COC	32.71 ± 0.90	32.55 ± 0.93	No difference	[60]
	COC	33.3 ± 0.93	32.9 ± 1.13	No difference	[69]
RDW (%)	COC	13.05 ± 1.35	12.89 ± 1.61	No difference	[60]

Note: The conclusions are derived from the respective authors listed.

Abbreviations: g/dl, gram per deciliter; pg, pico gram.

Platelet Parameters

Circulating platelets, coagulation factors, the endothelium of the blood vessel wall, naturally occurring anticoagulants (such protein C, protein S, and AT), and fibrinolysis (clot digestion) all interact to regulate hemostasis.⁵⁹ The platelet count assesses if there are enough platelets to achieve early hemostasis. There is a typical range of 150,000 to 450,000 platelets.⁶⁷

Use of OCs raises platelet count.^{65,67} The increase in the number of platelets may be explained by the fact that estrogen administration significantly increases the division and proliferation of HSCs in women during the reproductive years.^{24,25} Additionally, PLT count^{46,60,66,68,69} and MPV⁶⁶ among OC users were observed to have remained stable. The lack of substantial alterations might be attributable to the use of smaller sample sizes or the short duration of OC usage, which might not have produced differences in these parameters (Table 2).

White Blood Cell Parameters

White blood cells, also referred as leukocytes, are the body's defense against infection. Depending on the presence or lack of granules in the cell cytoplasm, they can be generically categorized as granulocytes or agranulocytes. Neutrophils (polymorphs), eosinophils, and basophils are examples of granulocytes. Monocytes and lymphocytes are examples of circulating agranulocytes. By phagocytosing pathogens, neutrophils and monocytes have a crucial part in the defense against acute bacterial infections. Lymphocytes help phagocytes by generating a regulated immune response.⁵⁹

Regarding their count, there was no difference between OC users and non-users in WBC.^{46,60,65,66} Additionally, there was no discernible difference between users and non-users in the WBC differential count, which includes LYM, MON, NEU, EOS, and BAS percentages⁶⁰ (Table 3).

Changes in Coagulation Profiles Among Oral Contraceptive Users

Platelets collect on the blood vessel endothelium as a result of vascular injury, and a group of coagulation factors designated by their roman numbers are activated. Von Willebrand factor serves as a carrier of factor VIII and promotes platelet interaction with the exposed endothelium. The final step in the coagulation pathway's activation is thrombin's conversion of fibrinogen into a fibrin gel. A sophisticated network of activating and feedback processes makes sure that proteases are formed to both activate and degrade the coagulation factors, avoiding the development of large-scale clots.⁵⁹

The fibrinolytic system, which is made up of the proenzyme plasminogen and its related activators and inhibitors, regulates the removal of fibrin. The tissue protease, T-PA, which is triggered concurrently with the initiation of the coagulation process, is the primary activator of plasminogen. The fibrinolytic system's final step is plasmin, which breaks

Table 2 Changes in Platelet Parameters Among Oral Contraceptive Users

Parameter	OC Used	Result		Conclusion	Author
		Users	Non-Users		
Platelet $\times 10^9/L$	COC	270.59	249.75	Increase	[65]
	COC	–	–	Increase	[67]
	COC	289.86	283.38	No difference	[46]
	COC	206.28 $\pm$ 39.08	196.65 $\pm$ 35.12	No difference	[60]
	COC	–	–	No difference	[66]
	COC	–	–	No difference	[68]
	COC	258 $\pm$ 100	294 $\pm$ 69	No difference	[69]
MPV	COC	–	–	No difference	[66]

Note: The conclusions are derived from the respective authors listed.

Abbreviation: L, liter.

Table 3 Changes in White Blood Cell Parameters Among Oral Contraceptive Users

Parameter	OC Used	Result		Conclusion	Author
		Users	Non-Users		
WBC $\times 10^9$ /L	COC	5.07	4.93	No difference	[65]
	COC	–	–	No difference	[66]
	COC	6.49	6.99	No difference	[46]
	COC	6.07 $\pm$ 1.29	6.19 $\pm$ 1.33	No difference	[60]
LYM%	COC	32.69 $\pm$ 3.83	32.81 $\pm$ 4.11	No difference	[60]
MON%	COC	8.04 $\pm$ 1.63	8.30 $\pm$ 1.82	No difference	[60]
NEU%	COC	56.12 $\pm$ 3.70	55.67 $\pm$ 4.14	No difference	[60]
EOS%	COC	2.35 $\pm$ 1.10	2.16 $\pm$ 0.83	No difference	[60]
BAS%	COC	0.91 $\pm$ 0.25	1.00 $\pm$ 0.34	No difference	[60]

Note: The conclusions are derived from the respective authors listed.

Abbreviation: L, liter.

down fibrin and fibrinogen into degradation products (FDPs) such fibrinopeptide A and D-dimer. Plasminogen activator inhibitor-1 is a potent inhibitor of T-PA and is mostly generated by endothelial cells.⁵⁹

The liver produces proteins C and S, which are physiological anticoagulants that depend on vitamin K. Through the neutralization of active coagulation factors V and VIII, they work as one of the primary regulators of hemostasis. Congenital or acquired protein C or S deficiency are both possible.⁷⁰ A glycoprotein called antithrombin predominantly inhibits thrombin while also inhibiting a number of activated coagulation proteins.⁷¹

Several investigations were made to clarify the effects of OCs on blood coagulation^{72,73} and it is important to emphasize here the changes to the coagulation profile brought on by using these drugs.

Fibrinogen

Fibrinogen level increases among users of OCs.^{46,65,67,74,75} The increase may be because of the effect of the estrogen hormone. The hormone has been reported to increase the level of various coagulation proteins including fibrinogen.⁵¹ On the contrary, fibrinogen level in OC pill users was found to be similar to non-users among Chinese women.^{76,77}

Activated Partial Thromboplastin Time

A test called activated partial thromboplastin time is used to check for anomalies in the intrinsic and general clotting systems as well as to track how well circulating heparin is preventing clotting. It assesses the activity of factors from the intrinsic and common pathways, including factors I, II, V, VIII, IX, X, and XI.⁷⁸

Shorter APTT has been demonstrated among users of OC.^{65,69,73,75,79} Shorter APTTs may occur with the administration of OCs if one or more of the clotting factors, including as prothrombin, factor VIII, factor X, fibrinogen, and prothrombin fragment 1+2, as well as due to decreases in the levels of protein S, AT, and activated protein C resistance.^{22,50,80} Few studies, meanwhile, claimed that there was no discernible difference in APTT levels between people who used and did not use OC.^{46,67,76}

Prothrombin Time

The extrinsic and common coagulation pathways are tested in the laboratory using the prothrombin time. It is the amount of time needed for platelet-poor plasma to clot after calcium, phospholipid, and tissue factor have been added. The component that is “extrinsic” to the plasma-based coagulation mechanism is the tissue factor. Patients on warfarin for

anticoagulation may experience isolated prolongations of the PT due to factor VII insufficiency, fibrinogen abnormalities, or both. It is also regarded as the most accurate screening method for coagulopathies caused by malfunction of the liver.⁸¹

Shortening of the PT^{46,65,67,73,75} has been demonstrated in OC users. Increases in the levels of activity of one of the clotting factors including prothrombin, factor VII, factor X, fibrinogen, and prothrombin fragment 1+2 and decreases in the levels of factor V, AT, protein S, and activated protein C resistance during the use of OCs might cause shorter PT.^{22,51} However, the PT of OC users resulted in no difference from the non-users according to other studies.^{69,76,77,79}

Thrombin Time

The term “thrombin time” (TT) refers to the amount of time needed for fibrin to polymerize into a solid clot when thrombin is present.⁶ It mostly reflects the fibrinogen concentration. However, it also reflects the fibrinopeptides’ cleavability and fibrinogen’s polymerization.⁸² When fibrinogen levels are low, fibrinogen is dysfunctional, or thrombin inhibitors such heparin or fibrin-split products are present, the TT is extended but remains normal in individuals with abnormalities in the intrinsic or extrinsic route.²⁰

Shortening of the TT has been reported among women that were using OCs.^{65,67,75,77} The shortening of TT may be because of the increase in the concentration of fibrinogen among OC users.^{51,80,82}

D-Dimer

The D-dimer antigen, a characteristic biological indicator of fibrin breakdown, is produced by the sequential activities of the enzymes thrombin, activated factor XIII, and plasmin.²¹ Oral pill users were shown to have considerably higher D-dimer levels than non-users.^{65,79,83} The enhanced fibrinolytic activity that prevents excessive fibrin deposition inside the vascular channels and its obstructive effects is reflected in the higher plasma D-dimer level. As independently reported by Winkler and Meijers et al, the enhanced fibrinolysis thereby physiologically counteracts the hypercoagulable condition and maintains normal circulation^{55,56} (Table 4).

Table 4 Changes in Coagulation Profile Among Oral Contraceptive Users

Profile	Type of OC Used	Result		Conclusion	Author
		OC Users	Non-Users		
PT in second	COC	11.46	13.79	Decrease	[65]
	COC	-	-	Decrease	[67]
	COC	13.4	13.6	Decrease	[73]
	COC	12.8 ± 1.5	13.7 ± 2.05	Decrease	[75]
	COC	10.35	10.78	Decrease	[46]
	Both	14	13.4	No difference	[77]
	Not indicated	14.8 ± 1.2	14.8 ± 2.1	No difference	[76]
	COC	11.8 ± 2.27	12.4 ± 1.08	No difference	[69]
	Not indicated	14	13.5	No difference	[79]

(Continued)

Table 4 (Continued).

Profile	Type of OC Used	Result		Conclusion	Author
		OC Users	Non-Users		
APTT in second	COC	27.99	32.49	Decrease	[65]
	COC	40.9	41.9	Decrease	[73]
	COC	32.6 ± 1.92	33.7 ± 1.52	Decrease	[75]
	COC	25.9 ± 2.61	28.3 ± 2.76	Decrease	[69]
	Not indicated	31.3	33	Decrease	[79]
	Not indicated	33.1 ± 3.9	34.3 ± 4.9	No difference	[76]
	COC	-	-	No difference	[67]
	COC	29.83	30.46	No difference	[46]
TT in second	COC	17.57	20.45	Decrease	[65]
	COC	-	-	Decrease	[67]
	COC	11.9 ± 1.81	12.6 ± 1.27	Decrease	[75]
	Both	9.1	9.8	Decrease	[77]
Fibrinogen in mg/dl	COC	387.10	276.85	Increase	[65]
	COC	-	-	Increase	[67]
	COC	625	260	Increase	[74]
	COC	347 ± 80.9	305.9 ± 45.99	Increase	[75]
	COC	327.47	296.57	Increase	[46]
	Not indicated	161.4 ± 64.3	161 ± 71	No difference	[76]
	Both	266	249	No difference	[77]
D-dimer in ng/mL	COC	813.36	257.04	Increase	[65]
	Not indicated	1000	Negative	Increase	[79]
	COC	350 ± 0.07	210 ± 0.07	Increase	[83]

Notes: Negative D-dimer value is when the D-dimer level is below a predetermined cut-off threshold. The conclusions are derived from the respective authors listed.

Oral Contraceptive Use Associated Coagulation Disorders

Women on combined OCs have consistently noted changes in blood coagulation that may be the cause of their elevated risk of thromboembolic illness.⁷³ The balance of hemostasis is impacted by estrogen, and its usage as Combination oral contraceptives⁵ are linked to an increased risk of arterial thrombosis⁵² and a greater risk of VTE, however the exact risk is uncertain.^{84,85}

The risk of VTE may vary across different OCs due to the progestin component.⁸⁴ The risk of thrombosis was increased more among users of OCs containing third-generation progestin, drospirenone, and cyproterone acetate than in users of second-generation tablets.⁸⁵ It is essential to comprehend that women utilizing COCs run the chance of developing VTE if there is a family history of the condition.⁸⁶ The risk of venous thrombosis decreases in COC users with sustained use and reduced estrogen dose. Progestogen-only drugs were not associated with an increased risk of venous thrombosis.^{81,87}

Conclusion

Despite studies that found no difference to be statistically significant, most OC users had higher Hb, PLT, and RBC counts than non-users did. However, other RBC characteristics as MCV, MCH, MCHC, and RDW showed no obvious difference. Moreover, WBC and its differential count had no significant change among the OC users in comparison to non-users.

In contrast to non-users, OC users' coagulation states, measured through tests including PT, APTT, and TT, were shortened, and their levels of fibrinogen and D-dimer tended to rise. An increase in coagulation proteins and a decrease in anticoagulant proteins may be the cause. These might increase the risk of thrombosis among OC users. When pre-exposure variables such as family history of VTE exist, the thrombotic risks might be further increased. Since VTE runs in families, caution should be used while giving these OCs to patients.

Oral contraceptives had a substantial impact on the hematological profile, particularly RBC count, platelet count, Hb, and coagulation states including APTT, PT, TT, fibrinogen, and D-dimer levels, albeit results were not consistent across studies. As a narrative review, our analysis did not demonstrate the pooled impact of OCs on these hematological parameters. Therefore, systematic and meta-analysis are advised to ascertain the combined impact of OCs.

Abbreviations

APTT, Activated Partial Thromboplastin Time; AT, Antithrombin; COC, Combined Oral Contraceptive; EC, Erythrocyte count; ER α , Estrogen Receptor- α ; EVF, Erythrocyte Volume Fraction; Hb, Haemoglobin; HSC, Hematopoietic Stem Cell; MPV, Mean Platelet Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; MCV, Mean Corpuscular Volume; OC, Oral Contraceptive; PAI-1, Plasminogen Activator Inhibitor Type-1; PDW, Platelet Distribution Width; TAFI, Thrombin Activatable Fibrinolysis Inhibitor; PT, Prothrombin Time; TT, Thrombin Time; T-PA, Tissue Plasminogen Activator; RBC, Red Blood Cell; RDW, Red Cell Dispersion Width; VTE, Venous Thromboembolism; VWF, Von Willbrand Factor; WBC, White Blood Cell.

Disclosure

The authors report no conflicts of interest in this work.

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Distribution Width of Red Blood Cells and Related Factors Among Patients with End-Stage Renal Disease in Addis Ababa, Ethiopia

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Background: RDW is critical to the clinical diagnosis and progression of ESRD. There is currently little data on the relationship between RDW and ESRD in sub-Saharan Africa. Because of this, the present study evaluates RDW in patients with ESRD and associated factors in Addis Ababa, Ethiopia.

Methods: The hospital-based cross-sectional study design was conducted on a total of 83 patients. RDW, MCV, SCR, BUN, GFR, FBS and serum albumin were determined. Blood pressure (mmHg), weight (kg), height (m), MUAC (cm) and BMI (kg/m²) were also measured. Data entry was via Epi-data version 3.4 and analyzed with SPSS version 26.0. A multivariate logistic regression analysis with a p-value < 0.05 at a 95% confidence interval was used to identify the associated factors of RDW.

Results: A total of 83 ESRD patients participated, with a response rate of 95.4%. RDW ranged from 15.5% to 23.6% with a mean of 17.40% ± 1.46%. Anisocytosis was present in 98.8% of patients. Of 83 patients, 66.3% were hypertensive, 20.5% had diabetes, and the remaining 13.3% had other conditions (glomerulonephritis and peripheral vascular disease). The mean GFR value was 5.20 mL/min/1.73 ± 1.58. RDW showed a significant association with GFR (AOR: 4.6, 95% CI [1.27, 20.74], P = 0.047), alcohol consumption (AOR: 13.4, P = 0.012, 95% CI [1.97, 22.62]), recurrent kidney disease (AOR=25.6, P=0.016, 95% CI [1.85, 53.71]) and use of medication (AOR=0.2, P=0.044, 95% CI [0.03, 0.95]).

Conclusion: RDW showed a significant association with GFR, recurrent kidney disease, alcohol consumption, and medication use in hemodialysis-dependent ESRD patients. The mechanisms of RDW disruption in ESRD patients need further investigation.

Keywords: red cell distribution width, end stage renal disease, glomerular filtration rate, serum creatinine, mean corpuscular volume, body mass index, blood urea nitrogen, mid-upper arm circumference

Introduction

RDW is part of the complete blood count which is used to quantify the size variation of red blood cells. Mostly, it is used for the diagnosis of anemia and other cardiovascular disorders.¹ The lower and upper limits of the RDW values ranged from (11.5–14.5%), but for women they ranged up to (11.5%–15.5%).² The RDW value was calculated by dividing the standard deviation of the erythrocyte volume by the mean corpuscular volume and multiplying by 100.³ The RDW value below the standard reference range is not clinically significant, while values above the normal range reflect the presence of anisocytosis (the presence of small and large erythrocytes or both).⁴

CKD is a structural or functional abnormality in the kidneys' ability to filter blood for at least 3 months. It is characterized by either kidney damage (proteinuria (> 30 mg/24 h or > 1 on a specific dipstick) or decreased GFR < 60 mL/min/1.73 m²).⁵ A previous study had shown that in patients with stage^{3–5} chronic kidney disease, higher RDW was associated with high morbidity.⁵ ESRD is becoming a public health concern worldwide.⁶ In addition, high RDW was associated with type 2 diabetes mellitus, CKD, and albumin in the general population.⁷

Hemodialysis, one of the alternative treatments in patients with ESRD, can decrease the average survival time of red blood cells due to compression and twisting of the cells.^{8,9} Even today, RDW has been used to diagnose various types of anemia. Higher RDW values indicate a shortened red blood cell lifespan due to poor erythropoiesis or increased red blood cell destruction.¹⁰ Over the past few decades, there has been increasing evidence that high RDW is a predictor of cardiovascular morbidity in the general population,¹¹ chronic heart failure,¹² coronary artery disease,¹³ stroke,¹⁴ and kidney transplant recipients¹⁵ and acute kidney injury treated with hemodialysis.¹⁶

In addition, it has been reported that high RDW is associated with the presence of type 2 diabetes mellitus, the progression of chronic kidney disease in patients with varying degrees of renal dysfunction,⁷ and albumin in the general population.¹⁷ The underlying mechanisms linking high RDW to all of these serious diseases are still unclear, but chronic inflammation, endothelial dysfunction, oxidative stress, and malnutrition have been suggested as possible causes.¹⁸ Type 2 diabetes and hypertensive diseases accelerate atherosclerosis, deterioration in renal function and increased morbidity.¹⁹ A higher RDW was associated with morbidity and a stronger predictor of disease worsening than other laboratory markers of anemia, such as transferrin saturation and ferritin levels in hemodialysis patients.⁵ Globally, it has been estimated that more than 500 million individuals have CKD, defined by either kidney damage or glomerular filtration rate (GFR) < 60 mL/min/1.73 m² for >3 months.²⁰ In Africa CKD imposes disproportionate human suffering and a catastrophic economic burden. Less than 2% of the patients with ESRD have access to renal replacement therapy, making ESRD a death sentence for most patients.²¹ According to WHO report,²² death due to kidney disease in Ethiopia reached 12,038 or 1.47% of total deaths. Patients with the end-stage renal disease usually have various comorbidities.²² A study done by Lippi et al, confirmed that the estimated glomerular filtration rate (GFR) progressively decreased when the RDW level increased in adult outpatients.²³ Another study²⁴ showed that a lower estimated GFR is related to a higher RDW level. Some of the significant risk factors for poor prognosis of ESRD were anemia and RDW which provides an additional prognostic marker for the progression of CKD.²⁴ In addition, a previous study also had shown that RDW significantly increases among CKD patients and has a negative relationship with the estimated GFR.²⁵ Evidence suggests that RDW may serve as a prognostic indicator in healthy individuals as well as specific patient populations including malignancy, renal failure, and cardiovascular disease independent of anemia.²⁶ RDW was associated with diabetes-associated complications including diabetic nephropathy. It is known that inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α , desensitize bone marrow erythroid progenitors to erythropoiesis, inhibiting red blood cell maturation and thereby promote anisocytosis.²⁶ A previous study also showed that RDW level was associated with early renal injury in hypertensive patients,²⁷ and with diabetes-associated complications like diabetic nephropathy.²⁸ A previous study involving 513 patients with essential hypertension reported a statistically significant association between RDW and systolic blood pressure or albumin-to-creatinine ratio after adjustment for potential confounders such as age, sex, and hemoglobin.²⁸ In the United States, about 28% of patients with clinically significant stage 3 or worse CKD are neither diabetic nor hypertensive, particularly those older than 65 years.²⁹ Because it is known that chronic complications induce inflammatory cytokines, such as interleukin (IL)-1, IL-6,²⁹ tumor necrosis factor (TNF)- α ,³⁰ desensitize bone marrow erythroid progenitors to erythropoiesis, inhibiting red blood cell maturation and thereby promote anisocytosis. ESRD is a destructive problem that lies in the medical, social, and economic areas which contributes to significant morbidity and mortality, and economic, social, and fundamental health problems in the community.³¹ CKD is common in developed and developing nations which is conventionally assessed in terms of both overall renal function (GFR) and the presence of kidney damage ascertained by either kidney biopsy or other markers of kidney damage such as abnormal urinary sediment, abnormalities on imaging studies, or the presence of a kidney transplant.³² According to the 2010 US Renal Data System Annual Report, the leading causes of CKD leading to ESRD in the United States were diabetes (153 per million population), hypertension (99 per million population), and glomerulonephritis (23.7 per million population).³³ In Sub-Saharan Africa, CKD more commonly affects individuals aged between 20 and 50 years, and the age of onset of ESRD is 20 years earlier in populations of African compared with other ethnic groups in western countries.³³ In developing countries, diabetes and hypertension now appear to be the leading causes of ESRD with a prevalence of about 30% and 21%, respectively, but glomerulonephritis and CKD of unknown origin account for a larger fraction of the total. For example, in a recent study of people with CKD detected by the International Society of Nephrology-sponsored screening programs 43% of people with CKD did not have diabetes or hypertension.³⁴ The prevalence of CKD in the northwest part of Ethiopia, defined by the estimated glomerular filtration rate <60 mL/min/1.73 m², was found to be 17.3% and 14.3% by MDRD and CKD-EPI

(chronic kidney disease epidemiology collaboration) equations, respectively. The proportion of stage 3 CKD by MDRD equation was 14.7%, whereas the proportions of stage 4 and stage 5 CKD were 2.2% and 0.4%, respectively.³⁴ In addition to this, a study done in public hospitals of Addis Ababa on the prevalence and associated factors among stage^{3–5} CKD diabetic patients were 79.4%, 16.2%, and 4.4% respectively by the Cockcroft Gault equation, whereas, it was 73%, 21.6%, and 5.4% respectively by the MDRD equation.³⁵ Elevated RDW reflects increased variations of red blood cell size which indicate altered erythrocyte life span or dysfunctional erythrocytes.³⁶ On the other hand, greater RDW was associated with a greater risk of morbidity in men compared to women, whereas no effect was observed by ethnicity.³⁷ In addition, RDW has been found as a predictor of morbidity in the general population³⁷ and in several conditions including acute and chronic heart failure,³⁸ acute pulmonary embolism and myocardial infarction,³⁹ peripheral arterial disease,⁴⁰ acute renal failure which required hemodialysis,⁴⁰ and kidney transplant recipients.⁴¹ RDW was increased above the normal reference range in ESRD patients, especially in the subgroup of patients with inflammation and malnutrition. It was positively correlated with hemodialysis duration and weight gain, whereas, negatively correlated with serum albumin. These findings may reflect the negative effects of inflammation and malnutrition on the association between mortality and elevated RDW in ESRD patients.⁴² A positive correlation between a high RDW and increased incidence of both macro and microvascular complications was seen in diabetic patients without marked vascular complications.⁴³ Several mechanisms can be postulated to explain the association between rising RDW and adverse outcomes. The most important one is inflammatory stress. RDW correlated positively with the log of the CRP concentration in the total population, and the correlation was stronger in the RDW-increased group. Similarly, RDW was positively associated with CRP levels in a study based on a nationwide database of people with diabetes.⁴³ People undergoing hemodialysis treatment suffer from numerous pathological processes along with kidney disease.⁴⁴ Patients with sustained, higher RDW demonstrated a significantly higher risk than did those with lower RDW (adjusted OR: 1.65, 95% CI: 1.02–2.67).⁴⁵ Patients with hypertension have elevated RDW. The possible pathophysiological mechanism that increases RDW among hypertensive patients is that hypertension is associated with inflammation. However, whether inflammation is a cause or effect of hypertension is not well understood.⁴⁶ RDW increased with increased age, obesity, smoking, and alcohol consumption.⁴⁷ Researchers have shown that inflammatory markers, deficient vitamin B-12, and folic acid were more common in the elderly as compared to younger age.⁴⁸ Females were found to have a statistically significant higher RDW value as compared to males. The reason behind this is that women are more likely to have a folic acid deficiency and menorrhagia (which is one of the most common complaints of females of reproductive age) which might result in an increased RDW level.⁴⁹ Multiple mechanisms may play a role in the relationship between RDW and prognosis in critically ill alcoholic patients. The most accepted one is that alcohol metabolites like acetaldehyde, increase the generation of free radicals, such as reactive oxygen and nitrogen species. These free radicals affect the normal development of red blood cells, reduce their oxygen-carrying capacity, and shorten the life span of these cells.⁵⁰ RDW values were found to be higher among smokers. The pathophysiological mechanism explaining the association of RDW with smoking is that chronic subclinical inflammation appears to be the driving factor. Hs-CRP levels, a well-established surrogate marker of inflammation, as well as numerous other inflammatory markers such as interleukin-6 and soluble tumor necrosis factor- α , VCAM-1, ICAM-1, and E-selectin have been independently associated with smoking.⁵¹ Chronic kidney disease affects young adults aged 20–50, in sub-Saharan Africa and is primarily due to hypertension and glomerular diseases. Unlike developed countries, where chronic kidney disease presents in middle-aged and elderly patients is predominantly due to diabetes mellitus and hypertension.⁵² In a study done among renal patients that attend Dessie referral hospital, the prevalence of CKD, was 3.0% had stage I, 11.9% had stage II, 9.5% had stage III, 3.8% had stage IV and 1.6% had stage V CKD respectively.⁵³ High RDW group patients had significantly higher BMI, lower hemoglobin, higher urea, and creatinine, lower estimated glomerular filtration rate (eGFR), high albumin, and a longer period of T2DM diagnosis compared to a low value.⁵⁴ Increased RDW can be an appropriate indicator for various types of diseases like inflammatory bowel disease (IBD), cardiovascular disease (CVD), pulmonary disease (PD), Cancer, and cerebrovascular accident.⁵⁵ Inflammatory cytokines may directly inhibit erythropoietin-induced erythrocyte maturation, which leads to an increase in RDW. It is also known that inflammatory cytokines, such as interleukin 1 or interleukin 6, upregulated hepcidin, which regulates iron homeostasis by inhibiting iron absorption from the intestine and iron release from reticuloendothelial stores.⁵⁶ A study demonstrated no association existed between RDW and sex in either gender.⁵⁷ Several genetic risks were previously associated with different traits and diseases and found that most of these, especially those related to cholesterol (both LDL and HDL), triglycerides, systolic blood pressure, BMI, diabetes, and

some inflammatory diseases were significantly associated with RDW.⁵⁸ However, the importance of RDW scores in screening, diagnosis, and progression of ESRD has not been well researched. Therefore, the intention of this study was to assess RDW and associated factors in ESRD patients.

Methods and Materials

Study Area and Time Period

This study was conducted from February to September 2021 at St. Paulo's Hospital Millennium Medical College, Menelik II Referral Hospital and Zewditu Memorial Hospital.

Study Design and Population

A hospital-based, cross-sectional study design was used and study participants were all ESRD patients who were followed up for hemodialysis during the study period.

Determination of Sample Size and Sampling Technique

The sample size was calculated using the formula for individual population proportions, assuming the prevalence of ESRD in Addis Ababa to be 4.4% and 5.4% using the Cockcroft Gault and MDRD equations, respectively.³⁵ A prevalence of 5.4 and a marginal error of 0.05 were assumed to calculate the sample size.

$$n = (Z\alpha/2)^2 p(q) / d^2 = (1.96)^2 (0.054)(0.946) / 0.0025 = 78.5 = 79$$

n = minimum sample size required for the study

z = standard normal distribution (z = 1.96) with 95% confidence interval

p = sampling proportion of success (0.054),

q = sampling proportion of failure (0.946),

d = tolerable error margin = 5% (0.05).

Allowing for a 10% non-response rate, the final sample size was 87.

A systematic random sample was used to recruit study participants. A proportionate allocation was made for each hospital. From St. Paulo's = (500, 87)/988=44, from Minilik = (188, 87)/988=17, from Zewditu = (300, 87)/988=26.

Inclusion and Exclusion Criteria

All ESRD patients between the ages of 18 and 70 years who attended selected hospitals during the data collection period were included in this study. However, those suffering from congestive heart failure, anemia, blood cell cancer, chronic liver disease, and inflammatory bowel disease, recent infections including Covid-19 and refusing to participate were excluded.

Variables

RDW was the dependent variable in this study. However, socio-demographic characteristics, behavioral factors, chronic illnesses, medication, and blood cell parameters other than RDW were independent variables.

Data Collection Tools and Measurement

Socio-demographic characteristics and medical history were collected from the patients using a questionnaire. Blood pressure (mmHg); using mercury sphygmomanometer; weight (kg), height (m); using digital weighing scale; and mid-upper arm circumference (cm) using tape were measured. The body mass index (BMI) (kg/m²) was calculated from the weight and height of the study patients.

Laboratory Tests

Five milliliter blood samples were taken from each patient by the laboratory technician. Blood taken from each patient was separated into two test tubes: an EDTA tube for CBC by using MINDRAY BC-5130 Hematology Analyzer and RDW and mean corpuscular volume were determined according to the standard reference range of the machine. SST for

serum chemical analysis was used and blood in SST was centrifuged at 3400 RPM for 15 minutes for blood urea and albumin analysis.

Serum creatinine, blood urea level, and serum albumin were measured by Cobas C 311 (Roche, India). Fasting blood glucose level was also measured by a glucometer. GFR was calculated using the diet modification formula for kidney disease. Physical measurements and blood sample collection were conducted thirty minutes before they start dialysis.

Serum creatinine, blood urea level, fasting blood glucose level (glucometer) and serum albumin were also measured. GFR was determined using (the MDRD eGFR formula). Physical measurements and blood sample collection were conducted thirty minutes before they start dialysis.

Data Quality Control

Data were collected by trained health officials and nurses under the supervision of the principal investigators. All variables were filled daily in data extraction format. The laboratory producers were handled by laboratory technologists and all the tests were standardized.

Operational Definition

End-stage renal disease: $\text{GFR} < 15 \text{ mL/min/1.73 m}^2$ using the MDRD-GFR formula.

Anisocytosis: erythrocyte distribution $\geq 16\%$.

Medium anisocytosis: red cell distribution width between ($\geq 16\% - \leq 17.4\%$).

High anisocytosis: red cell distribution width $> 17.4\%$.

Statistical Analysis

Data entry was done with Epi-data (version 3.4) and analyzed with SPSS (version 26.0). Associations between variables were determined using logistic regression. A p-value < 0.25 during bivariate logistic regression analysis was subjected to multiple logistic regression, and a p-value < 0.05 during multivariate analysis at a 95% confidence level was considered statistically significant.

Ethical Approval

Ethical approvals for the study were obtained from the Addis Ababa University, Department of Medical Physiology and the Addis Ababa Public Health Research and Emergency Management Directorate with ethical approval reference number (A/A/13173/227). Patients were informed about the aims and benefits of the study before we began data collection. Written consent was obtained from each participant. Patient privacy and confidentiality has been maintained as we assign code numbers. This study is in line with the Declaration of Helsinki.

Results

Socio-Demographic Characteristics

A total of 83 adult ESRD patients took part in this study with a response rate of 95.4%. The average age of patients were 42 ± 11.27 , with a range of 20–68 years. Among the study patients, 69.0% were males, 54.2% were married, 45.8% had college or university education, 77.1% were from urban areas and 56.6% had a monthly salary (ETB) of less than 5462 (Table 1).

Clinical and Anthropometric Characteristics

RDW was ranged between 15.5% and 23.6% with a mean value of $17.40\% \pm 1.46$. Almost all patients (98.8%) had anisocytosis. Out of 83 patients, 55 (66.3%) were hypertensive, 17 (20.5%) had diabetes, and the remaining 11 (13.3%) had other diseases (glomerulonephritis and peripheral vascular disease) (Table 2). The mean value of GFR and MUAC was ($5.20 \text{ mL/min} \pm 1.58$) and ($28.32 \text{ cm} \pm 2.99$) (Table 3).

Table I Socio-Demographic Characteristics

Variable	Frequency N (%)
Age group	
18–42	40 (48.2)
43–70	43 (51.8)
Sex	
Male	57 (69)
Female	26 (31)
Marital status	
Single	24 (28.9)
Married	45 (54.2)
Widowed/ Divorced	14 (16.9)
Occupation	
Teacher	7 (8.4)
Soldier	2 (2.5)
Merchant	29 (34.9)
Others	45 (54.2)
Educational status	
No education	10 (12)
Primary school	10 (12)
Secondary school	25 (30.2)
College or University	38 (45.8)
Residency	
Urban	64 (77.1)
Rural	19 (22.9)
Monthly income	
<5462	47 (56.6)
≥5462	36 (43.4)

Behavioral Characteristics

In this study, 9.6% of patients were smokers, 53.0% were alcoholics, 15.3% had a history of using traditional medicine, 4.8% use traditional medicines currently and 31.3% habitually chew Khat (Table 4).

Association Factors of RDW

Of patients with moderate anisocytosis, 25 of them had GFR < 5.20 mL/min and 30 had GFR > 5.20 mL/min. whereas of those with high anisocytosis, 20 had GFR < 5.20 mL/min and 8 had GFR > 5.20 mL/min. GFR (AOR=4.6, P=0.047, 95% CI [1.27, 20.74]), alcohol consumption (AOR=13.4, P=0.012, 95% CI [1.97, 22.62]), recurrent kidney disease (AOR=25.6, P=0.016, 95% CI [1.85, 53.71]), and use of antihypertensive and antidiabetic medications (AOR=0.2, P=0.044, 95% CI [0.03, 0.95]) showed a significant association with RDW (Table 5).

Discussion

In this study, 66.3% of patients with ESRD had a prior diagnosis of hypertension and 20.5% had diabetes mellitus (Table 2), indicating that it may be hypertension that contributes to ESRD. In contrast to this study, a previously conducted study showed that 30% of patients with ESRD had diabetes mellitus, while only 21% of them had hypertension.³⁴ In this earlier study, glomerulonephritis and CKD of unknown cause are a major cause of ESRD. In the United States, approximately 28% of elderly (over 65 years old) patients with clinically significant stage^{3–5} chronic kidney disease were neither diabetic nor hypertensive.²⁹ This discrepancy between studies could be because the

Table 2 Frequency Distribution of Clinical Parameters

Variables	Category	Frequency N (%)
Mid-upper arm circumference (cm)	High	68 (81.9)
	Normal	15 (18.1)
	Low	0 (0.0)
Body mass index (kg/m ²)	High	23 (27.7)
	Normal	52 (62.7)
	Low	8 (9.6)
History of repeated kidney infection	Yes	59 (71.1)
	No	24 (28.9)
Blood urea nitrogen (mg/dl)	High	83 (100.0)
	Normal	0 (0.0)
	Low	0 (0.0)
Serum creatinine (mg/dl)	High	83 (100.0)
	Normal	0 (0.0)
	Low	0 (0.0)
Serum albumin (g/dl)	High	22 (26.5)
	Normal	58 (69.9)
	Low	3 (3.6)
Glomerular filtration rate(mL/min)	<5.2	45 (54.2)
	≥5.2	38 (45.8)
Mean corpuscular volume(fl)	High	0 (0.0)
	Normal	77 (92.8)
	Low	6 (7.2)
Red cell distribution width (%)	≥16%	82 (98.8)
	<16%	1 (1.2)
Family history of chronic kidney disease	Yes	22 (26.5)
	No	61 (73.5)
Chronic diseases	Diabetes Mellitus	17 (20.5)
	Hypertension	55 (66.3)
	Others*	11 (13.3)
Medications taken	Nifedipine	21 (25.3)
	Enalapril	19 (22.9)
	Metformin	9 (10.8)
	Insulin	6 (7.2)
	Other drugs**	28 (33.7)

Notes: *Glomerulonephritis and Peripheral vascular disease, **Ciprofloxacin, Lisinopril and Ramipril.

Table 3 Mean Values of Clinical Parameters

Clinical Parameters	Mean	SD
Glomerular filtration rate (mL/min)	5.20	1.58
Red cell distribution width (%)	17.40	1.46
Mean corpuscular volume(fl)	88.80	5.62
Fasting blood sugar (mg/dl)	109.92	36.72
Mid upper arm circumference (cm)	28.32	2.99
Serum albumin (g/dl)	5.02	1.22
Body mass index (kg/m ²)	22.90	3.39
Serum creatinine (mg/dl)	9.84	2.39
Blood urea nitrogen (mg/dl)	130.54	35.65

Table 4 Behavioral Characteristics

Variables	Categories	Frequency N (%)
Smoking habits	Yes	8 (9.6)
	No	75 (90.4)
Drinking alcohol	Yes	44 (53)
	No	39 (47)
Khat chewing	Yes	26 (31.3)
	No	57 (68.7)
Drinking coffee	Yes	79 (95.2)
	No	4 (4.8)
Previous use of traditional medication	Yes	13 (15.3)
	No	70 (84.7)
Current Use of traditional medications	Yes	4 (4.8)
	No	79 (95.2)

Table 5 Bivariate and Multivariate Logistic Regression Analysis of RDW and Other Variables

Variables		RDW		COR (95% CI)	AOR (95% CI)	P-value
		Moderate	High			
Glomerular filtration rate	≥5.2	30	8	1	1	0.047*
	<5.2	25	20	3.0 (1.13, 7.97)	4.56 (1.27, 20.74)	
Drinking alcohol	No	31	8	1	1	0.012*
	Yes	24	20	3.23 (1.22, 8.59)	13.39 (1.97, 22.62)	
History of repeated kidney diseases	No	20	4	1	1	0.016*
	Yes	35	24	3.43 (1.04, 11.29)	25.6 (1.85, 53.71)	
Medication taken	No	16	12	1	1	0.044*
	Yes	39	16	0.55 (0.21, 1.41)	0.16 (0.03, 0.95)	
Smoking status	No	52	23	1	1	0.211
	Yes	3	5	3.77 (0.83, 17.11)	3.13 (0.52, 18.67)	
Khat chewing	No	41	16	1	1	0.332
	Yes	14	12	2.19 (0.84, 5.76)	1.84 (0.54, 6.29)	
Traditional medication	No	49	21	1	1	0.102
	Yes	6	7	2.72 (0.82, 9.07)	2.99 (0.81, 11.11)	
Age	<42	28	12	1	1	0.460
	≥ 42	27	16	1.38 (0.55, 3.46)	0.68 (0.25, 1.86)	
Sex	Male	37	20	1	1	0.615
	Female	18	8	0.82 (0.30, 2.22)	0.74 (0.23, 2.37)	

Note: *Significant association.

Abbreviations: RDW, red cell distribution width; COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval.

prevalence of hypertension in Ethiopia was high compared to diabetes and other glomerular diseases. In this study, reduced GFR (AOR=4.6, P=0.047, 95% CI [1.27, 20.74]), repeated renal infection (AOR=25.6, P=0.016, 95% CI [1.85, 53.71]), medication use (AOR=0.2, P=0.044, 95% CI [0.03, 0.95]), and alcohol consumption (AOR=13.4, P=0.012, 95% CI [1.97, 22.62]) were significantly associated with a high RDW (Table 5). In this study, low GFR was four times more likely to develop high RDW compared to high GFR. This result is consistent with other studies.⁵ A cross-sectional study was conducted in adult outpatients and showed an inverse association between RDW and renal function.²⁴ The increase in RDW in patients with ESRD could be attributed to inflammation, stress response, malnutrition, and other comorbidities.⁴⁹ According to a previous study,⁴⁹ Inflammation plays an important role in increasing RDW in patients with ESRD. In addition, inflammatory cytokines such as interleukin-1 and interleukin-6, unregulated hepcidin that regulates iron hemostasis, are involved in RDW increase (67).

The second mechanism contributing to the higher RDW in patients with ESRD may be due to chronic hypoxia, oxidative stress, and endothelial dysfunction. A previously conducted study showed that RDW was autonomously associated with endothelial dysfunction and oxidative stress, which increases in patients with CKD, and oxidative stress can reduce red blood cell survival, inducing the release of premature and heterogenic shaped red blood cells.⁴⁸

The third mechanism could be due to the increase in blood pressure and hyperglycemia in patients with ESRD. One of the risk factors for elevated blood pressure is wall tension. Wall tension contributes to the loss of normal red blood cell shape, resulting in increased levels of RDW.⁵⁴ Long-term hyperglycemia contributes to loss of red blood cell size, which is worse in diabetics with kidney damage. The loss of the normal size of the RBCs further increases the fragility of the erythrocytes.⁵⁰

In this study, there was a significant association between RDW and recurrent kidney disease in patients with ESRD. Repeat kidney infection was twenty-five times more likely to develop high RDW than a single non-repeated infection (Table 5). This is similar to a previous study that reported that patients with many different chronic diseases had a higher baseline RDW.⁵³ The other factor that showed a significant association with the increase in RDW levels in this study was alcohol consumption. In patients with ESRD who consumed alcohol, the RDW level was 13 times higher than in patients who did not consume alcohol (Table 5). Although there is limited study of the association between alcohol consumption and RDW, alcohol metabolites such as acetaldehyde, which increase free radical formation such as reactive oxygen and nitrogen species, could be the possible mechanism. These free radicals interfere with the normal development of red blood cells, reduce their oxygen-carrying capacity and shorten the lifespan of these cells.⁵⁰ In this study, taking antihypertensive and antidiabetic medication also had a significant association with decreased RDW. This study is similar to a study done in China that, insulin therapy, beta-blockers, and ACE-inhibitors were protective in decreasing the level of RDW.⁵¹ A significant association between serum albumin and RDW was not seen in this study, but the previous study reported that RDW was negatively correlated with serum albumin.⁴² In the other study, patients in the high RDW group had significantly more albumin and a longer period of T2DM diagnosis compared to a low RDW group.⁵⁴ Although this difference occurred between studies, albumin and pre albumin are very poor predictors of nutritional status. Possible explanations for this were, first, that serum proteins such as albumin and pre albumin have not been included as defining characteristics of malnutrition, since evidence shows that serum levels of these proteins do not change in response to changes in nutrient intake, which shows no sensitivity. Specificity and reliability. Second, since serum albumin levels were time-averaged values over the year of hemodialysis, values from just a single measurement may not be representative of long-term nutritional status in this study population. The discrepancy in results could be due to differences in sample size, sociodemographic factors, the presence of comorbidity, and frequency of measurement of laboratory parameters, only once in this study but more than twice consecutively in other studies to investigate the relationship between nutritional statuses and to see RDW. In contrast to the previous study,⁵⁵ RDW was not associated with age and gender (Table 5). In contrast, another study showed that there is a positive relationship between RDW and age. This is because older people are more likely to be deficient in nutrients than younger people who have comorbidities and those who have had inflammation.²² The discrepancy between these results could be due to variations in sample size and socio-demographic characteristics. Not enough older people participated in this study. Although the frequency of males with high RDW was higher than females in this study, gender has no significant association with RDW. Similar to this finding, another study showed that there was no association between RDW in either sex.⁵⁷ Conversely to this finding, a larger RDW was associated with a higher risk of morbidity in males compared to females.³⁷ On the other hand, a study has shown that women have a statistically significantly higher RDW value compared to men.⁴⁹ Women are more likely to have folic acid deficiency and menorrhagia (one of the most common complaints in women of childbearing age), which can lead to increased levels of RDW.⁴⁹ This is because only twenty-six female patients with end-stage renal disease were involved during the study period. Blood pressure, body mass index, diabetes, and inflammatory diseases were significantly associated with RDW.⁵⁸ A previous study of 513 patients with essential hypertension reported that a statistically significant association was found between RDW and blood pressure and albumin after adjusting for potential confounders such as age and gender.²⁵ But in this study, no significant association was observed between RDW and hypertension, diabetes mellitus, and BMI. The differences between these studies, particularly those related to factors considered risky for this study, have been matched in other studies. The other reason could be that the sample size (83) was small in this

study compared to another study (513).²⁵ Smoking had no significant association with RDW in this study (Table 5). In contrast to this study, higher RDW values were found in smokers.⁵¹ The mechanism that explains RDW's relationship to smoking is that chronic inflammation appears to be the driving factor. Hs-CRP levels, a well-established surrogate marker of inflammation, as well as numerous other inflammatory markers such as interleukin-6 and soluble tumor necrosis factor-alpha, VCAM-1, ICAM-1, and E-selectin have been independently associated with smoking.⁵¹ Although this explanation was accepted, the differences between the studies could be due to the fact that the number of patients who smoked cigarettes was small compared to others. In conclusion, RDW showed a significant association with GFR, recurrent kidney disease, alcohol consumption, and use of antidiabetic and antihypertensive drugs. As shown by the adjusted odds ratio, reduced GFR, recurrent kidney disease, and alcohol consumption were more likely to result in a high RDW, while medication taken was the protective factor. The mechanisms of RDW disruption in patients with ESRD need further investigation.

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Author Contributions

All authors made significant contributions to the reported work, whether in conception, study design, implementation, data collection, analysis and interpretation, or all of these areas; were involved in drafting, revising, or critically reviewing the article; give final approval of the version to be published; agreed on the journal to which the article was submitted; and agreed to be responsible for all aspects of the work.

Disclosure

This paper was done for the fulfilment of Master's Degree in Medical Physiology from Addis Ababa University. Since it is a must to give a full thesis after successful defense for Addis Ababa University Library to put it online for the purpose of reading for students. So that it is available online in Addis Ababa University Library Circulation. But it is our original work paper which is not a published yet. The authors declare no conflicts of interest in relation to this, and all authors are responsible for the content and writing of this document.

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Comparison of Washing Efficiency and Recovery of Blood Cells Between Centrifugation, Coarse Filtration and Microfiltration Techniques to Prepare Autologous Blood for Transfusion

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Purpose: Cell salvage is the process by which blood lost in surgery is collected and washed or filtered to produce autologous blood for re-transfusion to the patient. Cell salvage aims to reduce the need for donor blood. Centrifugal cell salvage washing technique is a preferred medical treatment in order to retain lost red blood cells (RBCs) without contaminants. Although this technology very efficiently collects and washes shed blood, it is costly and often impractical or unavailable, especially in middle- or low-income countries. This study assessed two innovative filter devices as an alternative to centrifugal cell salvage technology: a coarse collection filter device (Hemafuse) and a microfiltration device (HemoClear). In contrast to centrifugal technology, both filter devices do not require electricity, nor costly equipment and extensive training. We compared the effectiveness of these filtration technologies to remove plasma constituents and recover and concentrate the cellular components with centrifugal technology (autoLog[®] device).

Methods: Whole blood was processed with each technology according to the device manufacturer's instructions. Before and after processing, the blood products were analyzed for supernatant solutes and cellular composition.

Results: The centrifugal technology confirmed its efficacy to remove potentially harmful solutes and capture red blood cells. The microfiltration technology (HemoClear) reached comparable levels of removal of solutes, with a potential advantage over centrifugal technology in the ability to also recover platelets. The coarse filtration technology (Hemafuse) had no washing capacity but, like the microfiltration technology, has the advantage of recovering platelets.

Conclusion: Innovative filtration devices represent an alternative to centrifugal technology in the preparation of autologous blood for reinfusion. The HemoClear technology for the first time enables the recovery of washed platelets and red blood cells. Clinical trials will have to be performed to investigate the clinical value of this new autologous blood product.

Keywords: cell salvage, blood salvage, cell salvage technology, cell saver, blood filter

Introduction

Globally there is a high demand for blood and blood products for transfusion.¹ The World Health Organization (WHO) reported 116 million units of red blood cell transfused worldwide. The WHO also reported a red blood cell shortage of nearly 17 million units.² Despite an overall increase in the number of blood donations collected in most WHO regions and World Bank Income groups, especially in lower- to middle-income countries, the number of blood collections is too low to meet the demand.¹ Most efforts to increase the availability of blood and blood products have focused on allogeneic blood donations.³ In Kenya, for example, non-remunerated blood collection drives were largely dependent on schools. This system has been hugely affected by the closure of schools during the COVID-19 pandemic, which has further increased blood shortages. Autologous

blood salvage systems have been proven to relieve the strain on donor blood systems.⁴ Cell salvage can effectively reduce both exposure to allogeneic blood and the volume of allogeneic blood transfused.⁵

Infusion of allogeneic red blood cells after significant blood loss is a valuable medical practice. Cell salvage, in which the patients' own blood is recovered during or after surgery and re-infused into the patient, is increasingly used.^{6,7} After collection and separation, the blood cells are concentrated and re-infused, while the residual plasma fluid, containing non-cellular components such as proteins, cations and pathological elements such as cytokines, chemokines and bacteria, is discarded.

A range of devices are available to assist in the salvage of the patient's own blood.⁸ The most commonly used device in high-resource settings is the centrifugal cell saver. The centrifugal cell saver washes and concentrates the RBCs efficiently. However, this device is often not available in lower-resource settings as consumables are expensive, and maintenance resources such as power supply, spare parts or skilled personnel are lacking. The Hemafuse (Sisu Global Health, Baltimore, United States) is a low-cost, handheld, mechanical coarse filter device for intraoperative autotransfusion of blood collected from internal bleeding, intended to replace or augment donor blood in emergency situations.¹⁰ The Hemafuse is available in Kenya and has a syringe-like design and includes a suction line and tip to collect blood from the surgical field. The suctioned blood is transferred to a reinfusion blood bag through a coarse dead-end filter (170 μ m pores) by manual pushing down of the syringe plunger. Everything smaller than the pores, including the blood cells, passes through the filter, while large debris is filtered out.

The HemoClear device (HemoClear BV, Zwolle, The Netherlands) uses microfiltration technology to isolate blood cells from a liquid component.¹¹ This technology is used in Europe as a blood cell recovery filter. The device captures shed blood cells through highly specific (2–3 μ m) crossflow filtration and removes solutes by its washing capability, yielding a high-quality cellular product.⁹ The HemoClear system is driven by gravity and can be used at patients' bedside. In short, a volume of collected blood is transferred to the HemoClear system and diluted with saline before entering the filter compartment. In the filter, the diluted blood flows along the filter membrane surface. Everything larger than the pores, including the blood cells, stays on the entry side of the membrane while everything smaller than the pores, including non-cellular components such as free hemoglobin, passes through the pores to the other side of the membrane. The compartments above and below the membrane are connected to two separate outlets. The outlet connected to the compartment above the membrane directs the blood cells to a reinfusion blood bag. The outlet connected to the compartment below the membrane directs the washing solution and non-cellular components to a waste bag.

This laboratory study was designed to compare the centrifugation (autoLog[®]), microfiltration (HemoClear) and coarse filtration (Hemafuse) techniques in their ability to remove non-cellular components and recover and concentrate the blood cells. Citrated whole blood was subjected to simulated salvage by the three technologies in accordance with each device manufacturer's instructions for use (Figure 1).

Materials and Methods

Blood Collection

All (non-remunerated) volunteer blood donors met standard donation criteria and gave their written, informed consent, in accordance with Sanquin Research's (Amsterdam, The Netherlands) guidelines and practices. This study was approved by the Institutional Medical Ethical Committee, in accordance with the standards laid down in the 1964 Declaration of Helsinki. A total of nine whole blood units, 500 mL $\pm$ 10%, were collected in quadruple, bottom-and-top collection systems containing 70 mL of citrate-phosphate-dextrose (CPD, Fresenius Kabi, Emmer Compascuum, the Netherlands) at the Sanquin Blood Center (Sanquin, Amsterdam, the Netherlands). The whole blood units were placed on butane-1,4-diol cooling plates (Compocool, Fresenius Kabi) to allow their temperatures to adjust to 20 to 24°C.¹² The processing of the whole blood with the devices was initiated at around 16 hours after collection.

Blood Salvage

Processing with the autoLog (Centrifugation)

Three whole blood units were transferred to the autoLog[®] Autotransfusion System (Medtronic, Eindhoven, the Netherlands) centrifugal system's collection canister through an outer port, ie, the whole blood did not pass through the canister internal coarse



Figure 1 Overview of evaluated cell salvage technologies including washed centrifugation (autoLog), coarse filtration (Hemafuse) and washed microfiltration (HemoClear).

filter. The whole blood units were separated by centrifugal force and washed with 750 mL of isotonic saline in an automated program that consisted of three consecutive washing rounds, in accordance with the manufacturer's instructions.

Processing with HemoClear (Microfiltration)

In accordance with the manufacturer's instructions for use, the HemoClear system was primed manually with isotonic saline before use.⁹ Three units of whole blood were attached to the HemoClear system and diluted with 500 mL of isotonic saline before being filtered. After the first round of filtration, the cellular component was filtered again to concentrate the cellular product.

Processing with Hemafuse (Coarse Filtration)

In accordance with the manufacturer's instructions for use, the Hemafuse pump was assembled and de-aired before use. Three whole blood units were attached to the Hemafuse system and filtered through the coarse filter by manual force.

Analyses

Each whole blood unit and processed cellular unit was analyzed for cellular composition including numbers of red cells, platelets, leukocytes and hematocrit, and for supernatant-free hemoglobin, total protein, glucose and potassium concentrations. Total loads of each parameter were calculated from the measured concentration and the volume of the product. The cellular and non-cellular recoveries were calculated from the total load in the whole blood and the processed product.

Volume

The volume of blood components was calculated from the net weight and the specific gravity: 1.026 g/mL for plasma, 1.100 for RBCs. Based on the hematocrit values, the specific gravity of whole blood and diluted whole blood was calculated.^{13,14}

Hematological Parameters

Hematological parameters (cell count, hemoglobin concentration, hematocrit and mean corpuscular volume (MCV)) were obtained using a hematology analyser (Advia 2120, Siemens Healthcare Nederland BV, Den Haag, the Netherlands).

Hemolysis was determined as described previously by de Korte et al.¹⁵ Briefly, cell-free supernatants were obtained by centrifugation of the blood product at $12,000 \times g$ for 5 min followed by an additional centrifugation of the supernatant at $12,000 \times g$ for 5 min. Free hemoglobin was determined by absorbance measurement of supernatant at 415 nm (Eon plate reader, Bio Tek, Bad Friedrichshall, Germany), with correction for plasma absorption if necessary. Hemolysis was expressed as a percentage of total hemoglobin present in the blood product after correction for hematocrit.

Extracellular Potassium and Glucose

Samples of whole blood and processed blood were assayed for extracellular K⁺ and glucose using a blood gas analyzer (RapidLab 1265, Siemens Healthcare, Nederland B.V.).

Total Protein

Total protein was measured using the biuret method on Architect clinical chemistry analyzer (Abbott, Abbott Park, ILL).

Thromboelastography (TEG) Properties

TEG assays were performed using a TEG 5000 hemostasis system and plain cups and pins (Haemoscope Corp., Niles, IL). Blood samples were recalcified, and the intrinsic coagulation pathway was stimulated with kaolin.¹⁶ Four values that represent clot formation were determined by this test: the reaction time (R value), the coagulation time (K value), the angle and the maximum amplitude (MA).¹⁷ The R value represents the time until the first evidence of a clot is detected. The K value is the time from the end of R until the clot reading reaches 20 mm and this represents the speed of clot formation. The angle is the tangent of the curve made as the K is reached and offers similar information to K. The MA is a reflection of clot strength.

Statistics

The results are expressed as mean values $\pm$ standard deviation. Paired two-sided Student's *t*-tests were performed to compare the whole blood measurements to the data acquired on the cellular and liquid components after processing. Significance was defined as $p < 0.05$. Based on past experience, a sample size of three whole blood units was expected sufficient to perform statistical analysis with power of at least 80%. The starting whole blood products were characterized by low variation (low standard deviation) as blood donors have to meet the standard donation criteria. Post hoc power calculations on the difference between whole blood and processed blood confirmed that the sample size of $n = 3$ for each parameter had a statistical power of at least 80%.

Results

Recovery of the Blood Cells

Volumes and mean cellular quantities for the pre-processed whole blood units, centrifuged (autoLog), microfiltered (HemoClear), and coarse filtered (Hemafuse) products are shown in Tables 1 and 2.

The whole blood units showed a normal cellular composition with a mean red blood cell concentration of $4.0 \pm 0.43 \times 10^{12}/L$, white blood cell concentration of $4.8 \pm 0.94 \times 10^9/L$ and platelet concentration of $175 \pm 31 \times 10^9/L$ (data not shown in table). The mean whole blood counts for red blood cells, white blood cells and platelets were $2.27 \pm 0.41 \times 10^{12}$, $2.56 \pm 0.64 \times 10^9$, and $105 \pm 23 \times 10^9$, respectively.

All three techniques resulted in efficient recovery of both WBC and RBC (Figure 2A). The amounts of RBC in the processed product did not significantly differ from the amounts in the whole blood. Mean platelet recovery for the

Table 1 Volumes and Cellular Quantities Before and After Whole Blood Processing with Each Technology

Parameter	Centrifugation (autoLog)		Microfiltration (HemoClear)		Coarse filtration (Hemafuse)	
	WB	Processed Product	WB	Processed Product	WB	Processed Product
Volume (mL)	549±0.3	281±30.4*	552±3.0	377±36.4*	552±1.6	510±38.6
Total WBC (x10 ⁹)	2.47±0.73	0.97±0.53* (39%)	2.56±0.64	2.06±0.45 (81%)	2.85±0.14	2.70±0.07 (95%)
Total RBC (x10 ¹²)	2.22±0.15	1.84±0.12 (83%)	2.27±0.41	2.13±0.35 (94%)	2.13±0.14	1.99±0.34 (93%)
Total Hb (g)	68±1.4	57±4.6 (85%)	66±4.6	61±3.6 (94%)	64±4.4	62±0.8 (97%)
Ht (%)	39.0±0.7	64.0±1.0*	37.4±2.4	52.2±5.1*	36.5±1.7	35.7±3.2
Total Plt (x10 ⁹)	89±10	4.0±1.1* (5%)	105±23	70±19* (67%)	96±20	88±17 (92%)

Notes: Values expressed as mean ± SD (% of recovery), n=3. *p<0.05 as compared to whole blood (Student's paired t-test).

Table 2 Quantities of Free Solutes Before and After Whole Blood Processing with Each Technology

Parameter	Centrifugation (autoLog)		Microfiltration (HemoClear)		Coarse filtration (Hemafuse)	
	WB	Processed Product	WB	Processed Product	WB	Processed Product
Total Glucose (mmol)	10.3±1.2	1.6±0.4* (16%)	11.9±1.3	2.2±0.2* (19%)	11.7±1.5	9.1±0.5 (80%)
Total protein (g)	32.4±1.7	0.7±0.1* (2%)	31.3±1.0	5.4±0.3* (17%)	30.7±2.1	28.2±4.0 (92%)
Total Free haemoglobin (mg)	10.1±8.9	74.6±8.8*	5.2±4.6	17.2±7.3	5.3±5.4	22.2±11.4
Haemolysis (%)	0.01±0.0	0.04±0.0	0.00±0.0	0.01±0.0	0.00±0.1	0.02±0.0
Total K ⁺ (mmol)	1.82±0.18	0.36±0.12* (19%)	1.82±0.32	0.74±0.08* (41%)	1.65±0.21	1.79±0.1 (110%)

Notes: Values expressed as mean ± SD (% of recovery), n=3. *p<0.05 as compared to whole blood (Student's paired t-test).

centrifugation technique was 5%. Both filtration-based technologies recovered a significantly greater amount of platelets, with the coarse filtration having the highest recovery of platelets, 92% versus 67% with microfiltration.

The mean hematocrit of the whole blood units was $37 \pm 1.8\%$. Microfiltration and centrifugation concentrated the red blood cells to a hematocrit of $52 \pm 5.1\%$ and $64 \pm 1.0\%$, respectively. The coarse filter did not result in concentration of the red blood cells, the obtained hematocrit, $36 \pm 3.2\%$, was comparable to the whole blood hematocrit (Figure 2B).

Removal of Non-Cellular Components

Washing performance as measured by non-cellular loads in whole blood and the produced products is shown in Table 2. The concentrations of supernatant glucose, potassium and total protein were measured as indicators of the devices'

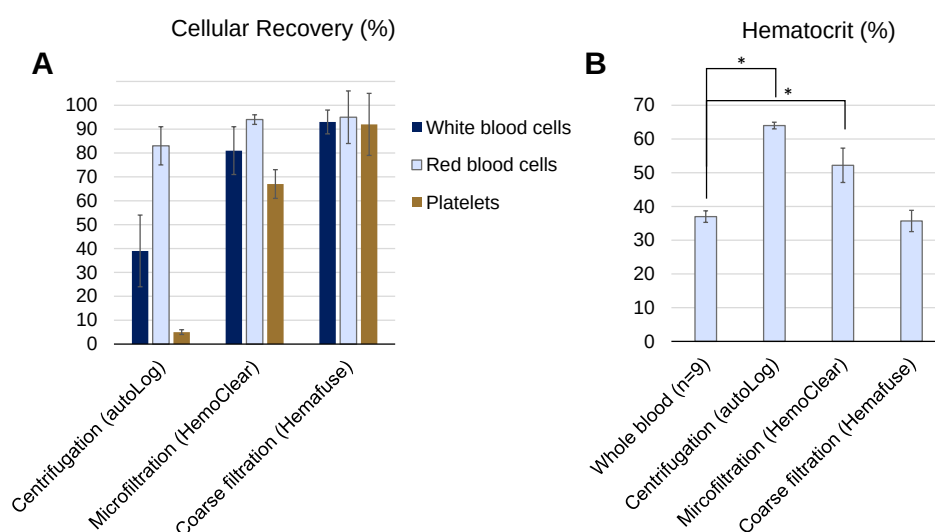


Figure 2 Recovery of the blood cells and hematocrit after processing with the different salvage technologies. (A) Cellular recovery. (B) Hematocrit (%). Values shown are mean ± SD (n=3). * p<0.05 as compared to whole blood (Student's paired t-test).

washing efficiency. Both the centrifugal and the microfiltration procedure effectively reduced the concentration of all solutes (Figure 3). Mean potassium concentrations in whole blood were low, around 3 mmol/L. After processing with both the centrifugal and microfiltration technologies, potassium concentrations were below 1 mmol/L (data not shown).

The coarse filtration (Hemafuse) device did not significantly reduce glucose, potassium, or total protein levels.

The mean-free hemoglobin concentration before processing was 11 ± 10 mg/L. The centrifugation procedure significantly increased mean-free hemoglobin concentration to 207 ± 22 mg/L. Processing with both microfiltration (37 ± 18 mg/L, $P < 0.001$) and coarse filtration (35 ± 19 mg/L, $P = 0.016$) resulted in significantly lower increase in free hemoglobin concentration (Figure 4). Also, total amounts of free hemoglobin after processing with microfiltration (17 ± 7.3 mg, $P = 0.001$) and coarse filtration (22 ± 11 mg, $P = 0.035$) were significantly lower as compared to the centrifugal device (75 ± 8.8 mg).

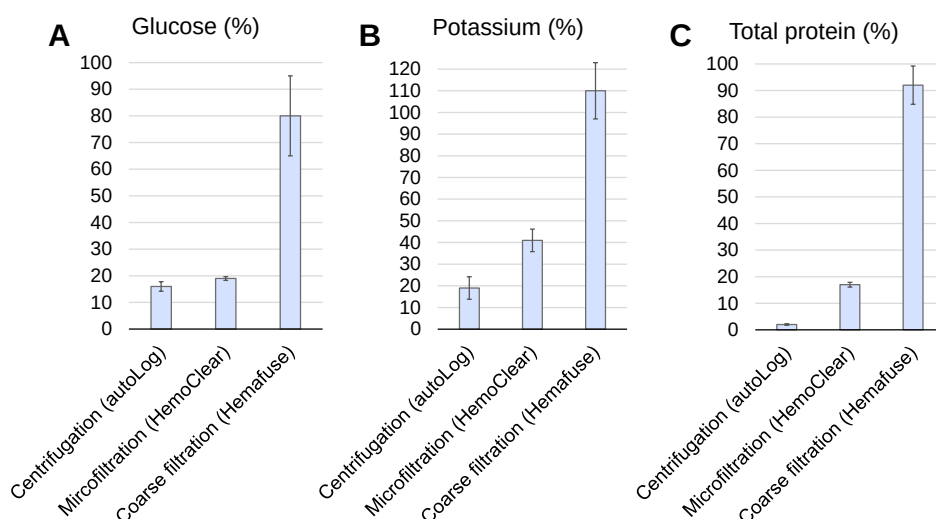


Figure 3 Recovery of solutes after processing with the different salvage techniques. Recovery of glucose (A), potassium (B) and total protein (C). Values shown are mean $\pm$ SD ($n=3$).

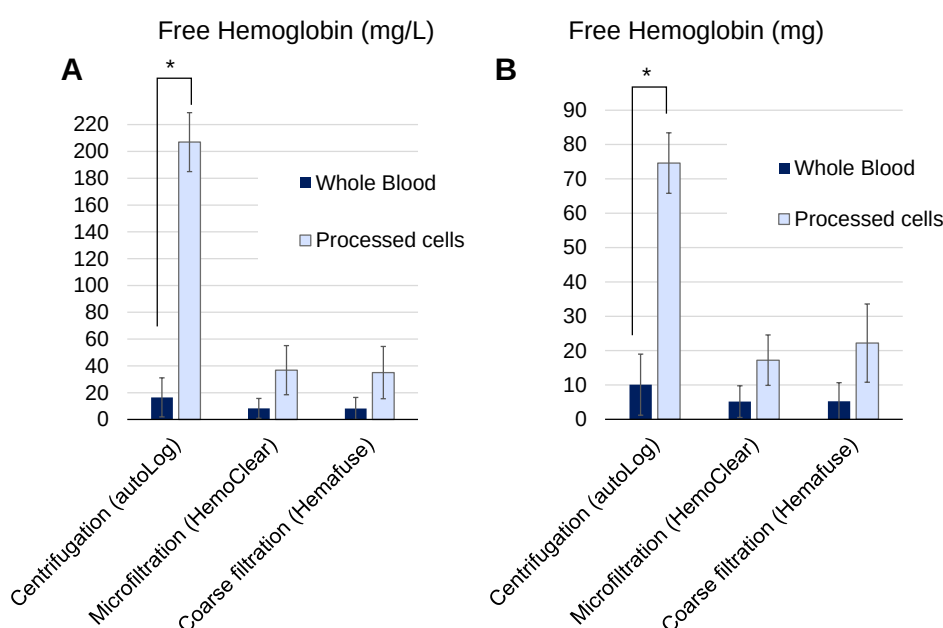


Figure 4 Release and removal of free hemoglobin. (A) Mean free hemoglobin concentration in milligram per liter. (B) Mean free hemoglobin total load in milligram. * $p < 0.05$ as compared to whole blood (Student's paired t-test).

Table 3 Thromboelastographic Properties Before and After Whole Blood Processing with Each Technology

	Centrifugation (autoLog)		Microfiltration (HemoClear)		Coarse filtration (Hemafuse)	
	WB	Processed Product	WB	Processed Product	WB	Processed Product
R (min)	8.9±0.3	52±9.6*	9.2±1.7	8.1±1.0	9.4±0.9	6.8±0.9*
K (min)	2.0±0.5	No coagulation	2.1±0.6	9.0±2.8*	2.3±0.4	1.8±0.4 *
Angle (°)	53±13.3		59±5.9	26±3.5*	52±7.6	63±6.0
MA (mm)	59±6.7		59±9.2	25±4.5*	64±1.7	66±2.0

Notes: Values expressed as mean ± SD (Recovery), n=3. *p<0.05 as compared to whole blood (Student's paired t-test).

Thromboelastography Properties

The mean thromboelastography properties for pre-processed whole blood units and microfiltered, coarse filtered and centrifuged products are shown in Table 3. For the units processed with the centrifugal device, no coagulation was measured. For the coarsely and microfiltered units, the R times, were comparable, 6.8 ± 0.90 min and 8.1 ± 1.0 min, respectively. Both were slightly, but significantly lower as the values obtained in whole blood. For the microfiltration technology, the mean K time, 9.0 ± 2.8 minutes, was significantly prolonged compared to the pre-processed mean time of 2.1 ± 0.6 minutes. In contrast, the angle and maximum amplitude were significantly decreased. After the coarse filtration, the K time slightly, but significantly, decreased to 1.8 ± 0.35 minutes. The angle and maximum amplitude remained comparable to the pre-processed values.

Discussion

In order to safely re-infuse shed blood cells, medical guidelines recommend the shed blood is washed with saline to remove harmful solutes.¹⁸ Centrifugal technology has been shown to effectively reduce the non-cellular load by about 90%.^{8,19,20} The centrifugal autoLog[®] procedure resulted in a removal of 98% of the total protein level. The reduction of smaller solutes was less effective, with about 20% of the small-molecule glucose and ion potassium left in the concentrated red cell product.

The HemoClear microfiltration technology achieved equal washing performance compared to the centrifugal autoLog[®] procedure and reduced the non-cellular components to a significantly greater extent than the coarse filtration technology (Hemafuse). Hemafuse showed no reduction in solute load. This finding is in line with expectations because the Hemafuse protocol does not include washing with saline. The Hemafuse procedure, using a 170 micron coarse filter, is effective in removing large debris but does not catch the smaller biologically active proteins, cations or small molecules.

A clinically relevant finding is the recovery of platelets by both filter technologies. Contrary to the centrifugal procedure, the filters recovered the majority of platelets together with the red cells. This is an important finding because administration of both red cells and platelets represents the most common co-transfusion.²¹ Storage of allogeneic platelet concentrates is limited to 5 to 7 days at room temperature.²² Therefore, maintaining hospital platelet concentrate inventory is logistically difficult and highly resource-intensive.^{23,24} Furthermore, platelet transfusion entails several risks to the patient, including allergic and febrile nonhemolytic reactions and sepsis. When platelet salvage is an option, these risks might be avoided.²⁵ The possibility to recover platelets with the red cells from shed blood could reduce pressure on blood banks to maintain adequate platelet stocks.^{1,26}

Based on the TEG results, the centrifugation washing procedure showed the complete depletion of coagulation. This is in agreement with the very low levels of platelets in the washed product. The results for the filtration technologies suggest that in the washed microfiltration product there still was coagulation. The decrease in cloth strength (MA) in the filtered product as compared to whole blood is most likely due to the removal of plasma coagulation factors such as fibrinogen. In the coarsely filtered product, coagulation was increased as reflected by a decrease in R and K times, suggesting some activation of the platelets. The TEG maximal amplitude was not influenced by the coarse filtration, and the lack of reduction of plasma components by this procedure might be involved in this. In a previous article on the microfiltration technology (HemoClear).⁹ It was shown that this processing method did not result in activation as measured by the expression of P-selectin (CD62P) on the surface of platelets. For the coarse filtration technology (Hemafuse) it should be investigated whether the recovered platelets are activated in processing.

Being processed shortly after donation, the whole blood free hemoglobin levels were very low at the start of processing. This finding is not representative of the practical cell salvage situation in which pre-processed shed blood contains substantial-free hemoglobin levels. In a previous article, the microfiltration HemoClear system was compared to a centrifugal device on reduction of high-free hemoglobin levels.²⁷ In this study postoperatively blood shed after elective cardiac surgery was collected over an extended period of 18–20 hours before processing with both technologies in a parallel set-up. The extended collection period yielded a mean-free hemoglobin concentration of 0.21 ± 0.14 mmol/L in the pre-processed samples. Processing with the centrifugal technology and microfiltration HemoClear device reduced the free hemoglobin load to statistically comparable values of 12.9% and 15.5%, respectively. The free hemoglobin levels reported in the current study might provide insight into the release of hemoglobin induced by the technologies, rather than their washing performance. The centrifugal procedure significantly increased free hemoglobin levels. This would be in line with previous findings that showed that centrifugal force exerted upon red cells results in sublethal injury and hemolysis.^{28,29}

In addition to this blood quality investigation, further research should be performed to evaluate the filtration technology's usability and cost-effectiveness.

Conclusions

Three technologies available for the preparation of autologous blood for transfusion were evaluated on the recovery of cellular and non-cellular components. The recovery of blood cells is the basis of effective autologous blood transfusion, whereas the removal of unwanted non-cellular contaminants is an imperative for safe cell salvage. Based on the results, we conclude that washing of blood cells with saline is necessary to remove non-cellular components and enable safe reinfusion. Both the centrifugation (autoLog) and microfiltration (HemoClear) technologies have a washing feature and effectively reduce the various non-cellular solutes. Whereas centrifugal technology only recovers the red blood cells, filtration technology unlocks the possibility to recover both platelets and red blood cells. Clinical trials should be performed to quantify the value of platelet salvage. The HemoClear microfiltration system would be a relatively simple procedure to produce washed blood cells of comparable quality as obtained with the centrifugal device, with as additional advantage the recovery of platelets.

The coarse filtration procedure with HemaFuse without a washing feature does not result in reduction of free solutes. The HemaFuse coarse filtration device presents a straightforward technique to filter out large debris from blood but is not suitable for salvage of blood cells contaminated with solutes such as free hemoglobin or bacteria.

Abbreviations

CPD, citrate-phosphate-dextrose; Hb, hemoglobin; Ht, hematocrit; K⁺, potassium; KEMSA, Kenya Medical Supplies Authority; MA, maximum amplitude; MCV, mean corpuscular volumes; Plt, platelet; RBC, red blood cell; TEG, thromboelastography; WBC, white blood cell.

Data Sharing Statement

Raw data files are available upon request from the corresponding author.

Ethical Approval

This study was approved by Sanquin's Research Medical Ethical committee, in accordance with the standards laid down in the 1964 Declaration of Helsinki.

Consent for Publication

HemoClear BV consents to the publication of these data.

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Disclosure

James Amenge purchased hemafuse devices and for presenting the study result in a virtual internal conference on bloodless surgery. He received manufacturer's training on hemafuse devices and used the hemafuse device in a patient. Dion Osemwengie is an employee of HemoClear BV. Arno Nierich holds stock in HemoClear BV. The other authors report no conflicts of interest in this work.

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Gene Therapy and Hemophilia: Where Do We Go from Here?

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Abstract: Gene therapy for hemophilia using adeno-associated virus (AAV) derived vectors can reduce or eliminate patients' disease-related complications and improve their quality of life. Broad implementation globally will lead to societal gains and foster health equity. Several vector products each for factor IX (FIX) or factor VIII (FVIII) deficiency are in advanced clinical development. Safety data are reassuring. Efficacy data for up to 8 and 5 years, respectively, vary considerably among vector types and among individuals, but indicate significant reduction in bleeds and factor use. Products will soon be approved for marketing. This review highlights the relevant considerations for implementation of hemophilia gene therapy, specifically across a broad range of socioeconomic backgrounds globally, based on recent publications and our own experience. We address the current efficacy and safety data and relevant aspects of vector immunology. We then discuss pertinent implementation steps including pre-implementation and readiness assessments, considerations on cost, cost-effectiveness and payment models, approaches to education and informed consent, and the operational needs as well as the need for monitoring of health outcomes and implementation outcomes. To prevent a lag or complete lack of establishing access to this life-changing therapy option for all patients with hemophilia worldwide, adaptable pathways supported by collaborative and international efforts of all stakeholders are needed.

Keywords: global health, health equity, cost-effectiveness, adeno-associated virus vector, factor VIII, factor IX

Introduction

Hemophilia A and hemophilia B are monogenic, x-linked bleeding disorders that occur due to a decrease or lack of function of the respective clotting factor, factor VIII (FVIII) or factor IX (FIX), respectively.^{1,2} Persons with hemophilia (PWH) experience frequent provoked or spontaneous bleeds into joints, or soft tissue, and, occasionally, intracranial or internal bleeds. Chronic hemarthropathy or recurrent intracranial bleeding can lead to lifelong disability or early death.^{3,4} The standard of care for bleed treatment or prevention includes factor replacement with plasma-derived or recombinant concentrates by intravenous infusion at intervals between every day to every 2 weeks, or a recently developed non-factor product given subcutaneously every 1–4 weeks.⁵

The standard of care varies for PWH in different socioeconomic environments. In high-income countries (HIC), standard dose prophylaxis is recommended, which aims to reduce bleeding episodes and prevent or at least minimize joint damage, significantly improving patients' quality of life. HIC health systems are well equipped to regularly supply these high-cost products and, therefore, consume more than 60% of the global factor supply.⁶

While these agents and treatment approaches are available in most HIC, access varies for PWH in resource-limited socioeconomic environments. In many low- and middle-income countries (LMIC), where more than 75% of PWH live, constrained resources limit the options, and clotting factor concentrate is not available or not affordable. Often PWH have to rely on fresh frozen plasma or cryoprecipitate, which make it harder to reach the desired factor level due to the risk of volume overload in addition to the risk of infection with blood-borne diseases. The financial burden associated

with factor concentrate in these settings has forced guidelines predominantly in LMICs to recommend low-dose prophylaxis or even low dose on-demand treatment to control bleeding episodes.⁵ Hence, the variability in the treatment approach and intensity across countries contributes drastically to the discrepancy in the quality of life and life expectancy for hemophilia patients globally.^{7,8}

AAV-mediated gene therapy for hemophilia A or B has the potential to cure the disease after a single peripheral vein infusion.⁹ The clinical development of several FVIII or FIX gene transfer products is now at the critical junction of moving from the clinical trial environment to marketing and commercial availability. Although regulatory approvals are still pending, preparations for provider and patient education, clinical infrastructure, and manufacture scale up including pricing and reimbursement plans are well on their way in high-income countries (HIC). In contrast, there is minimal preparative activity in low- and middle-income countries (LMIC); only 7 of 137 (4%) LMIC (Brazil, Bulgaria, China, India, South Africa, Thailand, Turkey) have been participating in hemophilia gene therapy trials, and only China is sponsoring its own trials.¹⁰ Therefore, most LMIC have only limited knowledge and experience, in addition to their lower economic capacity, regarding introducing these new and expensive drugs. The recently announced proposed price tag of US\$ 1,000,000–3,000,000/dose has the potential to worsen the existing health outcomes gap among PWH worldwide.

Gene therapy offers an exciting opportunity to reduce health inequity in PWH globally and contribute to improved quality of life, societal and global gains. Adaptation of gene therapy implementation in LMIC versus HIC is essential and requires the use of an implementation science framework. This review highlights the needs to be addressed for equitable, global implementation of hemophilia gene therapy. After summarizing the current evidence from hemophilia A and B gene therapy trials, we address the important considerations in this process: pre-implementation assessment and readiness; education of patients and providers; establishment of infrastructure and clinical delivery processes; monitoring for implementation and health outcomes; and cost, cost-effectiveness and payment models.

Current Experience with AAV-Mediated Gene Therapy for Hemophilia A and B

Efficacy and Safety of Gene Therapy Products in Development

Three initial clinical trials of AAV-mediated gene therapy in patients with hemophilia B laid the groundwork for today's 10 or more gene transfer products in ongoing development (Tables 1 and 2). The first trial administered an AAV2-FIX vector intramuscularly with no clinical complications, but without sufficient expression levels in plasma, although persistence of local transgene expression in muscle cells, as assessed by immunohistochemical staining of transduced myocytes, was present after 3.7 years.^{11,12} A subsequent study of AAV2-FIX delivered via intrahepatic artery injection, showed only temporary FIX expression, up to 12% in one participant, but uncovered the possible role of vector-induced, CD8 T-cell mediated immune response targeting transduced liver cells causing reduction or loss of factor expression post infusion.^{13,14} Subsequently, the trial of an AAV2/8-FIX vector introduced close monitoring post infusion for vector-induced transaminitis and, as indicated, the use of transient immunosuppressive treatment with oral prednisone or prednisolone. This study has reported stable long-term FIX expression for at least 8 years without late toxicity.^{15–17}

Following these trials, nine different AAV-FIX vectors have entered Phase 1/2 trials of which five are currently continuing in active research and development phases, four of these using a higher specific activity variant of FIX, termed FIX-Padua: AMT061 (etranacogene dezaparvovec, etranadez), SPK9001 (fidanacogene elaparvovec), FLT180a (vebrinacogene setparvovec), BBM-H901, and VGB-R04 (Table 1).^{10,18} AMT061 achieved a mean FIX activity of 36.9% ($\pm$ 21.4, range 4.5–122.9) at 1.5 years in 54 Phase 3 participants using a dose of 2×10^{13} vg/kg; preliminary data show that the initial vector construct, a wild-type FIX-containing vector AMT060, has been stably expressed without long-term toxicity up to 5 years.^{19–22} AMT061 is currently under accelerated review by the European Medicines Agency. SPK9001 therapy has resulted in mean FIX activity of 22.9% ($\pm$ 9.9) at a dose of 5×10^{11} vg/kg in 15 Phase 2 subjects after 1 year.^{23,24} FLT180a treatment has resulted in FIX activity levels between 50% and 180% at a dose of 8.3×10^{11} vg/kg in 4 subjects having received the final dose level of the phase 1/2 trial.²⁵ Interestingly, the final vector doses vary up to 40-fold between the different vectors. Overall, 20–70% of the patients experienced vector-induced transaminitis requiring

Table I Clinical Trial Results for AAV-Mediated Gene Therapy for Factor IX Deficiency

Vector Product	NCT	Trial Phase	Vector Dose	Number Treated	FIX (%) at Year: 0.5–1y	1.5–2y	2.5–3y	4y	5y	6y	Transaminitis Rate	Reference
<i>CHOI/Avigen</i>												
rAAV2-FIX (intramuscular)		I	0.2 to 1.8 ×10 ¹²	8	0							[11,12]
<i>CHOI/Avigen</i>												
rAAV2-FIX-WT (intra-hepatic artery)	NCT00515710	I/2	0.08 to 2×10 ¹²	7	0							[13]
<i>St. Jude/UCL</i>												
rAAV8-FIX-WT	NCT00979238	I/2	2×10 ¹²	6	1.4–7.2	5.1±1.7				5.1	4/6 (67%)	[15–17]
<i>Uniqure/CSL Behring</i>												
AMT060, rAAV5-FIX-WT	NCT02396342	I	2×10 ¹³	5	6.9 (2.6–11.3)	7.1	8.4	7.4	5.2		2/5 (40%)	[19,20]
AMT061, (etranacogene dezaparvovec, rAAV5-FIX-R338L)	NCT03489291	2	2×10 ¹³	3	47 (33–57)		50 (37.1–58.6)				0/0 (0%)	[21]
	NCT03569891	3	2×10 ¹³	54	39±18.7 (8.2–97.1)		36.9±21.4 (4.5–122.9)				0/0 (0%)	[22]
<i>Pfizer/Spark</i>												
SPK9001, (fidanacogene elaparvovec, SPK100-FIX-R338L)	NCT02484092	2	5×10 ¹¹	15		22.9±9.9					3/15 (20%)	[23,24]
	NCT03861273	3	n/a	n/a								
<i>UCL/Freeline</i>												
FLT180a, (AAVS3-FIX-R338L)	NCT03369444	I	8.3×10 ¹¹	4	50–180						n/a	[25]
	NCT05164471	I/2	7.7×10 ¹¹	1							n/a	
<i>IHBDH/SBDB</i>												
BBM-H901, AAV843-FIX-WT	NCT04135300	I/2	5×10 ¹²	10							n/a	

(Continued)

Table I (Continued).

Vector Product	NCT	Trial Phase	Vector Dose	Number Treated	FIX (%) at Year: 0.5–1y	1.5–2y	2.5–3y	4y	5y	6y	Transaminitis Rate	Reference
<i>HBDH/FIX Variant</i>												
VGB-R0-4	NCT05152732	2	n/a	n/a							n/a	
<i>Dimension/Ultragenics</i>												
DTX101, AAVrh10-FIX-WT	NCT02718915	I	5×10^{12}	3	0						n/a	[27]
<i>Baxalta/Takeda</i>												
BAX335, rAAV8-FIX-R388L	NCT01687608	I	3×10^{12}	2	45.3 (32–59)	20 (n=1)					n/a	[26]
<i>Takeda/Baxalta/Shire</i>												
SHP648, rAAV8-FIX-R388L	NCT04394286	I	n/a									

Table 2 Clinical Trial Results for AAV-Mediated Gene Therapy for Factor VIII Deficiency

Vector Product	NCT	Trial Phase	Dose	Number Treated	FVIII (%) at Year: 0.5y	1y	2y	3y	4y	5y	Transaminitis Rate	Reference
<i>Biomarin</i>												
BMN270, Roctavian	NCT02576795	I/2	6×10 ¹³	7	-	93±48	36	33	-	11.6±12.2	7/7 (100%)	[30–32]
			4×10 ¹³	6	-	21	15	-	5.6		4/6 (66%)	[30–32]
	NCT03370913	3	6×10 ¹³	134	-	42.9±45.5	24.4±29.2				106/134 (79%)	[29]
	NCT03392974	3	4×10 ¹³	1	-							
<i>Spark</i>												
SPK8011, AAV-LK03-coBDD-F8	NCT03003533	I/2	5×10 ¹¹	2	-							[35]
			1×10 ¹²	3	-							[35]
			1.5×10 ¹²	4	-	All four doses:						[35]
			2×10 ¹²	9	-	6.9±3.8 (3.0–14.3)*					13/18 (72%)	[35]
SPK8016, AAV-coBDD-F8	NCT03734588	I/2	5×10 ¹¹	4	-	10.4 (6.2–21.8)					3/4 (75%)	[36]
<i>UCL/St. Jude</i>												
GO8, scAAV2/8 HLP-FVIII-V3	NCT03001830	I	6×10 ¹¹	1	-	7					0/1	[39]
			2×10 ¹²	n/a	-							
			4×10 ¹²	n/a	-							
			6×10 ¹²	n/a	-							
<i>Pfizer/Sangamo</i>												
SB525, PF07055480, giroctocogene fitelparvovec	NCT03061201	I/2	3×10 ¹³	5	-	42.6	25.4				4/5 (80%)	[37]
	NCT04370054	3	3×10 ¹³	n/a	-							
<i>Bayer/Ultrogenix</i>												

(Continued)

Table 2 (Continued).

Vector Product	NCT	Trial Phase	Dose	Number Treated	FVIII (%) at Year: 0.5y	1y	2y	3y	4y	5y	Transaminitis Rate	Reference
Bay2599023, DTX201, AAVhu37-BDD-F9	NCT03588299	I/2	0.5×10 ¹³	2	-	≥5						[38]
			1.0×10 ¹³	2	-	2–10					1/2	[38]
			2.0×10 ¹³	4	10–45						2/2	[38]
			4.0×10 ¹³	n/a	-							
<i>Takeda/Shire/Baxalta</i>												
TAK754, SHP654, BAX888, AAV8-BDD-F8	NCT03370172	I/2	2×10 ¹²	n/a	-							
			6×10 ¹²	n/a	-							
			1.2×10 ¹³	n/a	-							

Note: *Two participants lost expression and their FVIII values are not included here.

transient reactive and/or prophylactic immunosuppressive treatment in the first months after vector infusion, except with the use of AMT061, an AAV5-FIX Padua construct (see discussion below). The trials of BBM-H901 and VGB-R04 have no results reported yet. Other vectors have failed due to early loss of expression possibly related to immune response by introduced CpG oligonucleotides (BAX335 or AAV8-hFIX)²⁶ or capsid-specific immune attack not responsive to immunosuppression (DTX101 or AAV-rh10-FIX),²⁷ or the program has been suspended for other reasons (SHP648). In summary, one vector showed stable expression at low levels for up to 8 years, two vectors report stable FIX levels in the mild hemophilia range up to 1.5 years, and the others are in earlier trial stages.

Seven FVIII-AAV-derived vector constructs have been in clinical evaluation since 2015 with six in ongoing research and development (Table 2).²⁸ The furthest developed vector, BMN270 or roctavian, has completed phase 3 enrollment of 134 participants administering a dose of 6×10^{13} vg/kg resulting in a mean FVIII activity of 42.9% (± 45.5) at 1 year, and 24.4% (± 29.2) at 2 years after infusion.²⁹ A similar decline of factor activity has been observed in the related phase 1/2 trial where the reduction of factor expression slowed over time.^{30–32} However, by end of the published observation period of 5 years the mean post-infusion FVIII level was 11.6% (± 12.2). This trend of declining FVIII expression is continuing.³² Therefore, in contrast to FIX, the durability of AAV-mediated FVIII gene therapy remains unclear. Several potential reasons are being considered, but related supporting data are limited.³³ These include characteristics of the vector construct including the oversized (for AAV) nature of the expression cassette, the type and conditions of the vector production platform, post-translational modifications, ongoing subclinical inflammation or damage of the liver, turnover of transduced hepatocytes, and instability of episomal AAV-DNA. A small number of liver biopsy tissue evaluations did not find evidence of stress to the endoplasmic reticulum.³⁴ Eighty-six percent of patients developed increased transaminases and 79% were treated with reactive transient immunosuppression, with resolution in 96% of the cases.²⁹ This vector product is currently under review by regulatory authorities in Europe and the United States (US). The other vector products have reported phase 1/2 trial results up to 2 years in less than 10 participants each for a given dose. SPK8011 and SPK8016 vectors achieved mean FVIII levels of 6.9% (dosing range $0.5\text{--}2 \times 10^{12}$ vg/kg) and 10.4% (at 2×10^{12} vg/kg) in 16 and 4 participants, respectively, with 13/18 and 3/4 patients experiencing abnormal liver function tests and, therefore, receiving oral steroids.^{35,36} Giroctocogene fitelparvovec, or SB525, treatment of 5 patients with 3×10^{13} vg/kg has resulted in 42.6% and 25.4% mean FVIII activity at 1 and 2 years post infusion, with 4 experiencing transaminitis and being treated with steroids.³⁷ BAY2599023 or DTX201 achieved FVIII activity in the range of 25–40% in 4 participants at 2 years using a vector dose of 2×10^{13} vg/kg.³⁸ The trials of the GO8 vector, scAAV2/8-HLP-FVIII-V3,³⁹ and the TAK754 and BAX888 vectors have reported only limited or no results yet. In summary, similar to FIX products, the vector doses reaching meaningful FVIII expression have varied widely between 0.2×10^{13} and 6×10^{13} vg/kg, a 30-fold difference. While most gene transfer products resulted in a state of mild hemophilia with significant reduction in bleed rate and factor use, only one product has been monitored up to 5 years and is showing a continuous decline in FVIII expression.

None of the vectors has been approved by regulatory agencies yet, because additional follow-up data were requested. For both, FVIII and FIX vectors, achieved levels vary significantly among vector types and among individuals given the same vector, indicating only limited predictability. Variability may be influenced by the subject's subclinical or clinical immune response to the vector, such as vector-induced transaminitis, and its response to immunosuppression.⁴⁰ However, other causes could include details of the vector construct, manufacturing methods, vector titering methods, transduction efficiency including AAV binding and internalization at cell surface, escape from endosomes, entry into the nucleus, DNA annealing or second-strand synthesis, episome and concatemer formation, mRNA transcription, and translation and post-translational processing – much of this is not well understood.^{34,41}

AAV Antibodies and AAV Vector-Induced Transaminitis

An appreciation for the immunologic aspects of adeno-associated virus (AAV) infection is critical for ensuring its successful use as a vehicle for affecting gene transfer. Yet, there is only a limited understanding of the role of cellular immunity and its contribution to the post-vector administration liver inflammation. The latter generally manifests as asymptomatic transaminitis in many, but not all, recipients of systemically administered AAV vectors, in a dose-dependent manner. Better understood is the humoral (antibody) response to naturally occurring infection with wild-

type AAV or recombinant AAV vectors used for gene transfer, which generates long term, serotype-specific neutralizing antibodies. The significant consequence is that these neutralizing antibodies, unless somehow abrogated, generally would prevent subsequent successful liver-targeted gene transfer with systemic, intravenous administration of recombinant AAV vectors of the same serotype.⁴² However, while pre-clinical animal models did not predict cross-reactivity of neutralizing antibodies between different serotypes, some degree of cross-reactivity was shown in studies on human blood samples, including from persons with hemophilia after AAV-mediated gene therapy.^{14,43–45}

Critical then, for most rAAV vector products, is to confirm the absence of serotype-specific neutralizing antibodies in PWH under consideration for enrollment on a gene therapy trial of liver-targeted, AAV-mediated transfer of the therapeutic gene encoding clotting factor VIII or IX. In fact, the lack of success of the early liver-targeted AAV-mediated gene therapy protocols was due, at least in part, to the failure to appreciate the problematic nature of low-titer pre-existing neutralizing antibodies in addition to the then-unanticipated potential of a cellular T-cell response via CD8 T-helper cells. The percentage of people with measurable neutralizing antibody titers varies between 20% and 70% depending on AAV serotype.^{46–48} A number of methods have been tested in preclinical studies in an attempt to circumvent these neutralizing antibodies including pheresis, immunosuppression, B cell depletion, immune signaling blockade, coating of AAV particles, modifying the immunogenic portions of the capsids and giving an excess of empty AAV particles to act as a decoy, but no strategy has clearly worked consistently well.

An exception may be vectors derived from AAV5. With AMT-061, an AAV5-FIX-Padua vector, only one participant with a very high anti-AAV5 antibody titer failed to show factor expression, while all other trial participants with low positive or negative titers did express factor successfully and had no evidence of vector-induced transaminitis.²² A phase 1/2 trial of BMN270, roctavion, an AAV5-FVIII vector, is currently further exploring the efficacy in patients with anti-AAV5 antibodies.⁴⁹

Not all serotypes have the same incidence of prior exposure, and the incidence varies with geographic location. Therefore, screening of specific potential recipients is required. A variety of methods have been used to screen for the presence of pre-existing neutralizing antibodies, using both in vitro and in vivo assays. To detect the presence of AAV-binding antibodies within a serum sample, the in vitro assay involves antibody capture in tissue culture plates that have been pre-coated with recombinant AAV vector of a specific serotype. The binding detection step is then achieved with commercial antibodies to human IgG performed after several washing steps. Although simple, inexpensive and high throughput, this method may not prove the identified antibodies to be functionally neutralizing. To detect neutralizing anti-AAV antibodies, two types of cell-based assays are used: in vitro (eg, human embryonic kidney 293 (HEK293) cells as targets for transduction) or in vivo (murine hepatocytes). These assays involve demonstrating inhibition of transduction of the target cells by human test serum given prior to administering the particular recombinant AAV vector.^{15,23,50} Transgene expression resulting in 70% or greater of expected factor activity is generally considered to demonstrate a lack of the presence of a neutralizing antibody. Another option is an in vitro assay in which proband serum is incubated with AAV-luciferase of a specific serotype and then added to cells. Luciferase is quantified to determine the level of transduction and resultant transgene expression compared to the control informing the titer of the neutralizing antibody. Although more expensive and time consuming, the in vivo assay using mice is generally preferred because it is a functional assay that demonstrates the presence of neutralizing antibodies. Typical negative and positive controls for these assays are serum samples from a human prior to and after successful transduction with AAV vector, respectively.

With most vector types, the prevention of transaminitis is approached with immunosuppressive therapy to modify immune reactivity by using prednisone or prednisolone prophylaxis with or without tacrolimus. Breakthrough transaminitis is typically treatable with a high-dose corticosteroid regimen if recognized promptly and managed with frequent monitoring of liver function tests such as 2–3 times per week for the first 6–9 months after vector infusion. This is a significant burden on the patient and provider clinic, but typically only occurs in this early post-infusion period. Point-of-care laboratory testing of liver function tests and remote clinical management should be explored in the future to support widespread access.

Long-Term Toxicity

No long-term liver toxicity has been reported after AAV-mediated gene therapy for hemophilia in clinical trials. Toxicity by vector genome integration into chromosomes and potential resultant oncogenesis remains an ongoing theoretical concern.⁵¹ AAV-mediated gene transfer primarily results in episomal persistence of vector genomes, but chromosomal vector genome integration has been described at very low frequency at random locations. In preclinical studies in different animal models, the oncogenicity of AAV-mediated gene transfer has been reported, but the significance of these findings in humans is not clear.⁵² A small number of patients after AAV administration, with diagnoses of hemophilia, acute intermittent porphyria and lipoprotein lipase deficiency, had liver biopsies revealing a low frequency of integrations, but no concern for integration at oncogenic sites and no evidence of abnormal pathology.^{34,53,54} To date, no AAV vector-related malignancy has been identified across all trials in hemophilia. A single case of hepatocellular carcinoma (HCC) in an AAV vector-treated patient with hemophilia B was determined highly unlikely associated with the vector, because the patient had multiple known clinical risk factors for HCC. Tissue evaluations found several HCC-related mutations and only a very low, random vector genome integration frequency at chromosomal locations unlikely to support vector-related oncogenesis.⁵⁵

Overall, long-term follow-up of efficacy and safety outcomes, including liver health and tissue evaluations when indicated, by participation of treatment centers and treated patients in globally accessible observational studies or registries, such as the one established by the World Federation of Hemophilia,⁵⁶ will be critical to further elucidate vector-related effects.

Considerations for Successful Implementation of Gene Therapy Worldwide

The new treatment paradigm of gene therapy for hemophilia offers the opportunity to prospectively plan for successful equitable access for all PWH globally. The latter will depend on an integrated approach to implementation adaptable to various settings. The adoption of an implementation science framework will guide the stakeholders and extended community. Drawing from multiple implementation constructs synthesized in the Consolidated Framework for Implementation Research (CIFR) and from our own experience in conducting clinical gene therapy trials, we will discuss the most relevant considerations in the following sections: the assessment of the pre-implementation context and readiness; a methodological approach to effective communication, education and training; cost and cost-effectiveness analysis for planning of sustainable pricing and reimbursement; resource-adjusted operational planning; and monitoring of health outcomes and implementation outcomes throughout the process.^{57,58}

Pre-Implementation Context Assessment and Readiness

For introducing a novel treatment concept, an interested and supportive environment is needed. All stakeholders should be identified, including the hemophilia providers, the target patient group which ideally includes either all local PWH or the potentially eligible patients, leaders of the patient advocacy groups, the healthcare institutions and local health authorities. A local leader of the process should be determined. This network of supporters may also include other groups currently supporting the care of patients in different ways, such as groups from programs of the World Federation of Hemophilia, pharmaceutical industry, or other local or international organizations. Depending on local preferences, additional legal or ethics consultants can provide valuable input.

An assessment of the regulatory and policy requirements may necessitate additional discussions with authorities and potentially need amendments or the establishment of new regulatory guidelines for drug manufacturing or procurement and approval. Policies and systems for regulation of clinical gene therapy are largely in place in North America and Europe, where most of the clinical trial activity has been conducted and pharma has established vector manufacturing. An industry guidance has been published by the Food and Drug Administration (FDA), and both FDA and European Medicines Agency (EMA) oversee robust regulatory processes.^{59,60} To foster global access, the WHO has published a White Paper draft in December 2021 addressing the need for regulatory convergence for cell and gene therapy.⁶¹ A recent

article supported by ASGCT discusses global regulatory challenges and tries to provide solutions considering the goal of providing access to gene therapy globally.⁶²

Notably, some LMICs are establishing their own regulatory and infrastructure frameworks for hemophilia gene therapy, either by developing and/or manufacturing their own vector products or participating in internationally sponsored gene therapy trials.^{63,64} These countries include Brazil, China, India, Nepal, Peru, South Africa, Thailand, Sri Lanka, and Vietnam, if not more. Beyond participating in international trials, another pathway of establishing a new regulatory framework for gene therapy in a given country is through interaction, exchange and harmonization of established framework templates between countries or regions and with the regulatory bodies in countries with more advanced frameworks such as in the US, Europe, and Japan.

Another important context dimension to explore are the financial resources in relation to cost and payment models. Current payment or reimbursement pathways may not be feasible and new arrangements and agreements will need to be created (see [Cost, Cost-Effectiveness, and Payment Models](#)). Further, any gap in current and required operational capacities needs to be evaluated and addressed as feasible to create a locally efficient process (see [The Iterative Process of Implementing Hemophilia Gene Therapy](#)). In a final step, checking the readiness for implementation should document the implementation plan, the availability of resources, and the commitment and motivation of all stakeholders to engage on the journey of change.

Addressing Barriers to Knowledge and Acceptability

The successful adoption and translation of gene therapy in LMIC will depend heavily on addressing barriers relating to knowledge and acceptability. Introducing multi-level interventions targeting healthcare providers, patients, caregivers and patient advocates to communicate the benefits and risks of gene therapy will clearly improve acceptance of gene therapy. Effective communication around the concept of a complex treatment option such as gene therapy may assuage fears and provide salient information to equip patients and providers in shared decision-making. The process of informed consent and steps taken to educate patients with hemophilia will set the stage for many other gene therapy products.

Various national and international societies such as the World Federation of Hemophilia (WFH), National Hemophilia Foundation (NHF), International Society of Thrombosis and Hemostasis and American Society of Gene and Cell Therapy (ASGCT) have generated a series of didactics including web-based videos and webinars, which can be used to improve gene therapy knowledge.^{65–68} These are excellent resources that provide basic information with excellent visuals for patients; however, most of the material is focused on educating providers. These resources lack detailed explanation in plain language and individualization for patients.

Gene therapy education should be approached at multi-level, focusing separately on providers and patients. Delivering education to providers has its own challenges such as different learning styles, competing professional priorities, implicit biases and lack of dedicated time. Physicians are often overburdened by a “culture of excellence” which leads to hesitancy in admitting knowledge deficiencies.⁶⁹ To design interventions that accommodate different level learners and to promote supportive learning environment, adult learner theory suggests that we focus on small group educational events to allow dialogue, interaction, and reflection.⁶⁹

Additionally, creation of education material for patients that can be used in obtaining informed consent needs to take into account health literacy, and take a deep dive in patients’ beliefs, fears, and expectations about the treatment. Although some progresses have been made in North America and Europe to map out patients’ perspectives and identify their fears, the same cannot be said about LMICs where no material has been published to our knowledge.

A summary of the conducted studies includes a small US-based qualitative study which used phone-based semi-structured interviews with 21 hemophilia A and B patients and found that prevention of bleeding and improvement in quality of life were the major advantages of gene therapy that patients looked forward to. Failure of gene therapy and financial burden of these treatments were amongst the biggest worry of the patients. The study called for clear information to be provided regarding safety and efficacy of gene therapy.⁷⁰ A multicenter study performed in the United Kingdom, North America and Australia conducted a discrete choice experiment to investigate the relative importance and differential preferences patients provided for gene therapy attributes via a survey of 183 hemophilia A and B participants. Patients prioritized reduction in bleeding rate (31%), and reduction in dose frequency and durability

of gene therapy (26%). Uncertainty regarding potential short- and long-term safety issues and impact on daily life were weighed at 17% and 11%, respectively.⁷¹ A qualitative study in Belgium with 20 hemophilia participants demonstrated a positive attitude towards gene therapy with 40% of patients “very willing” (N=8) or 35% “willing” (N=7) to receive gene therapy. Annual bleeding rate, factor level, uncertainty of long-term risk, impact on daily life and probability to stop prophylaxis were found to be the important factors to discuss.⁷² Review of this literature shows that despite the relatively similar cultural context, priorities and fears about gene therapy may differ considerably between individuals. These findings will probably vary significantly for participants in LMICs.

Ideally, creative, and practical approaches through multimedia and interactive formats can have numerous benefits for presenting information in more user-friendly ways, and can aid in optimizing discussion, through personalization and consolidation of the information to avoid excessive information. The multimedia format however should not be substituting robust conversation with patients about benefits, known and unknown risks, efficacy, and long-term effects of gene therapy.^{73,74} The education material must also address the psychosocial burden on the participant, which may affect a patient’s willingness to undergo gene therapy.⁷⁵ Multiple guidelines are published on how to discuss gene therapy with patients, provide lists of helpful vocabulary or pictorial representation of the basic process of gene therapy, and highlight topics of discussion related to safety and efficacy.^{76–78} These tools can help guide face-to-face discussions with each patient and should be individualized based on their priorities and fears (Table 3).

More research is required to evaluate the methods of obtaining informed consent and implementing educational programs regarding gene therapy in LMIC. Language barriers, cultural differences and socioeconomic disparities invariably pose challenges to the provision of clear and accurate information. Creating education packages that can be

Table 3 Guide for Physicians for Discussing Gene Therapy with Persons with Hemophilia

Topic	Sub-topics
Fundamentals of gene therapy	What is a vector? What is a functional gene? How is the vector delivered? How does the body react to the vector? Anti-vector antibodies
Expectations after gene therapy	
Efficacy	Treatment of bleeding events Frequency of bleeding events Pain management Factor expression (level and duration)
Safety (short- term)	Side effects (ie, transaminitis, inhibitor development, thrombosis, anti-vector antibodies) Treatment of side effects
Safety (long-term)	Risk of developing cancer or other disorders Possibility of unknown side effects Long-term monitoring and annual follow-up for many years
Eligibility for gene therapy	Severity of disease (factor level $\leq 2\%$) Adults or young adults Patients without inhibitors Patients without neutralizing antibodies to AAV capsid
Current clinical trials	Status of ongoing clinical trials Major findings of clinical trials
Other things to consider	Psychological functioning and emotional maturity Social support Family planning

rapidly adapted across cultures and countries with differing resources must account for variations in understanding of gene therapy across all stakeholders such as patients, caregivers, community leaders, and healthcare providers.

Cost, Cost-Effectiveness, and Payment Models

Pricing Approaches and Cost-Effectiveness Simulation Models

Although contextual factors that may limit adoption and acceptability of gene therapy in LMIC are critical, the prices proposed for gene therapy remain perhaps the largest impediment to global access. It is difficult to determine a price for gene therapy because of complex pricing considerations, the need to scale out or up, lack of transparency on initial research and development (R&D) investments and production costs, the combination of fixed indirect and variable direct costs, lack of agreement on whether prices of therapies should be value, procedure, or R&D based, and limited clinical evidence at the time payers need to make decisions.⁷⁹

Although multiple factors should be considered when pricing novel treatments, manufacturers often follow value-based pricing in which they choose the highest price point that the market could bear based on the comparative cost of the alternative approach.⁸⁰ The comparative outcome of cost-effectiveness models is often expressed in incremental cost-effectiveness ratio (ICER), which is calculated by dividing the additional cost of the new intervention compared to the standard one, by the additional effects generated by the new intervention. The effects are usually measured in quality-adjusted life years (QALYs), a unit of measure that combines the expected years of life lived (ie, life expectancy) with quality-of-life metrics. If the cost is cheaper and the QALYs exceed that of the standard intervention, then the new intervention is considered dominant. In the opposite scenario, the new intervention is considered dominated and should be rejected. In case the new intervention is more expensive, yet yields additional QALYs, then the ICER is compared against a country-specific threshold to determine whether or not it is cost-effective compared to the standard intervention.

As pricing remains tentative and clinical trials are ongoing, investigators have developed five cost-effectiveness models using simulated data to evaluate the cost-effectiveness of gene therapy in hemophilia. Four models evaluated gene therapy from the perspective of PWH in the US and one in the Netherlands.^{81–85} None of the models were conducted using perspectives from LMICs, demonstrating one more time the extent to which these countries have been overlooked. Our study⁸⁴ was the first and so far the only model focused on hemophilia B while the remaining four models assess the cost-effectiveness of treatment options for hemophilia A. Table 4 summarizes the key input parameters and the results of each of the five cost-effectiveness models. Although all five models reach the same conclusion, suggesting that gene therapy is less costly and more effective (dominant) compared to the alternative treatment, the final results – gene therapy lifetime cost and QALYs, comparator lifetime cost and QALYs – are very different. Driving these differences are several factors and key assumptions that should be carefully considered when interpreting the results of each model. Each of these parameters are also critical to understanding what, if any, role gene therapy may have to play in LMICs.

The first major determinant is the time-horizon, models with a 10-year time-horizon cannot be compared to those with a lifetime horizon. Furthermore, lifetime horizon models starting at 18 years of age are not comparable to those starting at 30. The perspective used while considering costs is another key factor. Societal perspective takes into account all incurred cost categories regardless of the payer, this includes productivity losses for both patient and caregiver. Conversely, healthcare perspective disregards all costs not paid by the healthcare system. Additionally, the discounting factor might play a role in the final results when accumulated over a long period of time. Different countries recommend different discounting factors. Finally, each model's parameters have a direct impact on the outcomes of the model and thus should be critically evaluated by the reader (Table 4).

Results of the five models are presented in Table 4. Machin et al and Ten Ham et al^{81,85} conducted a 10-year time horizon starting at approximately 30 years of age. Cook et al⁸² presented the latter as a scenario analysis which we use here for comparability purposes. These three models concluded that over the 10-year period, the gene therapy arm cost (in US\$) was \$1,022,249, \$2,526,897 and \$3,469,487, while prophylaxis with standard half-life FVIII cost was \$1,693,630, \$2,923,374 and \$8,502,702, respectively. These differences could be explained by the assumed price of the gene therapy vector at \$850,000, \$1,891,250 and \$2,000,000, the probability of developing bleeding under different treatment scenarios and the cost to treat a bleed when one occurred. Differences in costs to control and treat bleeds were

Table 4 Summary of Cost-Effectiveness Models Comparing Gene Therapy to Alternative Treatments for Hemophilia

	Machin 2018 ⁸¹	Cook 2020 ⁸²	Rind 2020 ⁸³ (ICER Report)	Bolous 2021 ⁸⁴	Ten Ham 2021 ⁸⁵
Model Outline					
Model type	Markov state-transition model	Microsimulation Markov model	Markov model	Microsimulation Markov model	Markov state-transition model
Disease	Severe hemophilia A	Severe hemophilia A	Severe hemophilia A	Severe hemophilia B	Severe hemophilia A
Intervention	Gene therapy (No brand specified)	Valoctocogene roxaparvovec	Valoctocogene roxaparvovec	Gene therapy (No brand specified)	Valoctocogene roxaparvovec
Comparator	FVIII prophylaxis	FVIII prophylaxis - SHL (base case) - EHL (scenario analysis)	FVIII prophylaxis - SHL - EHL	1) FIX prophylaxis 2) On-demand treatment - SHL (for both options) - EHL (for both options)	1) FVIII prophylaxis 2) Emicizumab
Model Specifications					
Time horizon	10 years starting at 30	Lifetime starting at 30	Lifetime starting at 18	Lifetime starting at 18	10 years starting at 31
Perspective	US healthcare perspective (Assumed 2018 USD)	US healthcare perspective (2019 USD)	US healthcare perspective (2019 USD)	US healthcare perspective (2020 USD)	Dutch societal perspective (2019 Euro)
Discounting	3%	3%	3%	3%	Costs 4% QALYs 1.5%
Model Input Parameters					
Gene therapy cost	\$850,000	\$2,000,000	\$2,500,000	\$2,000,000	€2,125,000 (\$1,891,250)
Clotting factor unit cost	\$1/IU	\$1.63/IU WAC	Advate net: \$1.08/IU Eloctate net: \$1.82/IU Net price was used in base case Advate WAC: \$1.69/IU Eloctate WAC: \$2.23/IU	Benefix WAC: \$1.41/IU Alprolix WAC: \$3.24/IU	FVIII: €0.89/IU (\$0.79/IU) (11% discount from WAC for base case) Emi: €2476 per 30 mg/mL vial (\$2204)
Mean weight	88.7 kg for 30-year-old	90.2 kg for 30-year-old	81.4 kg for 18-year-old	89.3 kg for 30-year-old	85 kg for 31-year-old

(Continued)

Table 4 (Continued).

	Machin 2018 ⁸¹	Cook 2020 ⁸²	Rind 2020 ⁸³ (ICER Report)	Bolous 2021 ⁸⁴	Ten Ham 2021 ⁸⁵
Dosing	Prophy: 33 IU/kg 3 times weekly Bleed: 50 IU/kg/d 2 or 4 days	Prophy: 40 IU/kg 3 times a week Bleed: 50 IU/kg 2 doses	Not stated	Prophy: 40 IU/kg twice weekly Bleed (minor): 50 IU/kg 3 doses per episode	Prophy: 30 IU/kg 3 times a week. Emi: 3 mg/kg biweekly. Bleed: 25 IU/kg then twice daily 15IU/kg for 3.5 days.
Effectiveness of gene therapy	Effective for 10 years. Success rate 90%, complications 1% for first 12 months.	90% mean 12.43 years and median 11.33 years. 10% mean 11.13 years and median 10.73 years. Move back to prophylaxis below 5 IU/dL. No joint bleeds above 15IU/dL	FVIII ≥1% for 12 years (7 and 15 years in conservative and optimistic scenarios)	31 years >3%, switch all patients to prophylaxis at 3% (worst case scenario 4 years)	Mean 11.5 years before dropping below 1% and switching to prophylaxis. 15% limited response (FVIII<5% after 2 years).
Results					
Gene therapy cost over entire time horizon	\$1,022,249	\$16,700,000 (10 years: \$3,469,487)	\$13,693,000	SHL: \$6,293,502 EHL: \$7,315,914	€2,839,210 (\$2,526,897)
Gene therapy QALYs	8.33 QALYs	18.07 QALY (10 years: 6.88 QALY)	19.091 QALYs	SHL: 23 QALYs EHL: 23.04 QALYs	7.03 QALYs
Comparator cost over entire time horizon	\$1,693,630	\$23,500,000 (10 years: \$8,502,702)	\$18,722,000	OD SHL: \$11,596,617 OD EHL: \$7,917,721 Pro SHL: \$15,109,058 Pro EHL: \$20,324,299	Prophy: €3,284,690 (\$2,923,374) Emi: €4,252,167 (\$3,784,428)
Comparator QALYs	6.62 QALYs	17.32 QALYs (10 years: 6.37 QALYs)	19.087 QALYs	OD SHL: 11.81 QALYs OD EHL: 12.20 QALYs Prophy SHL: 20.95 QALYs Prophy EHL: 21.57 QALYs	Prophy: 6.38 QALYs Emi: 6.90 QALYs
ICER	Dominant	Dominant	Dominant	Dominant	Dominant

Note: ICER is calculated by the following formula: $\frac{\text{Costs}_{\text{New Intervention}} - \text{Costs}_{\text{Standard Intervention}}}{\text{QALYs}_{\text{New Intervention}} - \text{QALYs}_{\text{Standard Intervention}}}$.

Abbreviations: ICER Report, Institute for Clinical and Economic Review Report; SHL, standard half-life; EHL, extended half-life; USD, United States dollar; QALYs, quality-adjusted life years; WAC, wholesale acquisition cost; Emi, emicizumab; OD, on-demand treatment; Prophy, prophylaxis; ICER, incremental cost-effectiveness ratio of the intervention being investigated versus the alternative, in costs per added QALY.

driven by major variations in 1) the dosing protocol which varied in the number of units and number of administered doses for prophylaxis and different types of bleeds (Table 4), 2) the assumed weight of the prototypical patient which ranged between 81.4 and 90.2 kg, 3) the FVIII unit cost which ranged between \$0.79/IU (discounted) and \$1.63/IU (Wholesale Acquisition Cost), and 4) the costing perspective and discounting rate used in the model. Ten Ham et al⁸⁵ was the only model that adopted a societal perspective meaning that more cost categories like productivity loss were considered. However, it was also the only model that discounted costs at an annual rate of 4% compared to 3% for the two other models.

For the three models ordered as above, the QALYs were 8.33, 7.03 and 6.88 for the gene therapy arm and 6.62, 6.38 and 6.37 for the prophylaxis arm, respectively. Effects attributed with the gene therapy arm differed based on the assumptions made regarding its treatment effects which lasted for a period that ranged between 10 and 12.4 years, and the quality of life assigned to patients after receiving it. Machin et al⁸¹ assigned a perfect health utility of 1 to patients who received gene therapy which is an overestimation.

On the other hand, our study⁸⁴ and the report generated by the Institute for Clinical and Economic Review (ICER)⁸³ both analyzed an 18 years old to death time horizon. However, the results are incomparable because each assessed gene therapy for a different type of hemophilia, hemophilia B for the former and hemophilia A for the latter, with very different assumptions regarding the long-term effectiveness of gene therapy. Lifetime cost associated with gene therapy was \$6,293,502 and \$13,693,000, respectively. The key model input that led to this significant difference in the results was the assumption made regarding the number of years during which gene therapy was sufficient to prevent bleeds, which was 31 and 12 years, respectively. The assumed price of gene therapy, \$2,000,000 and \$2,500,000, also had an impact, but played a less drastic role compared to the difference in effectiveness. Lifetime cost for prophylaxis with the standard half-life factor concentrate was \$15,109,058 and \$18,722,000 for FIX and FVIII, respectively. QALYs for gene therapy were 23.00 QALYs and 19.091 QALYs and those for prophylaxis using standard half-life factor concentrate were 20.95 QALYs and 19.087 QALYs, respectively. In the future, cost-effectiveness models that will be tailored for LMICs will need to consider totally different treatment approaches. In most of these countries, access to prophylaxis is scarce and, in many cases, patients rely on low-dose protocols and fresh frozen plasma or cryoprecipitate instead of factor concentrate. This means that the cost and yielded effects of gene therapy will be compared to significantly cheaper but also less effective alternatives than those available in HICs.

Challenges Encountered with the Traditional Health Economic Approach

In the case of hemophilia, recombinant clotting factor unit cost is distorted in the US and multiple other countries due to industry monopolization, exacerbated by failure of the government to regulate the price and by buyer insensitivity due to coverage by their insurance.⁸⁰ This distortion led to the high suggested commercial price of \$1,000,000–\$3,000,000 for hemophilia gene therapy, which was based on the cost of the alternative treatment in high-income countries, but did not take into account LMICs. In LMICs, available treatment options are limited and constrained by finances, therefore, a single-dose subsidized, gene therapy may significantly improve patients' outcomes if negotiations and international collaborations enabled a reasonable pricing.⁶⁴ For analyses using the ICER, analysts should adjust distorted clotting factor market prices in their calculations to represent social opportunity cost and avoid generating evidence demonstrating a false sense of cost savings.⁸⁰

Moreover, there is a need to reconsider economic evaluation methods as a whole because they may not be equally applicable to every type of medical intervention. Patients with rare genetic diseases present a unique set of conditions that warrant equally unique analytic approaches to estimating value for money.⁸⁶ Gene therapies have a number of particular characteristics, taken individually, none of them is exclusive to gene therapy; however, it is the confluence of these various characteristics that leads to specific methodological challenges making assessment of both costs and effects controversial.⁸⁷

Treatment effects are hard to evaluate when the only clinical data available are generated by small and single-arm trials, spanning a short period of time, lacking information on long-term durability or adverse consequences.^{87,88} This particularly imposes a challenge in conducting a lifetime horizon. Table 4 shows heterogeneity of model assumptions regarding the time horizon analyzed. Two of the 5 currently published models chose to limit their time horizon to 10

years starting approximately at 30 years of age, to minimize the extent of extrapolation needed. Nonetheless, one of the advantages of simulation models is the ability to envision plausible scenarios and use rational assumptions to predict future outcomes. Accordingly, the other three models chose 18 or 30 years old till death time horizons. These models relied on clinical trial data that span less than a decade, to predict the time at which decline in factor activity will necessitate switching back to prophylaxis.

To extend the time horizon even further, simulation models are yet to explore administration of gene therapy in the pediatric population which to-date remains an area for scientific aspiration. Theoretically, the earlier gene therapy is administered, the better the outcomes should be, in both costs and quality of life, given that joint damage develops in childhood. LMICs, in particular, could benefit from this, and payers could potentially have the incentive to invest in early years replacement therapy which would be less of a financial burden, yet would offer patients the opportunity to avoid long-term joint damage. Simulation models can play an important role in highlighting the potential gains yielded by achieving this scientific breakthrough, which in turn can help direct efforts in the right direction.

Another challenge is that there is no consensus over the definition of “cure”. This is superimposed by the limitations of traditional generic quality of life (utility) measures which may not capture the true impact of hemophilia.⁸⁸ On the other hand, costing is complicated by the perspective used in the evaluation whether it is only inclusive of healthcare costs or encompasses societal costs, the extent to which long-term cost-offsets should be counted against the large up-front payment and pricing of gene therapy given the uncertainty of treatment effect.⁸⁸

Special considerations are proposed to address these challenges such as using data from previous clinical trials of alternative treatments or real-world evidence to construct synthetic historic cohorts of patients as comparators, extrapolating the effects of clinical trials, and collecting post-approval real-world registry data.^{87,88} Additional suggestions include adding hemophilia-specific questions to generic quality of life surveys, using a comprehensive set of outcomes (coreHEM) beyond mere quality of life, conducting several time horizons, analyzing both healthcare and societal study perspectives, utilizing multiple discounting rates, including all treatment options available and exploring alternative payment models.

In November 2019, a new framework was proposed by the US-based, private, non-profit Institute for Clinical and Economic Review (ICER) for single or short-term therapies (SSTs).⁸⁹ To date, this is the first and only attempt to tailor a health economic approach for the purpose of gene therapy. This SSTs’ model assessed several scenarios assuming multiple clinically plausible outcomes, ranging from conservative to optimistic, in addition to the conventional base-case and sensitivity analyses to reflect uncertainty about the benefits of gene therapy. For cost offsets, several hypothetical “cost-sharing” scenarios were included. Moving forward, analysts should develop future simulation models assessing novel technologies, such as gene therapy, guided by the template carved out by ICER and the recommendations highlighted by Drummond et al,⁸⁷ Garrison et al⁸⁸ and others.

Payment and Reimbursement Schemes

After 30 years of official clinical trials, the total number of completed and ongoing gene therapy clinical trials exceeds 4000⁹⁰ with at least 22 gene therapies approved by drug regulatory agencies globally.⁹¹ Based on the pipeline and the clinical success rates of products, the FDA predicts approving 10 to 20 cell and gene therapy products every year by 2025.⁹² So far only a few have gained reimbursement in some countries and two (Glybera and Zynteglo) have since been withdrawn from the market due to market access and reimbursement challenges.⁷⁹ Fair pricing and functional reimbursement models are necessary to allow patients equitable access to the full potential of gene therapies.

Similar to cost-effectiveness models, reimbursement schemes should be conceived thoughtfully to ensure sustainability. Multiple gene therapies may enter the market simultaneously, and payers could face a steep, untenable financial burden that requires considering unconventional payment schemes.⁹³ Alternative payment models have been classified into two main categories: outcomes-based and finance-based schemes. The former links payments to an agreed upon target clinical outcome. Payments can be made either per achievable milestone, or by annuity if the desired result lasts, or by rebating part or all of the paid price if the outcome declines after initial full payment. In this type of model, an agreed upon clinically relevant outcome must be identified and consensus needs to be reached how to objectively measure it. This can be difficult and resource consuming, making it less feasible outside a highly controlled context.

Finance-based schemes have fewer logistical considerations; they are suitable when patient outcomes are predictable, but the number of potential patients is large or unpredictable.⁹³ Sometimes, a capitation is applied regardless of the number of beneficiaries. This can be implemented in a subscription fashion with a fixed amount covering an unlimited number of patients per year, or a volume-based approach in which the price per patient decreases after reaching a preset number. Other times, payments are spread over several years rather than paid up-front, or the risk is minimized by diluting it to multiple payers or transferring some of the liability to reinsurers.

In addition to the dilemma of payment schemes, the question of who will pay for this expensive new therapy remains a major concern. Given the devastating consequences of the disease and the burdensome costs associated with its treatment, ideally, funding should ultimately come as part of governmental responsibility. This would align with the goal of universal health coverage, a World Health Organization strategic priority. Unfortunately, out-of-pocket payments remain the main funding source in many LMICs, resulting in significant patient and family-level financial hardship. Country-specific strategies will need to be tailored to fit the local context. For example, in a high-income country like the US, formal drug discount programs such as that provided by section 340B of the Veterans Affairs act of 1993 help somewhat to control costs, and simultaneously allow for hemophilia center funding. Similar programs should be nestled within healthcare systems to fund gene therapy. In LMICs, it will be more challenging. However, scientifically sound cost-effectiveness models, budget impact analyses, and return on investment reports that would highlight the potential gains throughout the lifetime of the patient are all valuable tools that governments could rely on to make informed decisions. Moreover, while not sustainable over the long-term, existing hemophilia civil society organizations in the private sector such as global or local foundations must continue to play a vital funding and advocacy role to bridge existing gaps.

Unlike regulators who created novel approval pathways to ensure appropriate assessments of gene therapies, payers have not adapted distinct payment and reimbursement pathways.⁷⁹ Given the unique set of circumstances associated with gene therapies, most importantly that they usually require one or only a limited number of administrations and, thus, very high short-term cost have been quoted for them, gene therapies will necessitate novel payment and reimbursement models requiring multi-stakeholder engagement and risk-sharing arrangements between industry, patients, providers and payers.⁹⁴

The Iterative Process of Implementing Hemophilia Gene Therapy

The implementation plan for establishing gene therapy for hemophilia in HIC or LMIC will vary by country, region or even clinical centers and by existing level of experience through previous trial sponsorship, trial participation as a site, or having had only minimal to no exposure to clinical gene therapy activities. After the abovementioned pre-assessments, changes to existing infrastructure and processes may be needed, and the team may proceed along the sequential and iterative activities of planning, engaging, executing, and monitoring, evaluating and revising.

In theory, the clinical delivery of gene therapy is relatively simple. Screening for eligibility before and monitoring after vector infusion requires standard laboratory blood tests and the capacity for liver ultrasound. Factor activity assays should be established for easy accessibility and quick turnaround time, while the few other, non-urgently and infrequently required specialty lab tests can be arranged with remote specialty labs if not available locally. The possibility of use of immunosuppression after vector infusion requires screening of patients for history of hepatitis or other past or current infections. It also requires establishing access to the immunosuppressive medication, for most vector products a corticosteroid, but tacrolimus has also been used in some clinical trials. Required screening and monitoring evaluations and the use of immunosuppression may vary depending on the vector product.

The gene therapy administration itself first includes a pathway for vector procurement from the manufacturer and establishing a shipping process under frozen, temperature-controlled and trackable conditions. At the infusion center, the vector product requires storage in a -65°C freezer. Specific steps for thawing and reconstitution under sterile conditions (using a laminar flow hood) for administration will be provided by the manufacturer. In the US, biosafety level 1 is required for AAV vectors, defined for agents not known to consistently cause disease. It recommends the use of laboratory coat, gloves and eye protection, and standard microbiological practices within a room with doors for access control, easy to clean bench and sink for hand washing. However, the stakes are high since contamination or damage to

the product during thawing would be a grievous loss owing to its expensive list price. The administration to the patient is a peripheral vein infusion over about 1 hour in an outpatient setting with the ability to treat potential, infusion-associated allergic reactions.

Treaters will be required to have specific knowledge allowing skilled communication of the characteristics of all aspects of the treatment and follow-up. To maximize expertise of providers and optimize vector management for clinical administration but also provide broad access to PWH to gene therapy, a “hub-and-spoke” model has been suggested.⁹⁵ The “hub” center will perform the vector infusion and maintains a high level of skill and knowledge, whereas peripheral “spoke” sites or home hemophilia treatment centers (HTC) offer screening and conduct post-infusion monitoring in close collaboration with the “hub”.

Two very important responsibilities of the treater-patient team are the short-term and long-term follow-up collaboration. Short-term, the timely and effective prevention and/or treatment of vector-induced transaminitis is critical to avoid treatment failure and requires strict regular assessments one to three times per week for the initial 6–9 months after vector infusion, depending on the individual vector product. Long-term follow-up should evaluate for known and unknown outcomes, including liver health, provide comprehensive care for persistent, pre-existing hemophilia-related complications and provide ongoing psychosocial support to the patient whose hemophilia-related medical needs and life experiences have now been altered.

Health outcomes are important to assess on a regular basis throughout the process. The data generated will inform about safety and efficacy of gene therapy in the local setting and support further establishment of this novel treatment. At the same time, the monitoring of the methods and outcomes of implementation of this new treatment will inform necessary improvements and guide other centers in earlier stages. Specifically, the operational processes of vector management including shipping, storage, and reconstitution, the procedure of vector infusion, and the efficiency of follow-up monitoring and the compliance with these processes are important to be scrutinized for the patients to have a successful health outcome. Collecting and analyzing data on real-life cost and requirements for infrastructure and funding in each socioeconomic setting will be of great importance to develop a sustainable therapeutic offer.

Summary and Conclusion

Gene therapy has made great strides and has the potential to revolutionize treatment and transform the lives of a vast number of patients affected by hemophilia as well as other genetic diseases, through introducing a normal copy of the defective gene to restore its function for a prolonged time such as years or potentially decades.^{18,28,90} Current evidence indicates that eligible PWH can achieve a state of mild hemophilia or a normal clotting state for at least 5–8 years, if not longer, after a single-gene therapy infusion. For patients worldwide to realize the full potential of gene therapy, we have summarized the challenges needing to be addressed at the current stage of development. Specifically, relevant clinical, educational, regulatory, and financial aspects need to be tackled and at times reinvented to cultivate a broadly accessible landscape for this novel treatment technology, dissimilar to any previous one. Particularly, PWH in countries with limited access to current standard hemophilia treatments have an opportunity for significant improvements in mortality and morbidity. This review points to the directions to take for effective, international capacity building by all stakeholders to work and collaborate towards improved health equity for patients with hemophilia.

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Association of ABO and Rhesus Blood Types with Transfusion-Transmitted Infections (TTIs) Among Apparently Healthy Blood Donors at Bahir Dar Blood Bank, Bahir Dar, North West, Ethiopia: A Retrospective Cross-Sectional Study

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Background: Besides their clinical significance in blood transfusion medicine, ABO and Rh blood group antigens were found to be associated with many non-infectious and infectious diseases. This investigation aimed to assess the association of ABO and Rh blood group antigens with transfusion transmissible infections (TTIs).

Methods and Materials: A cross-sectional retrospective investigation was conducted on 27,027 blood donors at Bahir Dar blood bank, from March 24/2019 to October 21/2021. The blood sample was collected from each blood donor and tested for TTI markers (HBV, HCV, HIV, and syphilis), with ELISA, and ABO and Rh blood grouping was performed. Descriptive analysis was done for sociodemographic data, and a chi-square test was used to show the association between the ABO and Rh blood groups with TTI markers, and a P-value <0.05 was considered statistically significant.

Results: From 27,027 study participants, 18,911 (70%) were males, with a mean age of 25.2 years, and 49.4% of the blood donors were students. The overall TTI prevalence was 5.43%, of which 2.8% was HBV, 1.5% was syphilis, 0.8% was HIV, and 0.3% was HCV. Blood group O (41.4%) was the most common blood group followed by, A (29.6%), B (23.6%), and AB (5.4%). Ninety-one point seven percent of the blood donors were Rh (D) positive. All TTI markers (HBV: $p = 0.62$, HIV: $p = 0.77$, HCV: $p = 0.52$, and syphilis: $p = 0.94$) showed no significant association with ABO blood group. Rh blood type also showed no association with all TTI markers.

Conclusion: The prevalence of TTI markers was not significantly associated with ABO and Rh blood groups.

Keywords: ABO and Rh blood groups, blood donors, transfusion transmissible infections

Introduction

The surface of red blood cells (RBCs) contains different blood group antigens made up of polysaccharides and proteins. Along with their expression on red blood cells, ABO blood group antigens are also found on other cells and tissues like epithelium, sensory neurons, platelets, the vascular endothelium, breast ductal and lobular cells, and some malignant cells.¹ Among the blood group antigens identified, ABO and Rhesus (Rh) are the most widely studied. Besides their clinical significance in the blood transfusion and compatibility, ABO and Rh blood group antigens were found to be associated with many non-infectious and infectious diseases.^{2,3} Several studies revealed these blood group antigens were associated with diabetes mellitus, hypertension, gastric cancer, duodenal ulcer, breast cancer, malaria, and helicobacter pylori infection.^{2,4,5} Some other studies also reported that ABO and Rh blood group antigens were associated with

transfusion-transmitted infections (TTIs), which include Hepatitis B virus (HBV), human immunodeficiency virus (HIV), Hepatitis C virus (HCV), and syphilis.^{2,6,7}

Nowadays, the prevalence of TTI is increasing, especially in the developing world. This high prevalence of TTI incurs increased morbidity, mortality, and compromises the quality of life of the world's population. The acquisition of TTIs has become a vital obstacle, worthy of investigation, to maintain both the health and safety of human life.⁸

In Malawi, the prevalence of HBV, HCV, HIV, Syphilis was 3.6%, 1%, 1.9%, 2.6%, respectively, in 2015.⁹ A study conducted in Angola also reported even a higher TTI prevalence in which HBV (8.4%) infection covered a larger proportion, followed by syphilis (4.4%), HCV (3%) and HIV (2.1%).^{9,10} In Eritrea, the prevalence of HIV, HBV, HCV and Syphilis was 0.8%, 5%, 0.7% and 7.2%, respectively, and in Ethiopia, the overall prevalence of TTI was 6% (HBV = 3.9%, HCV = 0.6%, HIV = 0.5% and syphilis = 1.2%) in 2019.^{11,12}

Considering this high prevalence, the World Health Organization (WHO) recommended that, the blood and blood products collected for transfusion should be screened for HIV, HBV, HCV, and syphilis as a minimum requirement.¹³ Additionally, strict pre-donation screening and deferral of blood donors with high-risk behaviors is required to ensure safe blood and blood product.

The route of transmission of TTIs is principally through blood and blood products, sharing of needles to inject drugs, needle stick injury, and sexual contact. After their entrance into the host different factors like immune status of the host, load of the infectious agent, and route of transmission contributed to the development of an active infection. In addition to these factors, several studies also disclosed that ABO and Rh blood group antigens were associated with the development of active infection.^{14,15} The probable pathogenesis for the link between blood group antigens and disease susceptibility is that many organisms may bind to polysaccharides on the cell surface, but soluble blood group antigens may block this binding. On the other hand, different ABO antigens can prevent the species carrying it from being threatened by a pathogen using a given carbohydrate as a receptor. On the other saying, the ABO polymorphism leads to a polymorphic production of anti-A and anti-B natural antibodies, which potentially protect individuals from several infectious agents expressing A and B antigens.¹⁶

Several studies were conducted to show the possible link between ABO and Rh blood groups with TTI. For instance, in a study conducted by Tyagi et al, blood group A negative was associated with an increased risk of contracting HIV and HBV infection, while blood group B negative with syphilis.¹⁵ Another study also found maximum TTI sero-reactivity among B blood group donors (2.21%) followed by blood group A donors (2.16%), O donors (1.57%), and AB donors (1.17%), but was not statistically significant.¹⁷ In a study conducted in Gabon, no significant association was obtained between the prevalence of HIV, HCV, and syphilis and the ABO and Rh blood groups. However, HBV sero-prevalence was twice as high among non-O blood group donors compared with blood group O donors.⁷ A systematic review and meta-analysis study by Jing et al, indicated that blood group B was associated with a lower risk of HBV infection.¹⁴

Despite the presence of several studies on this project, the findings were not consistent, and as to our understanding, there was no such study conducted in Ethiopia. The lack of such information has initiated us to conduct this investigation. The main objective of this study was to assess the association between transfusion-transmitted infections and the ABO and Rh blood groups. The information obtained will be used by the governmental agencies to set firm blood donor selection criteria, and blood banks to pay attention when they enrolled their blood donors.

Methods and Materials

A cross-sectional retrospective investigation was conducted on 27,027 blood donors at Bahir Dar blood bank from March 24/2019 to October 21/2021. Bahir Dar blood bank is located in the capital city of Amhara regional state Bahir Dar, Ethiopia. Bahir Dar is found in northwest Ethiopia 565 km away from Addis Ababa, the capital city of Ethiopia.

All blood donors, included in our analysis, were voluntary non-remunerated blood donors. Five milliliters of blood samples were collected from all blood donors, from which tests for TTI markers (HBV, HCV, HIV, and syphilis) and ABO and Rh blood grouping were performed. The serum sample from each blood donor was tested with an enzyme-linked immunosorbent assay (ELISA). HBV surface antigen (HBsAg) and anti HCV antibody (anti-HCV) were tested with third-generation ELISA (wantai, Beijing Wantai Biological Pharmacy, China), HIV 1 and 2 antibodies and antigens

were tested with fourth-generation ELISA (Microlisa, J. Mitra and Co. Pvt. Ltd, India), and anti-Treponema pallidum antibodies were tested with DIALAB Syphilis IgG/IgM ELISA (Dia lab, Austria).

Forward and reverse ABO grouping was performed by slide method with commercially available antisera (anti-A and anti-B) (Mediclone, Mediclone Biotech, India) and by using 5% A and B cell suspension prepared at home, respectively. Rh typing was performed by test tube method with anti-D reagents, and Coombs test was performed to detect weak D antigen (Mediclone, Mediclone Biotech, India).

All donors' data including relevant socio-demographic, TTI marker result, ABO and Rh blood type were registered on the computerized blood donor data management system. Ethical clearance was secured from research ethics committee of Injibara University College of Health Sciences with reference number HSc-350. The data were collected after getting a seal of approval from Bahir Dar blood bank. The data were checked for completeness, and transferred to SPSS version 23 for further analysis.

Descriptive analysis was done for sociodemographic data, and a chi-square test was used to show the correlation between the ABO and Rh blood groups with TTI markers, and a P-value <0.05 was considered statistically significant. Bonferroni post hoc test was performed to detect which cells from the contingency table were 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 occupation 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 accessible only to the investigators.

Results

Of 27,027 study participants, 70% were males, and 58.5% were within 18–24 years age range (Table 1). The minimum and maximum ages were 18 and 67 years, with a mean age of 25.2 years, and 49.4% of the blood donors were students (Table 2). Blood group O (41.4%) was the most prevalent blood group followed by A (29.6%), B (23.6%), and AB (5.4%). Ninety-one point-seven percent of the blood donors were Rh (D) positive (Table 3). Of 27,027 blood donors, 1469 (5.4%) were positive at least for one of the TTI markers, from which HBV accounts 2.8% (767), syphilis 1.5% (402), HIV 0.8% (207) and HCV 0.3% (93) (Table 4).

Association of ABO and Rh Blood Group with TTI Markers

In this study, out of the 767 HBV positive cases, O (41.6%) was the most frequent blood group followed by A (29%), B (23%), and AB (6.4%). Among 207 HIV positive cases, blood group O (42.5%) blood donors were the most predominant succeeded by blood group A (27.5%), B (25.6%), and AB (4.4%). Of 93 HCV positive individuals, those with blood group O (46.2) were predominant followed by A (31.2%), B (17.2%), and AB (5.4%), and among 402 Syphilis reactive donors O (42.3%) blood group was predominant, followed by A (28.4), B (24.1%), AB (5.2%) blood group individuals. In HBV, syphilis, HIV, and HCV reactive case, 9.1%, 6.9%, 7.7%, and 11.8%, respectively, were Rh-negative (Table %).

Even though it was not statistically significant ($\chi^2 = 1.78$, $p = 0.62$), this study revealed the highest prevalence of HBV case in blood group AB. Blood groups B and O were found to have equally higher prevalence of HIV ($\chi^2 = 1.12$, $p = 0.77$). Relatively higher prevalence of HCV was seen in A and O blood groups ($\chi^2 = 2.24$, $p = 0.52$), but B and O blood groups were considered as having equally higher prevalence of syphilis ($\chi^2 = 0.37$, $p = 0.94$). Higher prevalence of HBV

Table 1 Age and Sex Distribution of Study Participants

Sex	Age Range						Total
	18–24	25–29	30–34	35–39	40–44	≥45	
Female	6442	871	337	227	123	116	8116 (30%)
Male	9377	3647	2951	1475	739	722	18,911 (70%)
Total	15,819 (58.5%)	4518 (16.7%)	3288 (12.2%)	1702 (6.3%)	862 (3.2%)	838 (3.1%)	27,027 (100%)

Table 2 Occupational Distribution of Study Participants

Occupation	Frequency	%
Civil servant	5658	20.9
Private worker	5171	19.1
Student	13,361	49.4
Unemployed	2128	7.9
Others	709	2.6
Total	27,027	100

Table 3 ABO and Rh Blood Group Distribution of Study Participants

Rh	ABO				Total
	A	AB	B	O	
Negative	695 (2.6%)	126 (0.5%)	478 (1.8%)	941 (3.5%)	2240 (8.3%)
Positive	7315 (27.1%)	1324 (4.9%)	5908 (21.9%)	10,240 (37.9%)	24,787 (91.7%)
Total	8010 (29.6%)	1450 (5.4%)	6386 (23.6%)	11,181 (41.4%)	27,027 (100.0%)

and HCV were seen among Rh-negative blood donors, while HIV and syphilis positivity rate was higher among Rh-positive blood group donors (Table 4).

Discussion

The relationship between ABO and Rh blood groups and numerous diseases has been investigated extensively by different scholars. The current study was conducted to show the association between TTIs (HBV, HCV, HIV, and syphilis) and ABO and Rh blood group antigens. On the current topic, several studies have been conducted, some of them reported a link between ABO and Rh blood types and TTIs,^{2,14} while others testified no significant association.^{17,18} In the current study the TTI prevalence was 5.4%, with HBV (2.8%) being the most prevalent, followed by Syphilis (1.5%), HIV (0.8%) and HCV (0.3%). Our finding was comparable with the output of studies conducted by Shiferaw et al,¹¹ Biadgo et al¹⁹ and Arif et al⁶ but relatively lower than the prevalence reported by Peliganga et al¹⁰ and higher than that of Prakash et al¹⁷ and Hroob et al.⁸ The observed difference in the prevalence of TTI markers may be due to differences in lifestyle and economic status of the study participants.

Table 4 Association of ABO and Rh Blood Group with TTI Markers

ABO	HBV		HCV		HIV		Syphilis	
	Negative (n, %)	Positive (n, %)	Negative (n, %)	Positive (n, %)	Negative (n, %)	Positive (n, %)	Negative (n, %)	Positive (n, %)
A	7788 (97.2)	222 (2.8)	7981 (99.6)	29 (0.4)	7953 (99.3)	57 (0.7)	7896 (98.6)	114 (1.4)
AB	1401 (96.6)	49 (3.4)	1445 (99.7)	5 (0.3)	1441 (99.4)	9 (0.6)	1429 (98.6)	21 (1.4)
B	6209 (97.2)	177 (2.8)	6370 (99.7)	16 (0.3)	6333 (99.2)	53 (0.8)	6289 (98.5)	97 (1.5)
O	10862 (97.1)	319 (2.9)	11,138 (99.6)	43 (0.4)	11,093 (99.2)	88 (0.8)	11,011 (98.5)	170 (1.5)

(Continued)

Table 4 (Continued).

ABO	HBV		HCV		HIV		Syphilis	
	Negative (n, %)	Positive (n, %)	Negative (n, %)	Positive (n, %)	Negative (n, %)	Positive (n, %)	Negative (n, %)	Positive (n, %)
Total	26,260 (97.2)	767 (2.8)	26,934 (99.7)	93 (0.3)	26,820 (99.2)	207 (0.8%)	26,625 (98.5)	402 (1.5)
	$\chi^2 = 1.78, p = 0.62$		$\chi^2 = 2.24, p = 0.52$		$\chi^2 = 1.12, p = 0.77$		$\chi^2 = 0.37, p = 0.94$	
Rh								
Positive	24,090 (97.2)	697 (2.8)	24,705 (99.7)	82 (0.3)	24,596 (99.2)	191 (0.8)	24,413 (98.5)	374 (1.5)
Negative	2170 (96.9)	70 (3.1)	2229 (99.5)	11 (0.5)	2224 (99.3)	16 (0.7)	2212 (98.9)	28 (1.1)
Total	26,260 (97.2)	767 (2.8)	26,934 (99.7)	93 (0.3)	26,820 (99.2)	207 (0.8)	26,625 (98.5)	402 (1.5)
	$\chi^2 = 0.73, p = 0.39$				$\chi^2 = 0.086, p = 0.77$		$\chi^2 = 0.94, p = 0.33$	

Abbreviations: HBV, hepatitis B virus; HIV, human immunodeficiency virus; HCV, hepatitis C virus.

In this study, even though it was not significant ($p=0.62$), the highest prevalence of HBV was found in blood group AB, whereas the lowest positivity rate was in blood groups A and B. Similarly, studies conducted by Prakash et al¹⁷ and Sreedhar Babu et al¹⁸ revealed no association between TTI markers and ABO blood groups. However, a study carried out by Liu et al² revealed the presence of a significant association between ABO blood group and the risk of TTI, in which blood group O was associated with an increased HBV infection rate. In the current investigation, blood groups B and O were found to have the highest prevalence of HIV and syphilis, but the association was not statistically significant. This finding was in concordance with a study conducted by Ngassaki-Yoka et al,⁷ which detected no association between ABO blood group and TTIs. Even though it was not significant, a comparatively higher frequency of HCV positivity was seen among A and O blood group donors. This discrepancy might be caused by characteristics of the study participants and the differences in blood type frequency distribution in different ethnic groups.²

This study also testified that Rh (D) type was not associated with all TTI markers' positivity rate. In line with our finding, investigations done by Bharadva et al²⁰ and Sreedhar Babu et al¹⁸ found no association, but the existence of a significant association was reported by Liu et al,² in which Rh-D-positive participants were at higher risk of HBV infection.

Conclusion and Recommendation

The main aim of this research work was to show the prevalence of TTI (HBV, HCV, HIV, and syphilis) and its association with ABO and Rh blood groups. According to our findings, the prevalence of TTI was not significantly associated with ABO and Rh blood group phenotypes.

Data Sharing Statement

All relevant data are included in this document.

Ethical Considerations

Ethical clearance was obtained from research ethics committee of Injibara University College of Health Sciences with reference number HSc-350. A permission letter was obtained from Bahir Dar blood bank. Since it was a retrospective analysis, written informed consent was not obtained from each study participant. To safeguard confidentiality, all blood donor data were collected after removing all personal identifiers by the blood bank data manager.

Consent for Publication

Consent for publication of this work was found from Bahir Dar blood bank.

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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 declared that no competing interests exist.

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Hemophilic Arthropathy: Barriers to Early Diagnosis and Management

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Abstract: Hemophilia is a congenital coagulopathy characterized by a deficiency of one of the clotting factors. It is characterized by the development of hematomas and hemarthrosis, either spontaneously or after minor trauma. The recurrence of hemarthroses leads to progressive and degenerative joint damage from childhood (hemophilic arthropathy). This arthropathy is characterized by disabling physical effects that limit the functionality and quality of life of these patients. Medical progress achieved over the last decade in the drug treatment of hemophilia has improved the medium and long-term prospects of patients with more effective and long-lasting drugs. The universal use of safer, more effective and prolonged prophylactic treatments may promote the prevention of bleeding, and also therefore, of the development of hemarthrosis and joint damage. A number of imaging instruments have been developed for the assessment of hemarthrosis and hemophilic arthropathy, using ultrasound, magnetic resonance imaging and simple radiology. Different physical examination scores and questionnaires allow the assessment of joint health, self-perceived activity and functionality of patients with hemophilia. The approach to these patients should be interdisciplinary. Assessment of the processes that affect pain in these patients and the development of pain education models should be implemented. Expert advice and information to patients with hemophilia should be based on individual functional prevention diagnoses, advice on available therapies and sports practice, as well as health recommendations.

Keywords: hemophilia, joint disease, pain, diagnosis, management

Hemophilia. Overview and Clinical Manifestations

Hemophilia is a genetic bleeding disorder caused by a missing or defective clotting factor.¹ Hemophilia is usually inherited via an affected X chromosome with a mutation of the FVIII or FIX gene. However, both genes are prone to new mutations and about 30% of all cases result from spontaneous genetic variants. Prospective studies have shown that more than 50% of newly diagnosed individuals with severe hemophilia have no family history of the disease.² Depending on the missing clotting factor, it can be classified hemophilia A (factor VIII) or hemophilia B (factor IX). Both types of hemophilia can be classified according to the plasma level of existing clotting factor: mild (5–40%), moderate (1–5%) or severe (<1%).³ The severity of bleeding in patients with hemophilia is proportional to the amount of residual factor activity in the blood. In patients with severe hemophilia, bleeding may occur spontaneously or with minor trauma. In patients with moderate or mild hemophilia, bleeding episodes occur after minor surgical interventions, requiring immediate treatment to prevent complications.⁴

Hemophilia is considered a rare disease. The estimated prevalence for hemophilia A is 24.6:100,000 live births, and 5.0:100,000 live births for hemophilia B.⁵

Uncontrolled bleeding in critical organs can lead to death in patients with hemophilia. However, the main clinical manifestation is the development of hemarthrosis. These intra-articular bleeds are more common in knees, ankles and elbows.¹ Similarly, the risk of hemarthrosis increases in cases of severe hemophilia with minimal trauma.⁶

Repeated bleeding in the same joint eventually leads to synovial hypertrophy, hemosiderin deposits, cartilage destruction and changes in the structure of the subchondral bone. Such hemosiderin deposits can stimulate further synovial hypertrophy and swelling.⁷ Several in vitro and in vivo animal studies have shown how blood has a direct effect on cartilage due to the combination of mononuclear cells and red blood cells present in the blood.⁸ The development of hemarthrosis induces chondrocyte apoptosis, which alters the cartilage matrix renewal. Hypervascularization occurs on the synovial tissue, initially synoviocyte hypertrophy, increasing the risk of new bleeding events and inflammatory changes.⁹ These inflammatory changes contribute to cartilage damage through the production of tissue-destroying enzymes and cytokines¹⁰ leading to joint destruction. A recent study by Zheng et al¹¹ has shown how iron is involved in cartilage injury through regulation of FGF23 and SOX9 expression in chondrocytes in patients with hemophilia who have developed hemarthrosis. A high level of COL-18N has been noted in patients with hemophilia A¹², which could be used as a potential marker to monitor the development of arthropathy, making it possible to adapt the best treatment to prevent further joint damage. The recurrence of hemarthrosis leads to the development of a degenerative and irreversible process known as hemophilic arthropathy.^{1,13} As a result, hemophilic arthropathy is accompanied by pain, loss of joint range and muscle atrophy, among other alterations.¹ Figure 1 shows the joint damage process induced by the development of hemarthrosis.

The *gold standard* in the prevention of bleeding and hemophilic arthropathy is the intravenous prophylactic administration of the missing clotting factor.¹ However, most patients with hemophilia worldwide can only access on-demand pharmacological treatment once bleeding has happened, with the consequent risk of joint damage.³ The main complication related to the treatment of hemophilia is the development of antibodies to clotting factor concentrates. These inhibitors react against the administered substitution factor, increasing the risk of serious bleeding and accelerating the degenerative damage caused by the recurrence of hemarthrosis.¹⁴

Prophylactic regimens are customized depending on the hemorrhagic phenotype, the pharmacokinetics of FVIII/FIX or the physical activity performed by the patient.¹⁵ However, the short half-life of these factor VIII/FIX concentrates implies the need for frequent infusions. In addition, the limitations offered by the minimum blood levels of FVIII/FIX do not ensure complete protection against the development of hemarthrosis in the event of moderate-intensity trauma.¹⁶

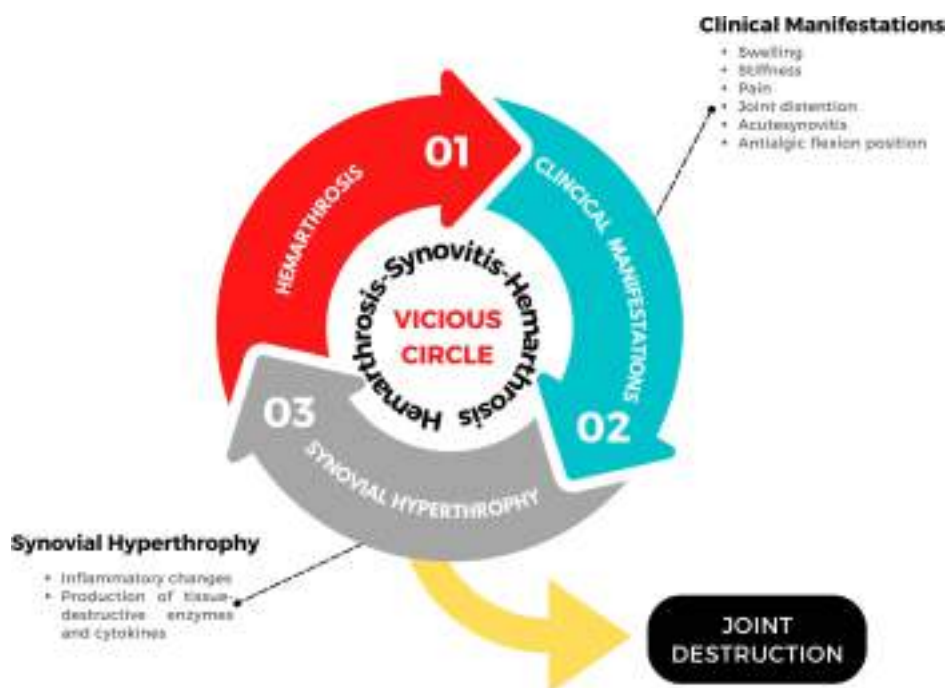


Figure 1 The hemarthrosis-synovitis-hemarthrosis vicious circle in hemophilia.

In the last ten years, new FVIII/FIX concentrates have been designed to prolong their half-life: extended half-life treatment (EHL).¹⁷ This extended half-life can reduce the frequency of intravenous infusions by 30–35%,¹⁸ maintaining a higher minimum level of FVIII (3%) than with conventional products.¹⁹ Similarly, in patients with hemophilia B, a considerable increase in the terminal half-life has been described in pediatric subjects (median: 36.9 h) and over 13 years of age (median: 49.9 h).²⁰

Search Strategy

Clinical trials were searched in various electronic databases: *PubMed/MEDLINE*, *Web of Science*, *Embase*, and the Cochrane Central Register of Controlled Trials (CENTRAL). For the drafting of the review, articles were extracted using the following keywords and search strategies: (hemophilia OR haemophilia), (joint disease OR haemophilic arthropathy), (pain OR pain management), and (diagnosis OR evaluation OR approach). Boolean operators (AND, OR, NOT) were used in the search.

Limitations in the Physical and Functional Approach and Evaluation in Patients with Hemophilia

The clinical symptoms of acute haemarthrosis are characterized by a rapid swelling where patients usually notice initial symptoms of stiffness, tingling and pain.²¹ Their physical examination reveals typical signs of swelling, hotness, redness of the surrounding skin, joint strain, muscle contracture and limitation of mobility.²² As a result of the hemorrhagic process, the joint becomes positioned in an antalgic flexion position, compensating for intra-articular pressure to reduce pain. Acute hemarthrosis do not usually cause secondary changes in the synovial cells, so limitations do not usually persist and the joint returns to its normal state.²³

Considering the hemarthrosis recurrence pattern in patients with hemophilia, hemarthrosis tends to become chronic, generating chronic synovitis, inflammatory arthritis and progressive hemophilic arthropathy.²⁴ This arthropathy is the cause of the progressive development of chronic pain, limitations in the range of motion and functional alterations causing disability in patients with hemophilia.²⁵ Due to the serious sequelae of hemarthrosis in patients with hemophilia, early diagnosis and treatment is essential to minimize the evolution of arthropathy, with the functional and physical limitations that this process entails. Hence the importance of an early diagnosis and treatment in order to minimize the degenerative evolution to hemophilic arthropathy with the functional and physical limitations that such a process would entail.²⁶

The World Federation of Hemophilia has been recommending for years the assessment of the degree of joint injury using radiological scoring systems and orthopedic exploration.²⁷ The use of diagnostic imaging techniques makes it possible to classify the joint degenerative process into different clinical and pathological stages.²⁸ The aim is to detect joint changes, determine their severity and evaluate the follow-up of therapeutic effects. The most commonly used techniques are ultrasound, simple radiology and magnetic resonance imaging (MRI).

Ultrasound is an inexpensive, easily available and excellent technique for determining the presence of soft tissue inflammation. In the same way, it allows detecting intra-articular fluid and evaluating the cartilage, being a highly useful tool for clinical follow-up and adapting treatments for bleeding and synovitis. Musculoskeletal ultrasound has shown high sensitivity in detecting low concentrations of intra-articular blood and in discriminating between bloody and non-bloody fluids. Thus, ultrasound is an ideal tool for the rapid detection of joint hemorrhage.²⁹ Martinoli et al³⁰ designed a simplified scoring system (HEAD-US) based on an additive scale to define the joint condition and offer a tool to assess the progression of the disease and monitor the outcome of treatment in follow-up studies. It has three markers for the main joints affected (knees, elbows and ankles): synovitis (score 0–2), cartilage (score 0–4) and subchondral bone (score 0–2), with a maximum score of 8 points per joint; the higher the score the more evolved the degenerative joint process. The literature has shown the importance of ultrasound in the early detection of arthropathic changes and its contribution to the clinical approach in patients with hemophilia.³¹ An association between the Joint tissue Activity and Damage Exam protocol and clinical and functional parameters has been observed,³² supporting the clinical value of musculoskeletal ultrasound as a complementary tool for the diagnosis and treatment of hemophilic arthropathy.

Simple radiology remains one of the most widely used methods for joint evaluation in hemophilic arthropathy, despite its limitations.³³ Its main disadvantage is that in the initial stages it underestimates the degree of joint pathology and alterations in soft parts such as synovitis due to its low sensitivity for detecting problems in the synovial membrane and cartilage. Simple radiology should not be the method of choice for early assessment of the disease or to determine the evolution of a short-term treatment.³⁴ Its main utility lies in the evaluation of adult patients with hemophilia who present with advanced joint pathology.³⁵ The scoring system recommended by the WFH is the Pettersson system. It assesses 8 parameters with a maximum score of 13 points; each radiological change is assigned a score according to its severity.³⁶ The higher the score, the poorer the joint condition.

MRI is the *gold standard* instrument for the study of joint changes in patients with hemophilia.³⁷ It has been shown to be the most useful tool in the analysis of soft tissues and osteochondral alterations, present in the initial stages of hemophilic arthropathy. However, its high costs, its lower accessibility, the time required for its evaluation and its difficult applicability in children, limit its use and periodic follow-ups to determine the evolution of acute and chronic processes.²⁶ The most widely accepted methods proposed for assessing joint damage with magnetic resonance imaging include the Denver scale³⁸ and the European scale.³⁹

The Denver group created a 5-phase joint evaluation scale assessing the presence of effusion or hemarthrosis, synovial hypertrophy with hemosiderin deposits, presence of cysts, bone erosions and cartilage destruction with joint impingement. The score is determined on the basis of the most significant observable deterioration with a range of 0 to 10 points. For its part, the European classification collects information on similar criteria with a more detailed reading procedure. The evaluation criteria are the existence or not of subchondral cysts, erosion, chondral lesion, joint effusion or hemarthrosis, synovial hypertrophy and hemosiderin deposition. This additive scale has a range of 0 to 28 points where the higher the score, the greater the joint destruction.

In relation to the diagnosis of the degree of joint injury by orthopedic examination, the World Federation of Hemophilia considers the use of different tools.

The *Hemophilia Joint Health Score* (HJHS) version 2.1, evaluates the joint condition of knees, ankles and elbows, in patients with hemophilia. This scale was designed to assess joint deterioration in pediatric patients with hemophilia,⁴⁰ although a study has recently been published that shows its validity in the adult population.⁴¹ This measuring instrument measures 8 items: *Swelling, Duration of swelling, Muscle Atrophy and Strength, Crepitus, Mobility and Joint Pain*. The scoring range of this additive scale is 0–20 points in each joint.

The Gilbert screening protocol⁴² is a tool recommended by the World Federation of Hemophilia for the measurement of joint deterioration. This protocol consists of 7 items linked to anatomical destruction, biomechanical alterations and deformity. Each item has a value of 0, 1 or 2 points, so that the final value is presented in a range between 0 and 12 points, in knee and ankle, and from 0 to 10 in elbow, a higher score indicating greater joint deterioration. Among its limitations we find that it may not be appropriate in the assessment of young children and in mild arthropathies as it is not very sensitive to slight joint changes.⁴³

The *Hemophilia Activities List* (HAL) is a questionnaire used for the measurement of participation and performance in daily activities of patients with hemophilia.^{44,45} With this instrument, the impact of the disease on self-perceived functional abilities in patients with hemophilia is evaluated, through 42 items grouped into seven domains: *Lying down, sitting, kneeling and standing; Functions of the legs; Functions of the arms; Use of transportation; Self-care; Household tasks; and Leisure activities and sports*. This scale has a score range from 0 to 100 points, where a low score indicates a greater impact of hemophilia on self-perceived functional abilities.

The *Functional Independence Score in Hemophilia* (FISH)⁴⁶ is a questionnaire developed to measure the degree of disability in patients with hemophilia with three categories: *Self-care* (assesses the ability to perform basic activities of daily living); *Transfers* (assesses the ability to sit and stand); and *Mobility* (walking and step climbing). Eight activities (*Eating; Dressing; Dressing; Transferring from chair; Squatting; Walking; Climbing stairs; and Running*) are measured and scored from 1 to 4 with a maximum score of 32 points. The purpose of this instrument is to measure what the person with hemophilia can actually do, and not what he or she should be able to do or might be able to do if the circumstances were different. It is also used to evaluate the change or evolution of these patients over time or after a therapeutic intervention.

ASSESSMENT	MEASURE (SCORE)	FEATURES	ITEMS	SCORE
IMAGING TECHNIQUES	MAGNETIC RESONANCE (DENVER SCALE)	GOLD STANDARD TO ASSESS JOINT DAMAGE	5	0-10
	MAGNETIC RESONANCE (EUROPEAN SCALE)	GOLD STANDARD TO ASSESS JOINT DAMAGE	6	0-28
	ULTRASOUND (HEAR-UI)	INDICATED FOR ASSESSMENT AND CONTROL OF THE JOINT DEGENERATIVE PROCESS	4	0-8
	WASH-QUEST (PETERSSON SCORE)	INDICATED FOR MONITORING ADVANCED HEMOPHILIC ARTHROPATHY	8	0-13
PHYSICAL/ FUNCTIONAL	HEMOPHILIA JOINT HEALTH SCORE (HJHS)	INDICATES FOR ASSESSMENT JOINT STATES	8	0-124
	SILBERT SCORE	INDICATES FOR MONITORING JOINT HEALTH (IMPAIRMENT)	7	0-12
	HEMOPHILIA ACTIVITIES LIST (HAA)	SELF-PERCEIVED FUNCTIONALITY	42	0-100
	FUNCTIONAL INDEPENDENCE SCORE IN HEMOPHILIA (FISH)	DEGREE OF DISABILITY	7	7-28
	HAEMOPHILIA & EXERCISE PROJECT TEST QUESTIONNAIRE (HEP-TEST-Q)	SUBJECTIVE PHYSICAL PERFORMANCE	32	0-100

Figure 2 Measuring instruments used in the radiological and physical assessment of patients with hemophilia.

For its part, the *Haemophilia & Exercise Project Test Questionnaire* (HEP-Test-Q) is a measuring instrument that evaluates subjective physical performance.⁴⁷ This Likert-like scale has five domains: *Physical condition* (comparing current physical activity to a year earlier), *Mobility*, *Strength and coordination*, *Resistance* and *Body perception*.

Figure 2 shows the main characteristics of the radiological and physical measuring instruments used in patients with hemophilia.

Evaluation and Approach of Pain in Patients with Hemophilia

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with or similar to that associated with actual or potential tissue damage.⁴⁸ Pain is recognized as a personal experience, influenced by biological, psychological and social factors. In the same way, it identifies nociception and pain, and addresses the learning of pain through life experience.

With this concept in mind, we must consider how to assess pain in a patient with hemophilia and how to treat it. Acute and chronic pain in patients with hemophilia has been evaluated with self-reporting tools.⁴⁹ One-dimensional (analog or numerical) scales have been used to quantify the intensity of pain, and some non-specific multidimensional scales such as the *McGill Pain Questionnaire* or the *Brief Pain Inventory*.⁵⁰

Specific (*Hemophilia-Specific Quality of Life Index* [Haem-a-QoL])⁵¹ and generic scales (*36 and 12-Item Short Form Health Survey* [SF-36 and SF-12])⁵² have been used to assess the quality of life of these patients. Other scales used are those that evaluate determining psychosocial factors in the experience of pain, such as catastrophism (*Pain catastrophizing scale*), kinesiophobia or fear of movement (*Tampa Scale of Kinesiophobia*), or anxiety (*State-Trait Anxiety inventory questionnaire* [STAI]).⁵³

The *Multidimensional Hemophilia Pain Questionnaire* [MHPQ]⁵⁰ is a specific scale for patients with hemophilia, developed from a biopsychosocial perspective. This scale evaluates different pain dimensions (location, duration, frequency, trigger factors, intensity, interference in the patient's life, management strategies used by patients and by professionals, and satisfaction). Although this scale is very comprehensive, it does not assess other psychosocial aspects such as kinesiophobia or catastrophism.

The use of specific scales improves sensitivity with respect to the study population. However, they prevent comparing the situation of these patients with other populations with similar pathologies. This makes it difficult to find normative values in a population without pathology with which to compare results.

The use of these scales and questionnaires offer valuable information regarding the patient's painful experience and should be part of the assessment process. However, they do not report aspects such as the functioning of the nervous system or the processing of information whose output is the patient's pain. Neither do these scales assess the descending

inhibitory mechanisms of the patient. It would further be important to establish if there is nociceptive information that is being perceived and relevant to the brain and its bodily representation.⁵⁴

The literature discloses multiple studies focused on the evaluation and treatment of pain in patients with hemophilia.⁵⁵ By contrast, no evaluation models are proposed that assess the processes that influence pain. A number of clinical practice guidelines propose a treatment based on analgesics⁵⁶ understanding pain explained by its intensity, and not as an experience influenced by brain inputs and outputs. Although Auerswald et al⁵⁷ pointed out the absence of a multidisciplinary approach by pain management specialists, much progress is still to be made. Health professionals treating patients with hemophilia should receive education in pain and this could facilitate a transition from the obsolete Cartesian stimulus-response approach to a biopsychosocial model, a more popular approach to pain today.

Acute pain in hemophilia is associated with the development of joint bleeding, although imaging techniques have shown how it can develop in the absence of hemarthrosis.⁵⁸ Acute pain linked to hemarthrosis and, therefore, clearly having a nociceptive mechanism, is treated conservatively according to the RICE protocol (*Rest, Ice, Compression, Elevation*). This approach does not differ from that used in most acute injuries that involve bleeding or swelling. However, doubts have been raised on the use of ice due to its interference in the clotting process.⁵⁸ Along with the administration of clotting factor replacement, the administration of analgesic drugs (paracetamol, acetaminophen, COX-2 inhibitors) and, where indicated, arthrocentesis is recommended to reduce bleeding as soon as possible.⁵⁵

The pharmacological approach to chronic pain includes the use of antidepressants, antiepileptic drugs, NSAIDs, opioids and paracetamol.⁵⁵ Ultimately, with the progression of the joint degenerative process, orthopedic surgery and the use of orthoses are widely supported therapeutic proposals. The above is useful when there are movement-related nociceptive mechanisms involved in the patient's pain.

In terms of the physiotherapeutic approach, various therapeutic alternatives have been proposed. Passive and analgesic interventions, such as the use of laser, massage, heat or cold, have shown limited evidence in the approach to chronic pain.^{59,60} Approaches aimed at causing structural changes or addressing nociceptive or tissue mechanisms such as manual therapy or myofascial release have shown good results in the short and medium term. Similarly, interventions focused on tackling central mechanisms through educational programs, and the approach to the patient's descending inhibitory mechanisms through aerobic exercise, and strength or proprioception have demonstrated relevant results.⁶⁰

However, different clinical guidelines and publications stress on the pharmacological approach to pain in patients with hemophilia, using analgesics and anti-inflammatory drugs.⁵⁵ In the same way, physiotherapy is outlined as a therapeutic tool focusing only on passive techniques, centered on the patient as a passive target. This approach limits the active role of the patient as an active participant necessary to achieve changes in the process and improve his or her painful experience. It should not be forgotten that psychosocial factors have an impact on the painful experience of hemophilia patients.⁵³

If chronic pain is understood as a disease in itself, and as something dysfunctional that will affect people's lives,⁶¹ progress must be made in the treatment of patients with hemophilia. Future research should consider the treatment of chronic pain in patients with hemophilia accounting for the mechanisms involved in the patient's pain. A summary of the approach to pain in patients with hemophilia is shown in [Figure 3](#).

Quality of Life and Psychosocial Factors in Patients with Hemophilia

The World Health Organization⁶² defines quality of life as "an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns". In the same way as pharmacological treatment is needed, objective health measures are required in aspects such as the joint condition and the quality of life of hemophilia patients. In recent decades, the assessment of quality of life has increasingly focused on the inclusion of items and domains that assess the doctor-patient relationship, in order to quantify the impact of the disease and its treatment. Over time, generic and specific hemophilia questionnaires have been designed and validated, which measure the impact of the disease and treatment from the patient's perspective.⁶³

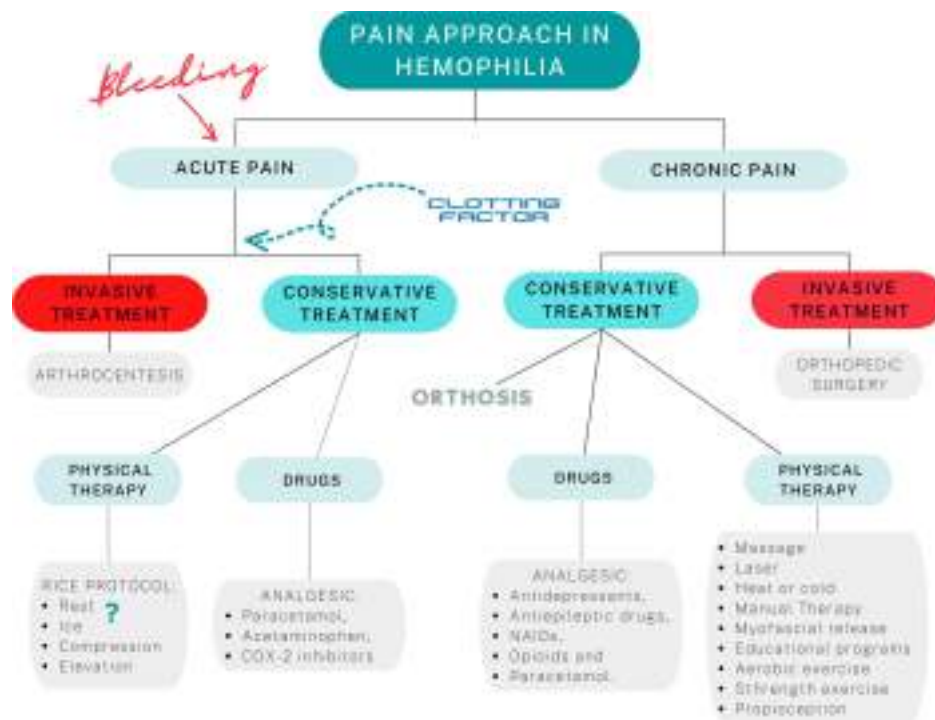


Figure 3 Multidisciplinary approach to pain in patients with hemophilia.

The psychosocial predictors of patients with hemophilia have a greater influence on the quality of life assessment than the clinical predictors. However, psychosocial treatment is deficient and poorly implemented, even in countries where pharmacological treatment is novel.⁶⁴

The severity phenotype of the pathology affects the quality of life of patients with hemophilia A and B, showing a poorer quality of life in patients with severe hemophilia, compared to those with mild or moderate phenotypes.⁶⁵ Patients with hemophilia who report pain have limitations in their mobility and autonomy, with increased anxiety, poorer quality of life and frustration due to restrictions in the performance of activities of daily living.⁶⁶ However, therapeutic advances in the approach to patients with hemophilia and the generalization of prophylactic treatment have allowed to improve life expectancy and health-related quality of life in these patients.⁶⁷

The development of hemophilic arthropathy and joint pain are predictors of a deteriorated perception of physical health⁶⁸ limiting the type of work and activities in these patients, especially when the lower limbs are involved, causing a high prevalence of mental health disorders.⁶⁹ The clinical situation and psychosocial variables affect the perceived quality of life of patients with hemophilia. The absence of a relationship between the patients' joint condition and their perceived quality of life prompts the need to investigate the effectiveness of interventions aimed at improving quality of life.⁷⁰

The literature that addresses other psychosocial factors such as catastrophism or kinesiophobia in patients with hemophilia is limited, thus limiting the knowledge of these factors and their possible approach. Kinesiophobia, catastrophism and anxiety are determining factors in the painful experience in patients with chronic pain and their approach may be key for the treatment of patients with hemophilia.⁵³ Therefore, addressing the psychosocial factors that affect pain should be a priority for the multidisciplinary team, to establish the association between intensity of pain and functionality.

New Perspectives in the Approach and Management of Patients with Hemophilia

The development of extended-life factors and strategies to replace the function of procoagulant factors through drugs administered subcutaneously, have meant a paradigm shift in the treatment of hemophilia. In the near future, gene therapy,

which has shown a long-lasting effect for increasing the levels of the missing factor, poses a favorable horizon towards the reduction and even elimination of hemorrhagic symptoms.⁷¹ Although gene therapy with adeno-associated vectors (AAV) has shown some extent of efficacy in patients with hemophilia A, it is somewhat limited due to AAV-induced cellular stress, immunogenicity and lower durability of gene expression. Recently, Kaminski et al⁷² linked altered liver-targeted gene transfer to maladaptive structural changes of the hepatic endothelium associated with FVIII deficiency in mice. Despite the limitations of the predictive value of preclinical immunogenicity testing, progress has been made in developing the principles of safe and effective genetic drug design in animal models.⁷³ In June 2022, the European Medicines Agency has recommended granting a conditional marketing authorization in the European Union for roxaparvec valoctocogene gene therapy for the treatment of severe hemophilia A in adults who do not have factor VIII inhibitors or antibodies against adeno-associated virus serotype 5 (AAV5). Several clinical trials are currently investigating the safety and efficacy of gene therapy in hemophilia A (NCT05454774) and hemophilia B (NCT05203679). These advances can change the natural history of the disease and, consequently, the clinical manifestations of hemophilia. This promising future in terms of the significant reduction of muscle and joint bleeding will result in the improvement of the perceived quality of life in these patients.

These changes in the medical approach mean that the future management of hemophilic arthropathy will likewise change radically. In many cases arthropathy development will be minor, if at all, being similar to that present in the general population. Therefore, the therapeutic possibilities are expected to be greater and more effective in pediatric and young patients in whom a deterioration of the locomotor system has not yet been established, and who will benefit from these new therapies.¹

Faced with this paradigm shift, future clinical strategies should be oriented in two directions: a) adult patients who are over the 30 years old and who have hemophilic arthropathy and, b) younger patients, beneficiaries of the new treatments and who have not developed arthropathy or to a minor extent.

In the case of patients with an already established arthropathy, we must refer to the existing scientific literature to follow the guidelines for safe and effective therapeutic programs. These should be aimed at controlling the symptomatology related to pain, joint mobility, decreased functionality and quality of life. The effectiveness of laser combined with exercise and hydrotherapy and dry exercise has been proven, in relation to the management of joint pain. Similarly, physical exercise can promote a reduction in pain perception, improving range of motion and muscle strength in patients with hemophilia.^{74,75} Other physiotherapy approaches, aimed at tackling tissue damage through myofascial therapy,^{76,77} joint traction⁷⁸ and home exercises.⁷⁵ They have also shown their safety and efficacy in decreasing the frequency of hemarthrosis, improving range of motion and perceived joint pain.

As noted for the evaluation and management of pain, the patient's psychosocial and individual aspects should give him or her an active role in the process of changing the painful experience.⁵³ This consideration, together with suitable education in pain based on neuroscience, offers a new therapeutic perspective in the management of chronic pain. In this way, the approach to hemophilic arthropathy would follow the assumptions already noted for other osteoarthritis processes.⁷⁹ Similarly, improvements have been identified in psychosocial variables related to pain catastrophizing and kinesiophobia.⁸⁰ García-Dasí et al⁸¹ observed an improvement in patients' pain after a cognitive behavioral therapy approach, although further studies are needed to provide more information on this type of approach.

In the younger population groups, linked to the new drug treatments, a preventive approach associated with suitable education and counseling for the patient and their family environment is proposed. Promoting healthy habits and physical activity in children with hemophilia is essential at each stage of development and growth. In the postnatal period, adequate psychomotor stimulation should be used to favor the child's motor and social skills. In preschool and childhood it is recommended to encourage play and exploration of activities that stimulate and promote movement. An interest in physical or sports activity is generated in school children, at which time the relevant decisions must be analyzed and made in relation to the type of activity to be carried out. In this section it is essential that the interdisciplinary team assesses to what extent the corresponding prophylactic measures favor the hematological coverage of these patients.

These approaches are of great importance. Patients with hemophilia show an increased risk of low bone mass during childhood, due to reduced physical activity and other factors such as increased urinary calcium loss and impaired bone

metabolism. These metabolic alterations predispose subjects to osteoporosis later on in life.⁸² This phenomenon, together with lower vitamin D levels compared to the general population,⁸³ is a risk factor in young people with hemophilia and may contribute to increased skeletal fragility.

A low level of physical activity is a risk factor for the reduction of lumbar and coxofemoral bone mass in people with hemophilia,⁸⁴ and can be prevented through physical activity from childhood.⁸⁵ In this population it is essential to educate in order to avoid sedentary lifestyle and obesity, which act as negative stimuli for the locomotor system. In many cases, this inactivity can be reinforced by the family environment through overprotective behaviors and hypervigilance. Physical activity is effective for improving the musculoskeletal clinical symptoms of hemophilia, with a low incidence of related adverse events. Similarly, physical activity can improve endogenous clotting factors after high-intensity activities that may reduce the risk of bleeding in patients with mild and moderate hemophilia,⁶⁰ as well as prothrombin time in adults with hemophilia,⁸⁶ with greater changes in those individuals presenting elevated prothrombin time during exercise.

Expert advice and information to patients with hemophilia should be based on individual functional prevention diagnoses, advice on available therapies and sports practice, and health recommendations. In this way, the safety and effectiveness of the activity and physical fitness of these patients can be improved, without increasing the bleeding rate and maintaining joint function.⁸⁷ It is recommended that such advice, the prescription of therapeutic exercise and the recommendation on sports activities, be guided by a physiotherapist of reference within the framework of the interdisciplinary group. However, it is essential that this measure be taken in consensus with the patient and his or her family. For this to be successful, it is essential to consider the specific features of the activity, offering all the available information to guarantee safety, and promote the prevention of exercise-related bleeding. This advice can be based on that guidelines provided by Bérubé et al⁸⁸ who pointed out that behaviors, attitudes and subjective rules present in the educational and formative processes of children largely explain the intention to engage in physical activity in the future. Figure 4 shows a summary of the new perspectives in the approach and management of patients with hemophilia.

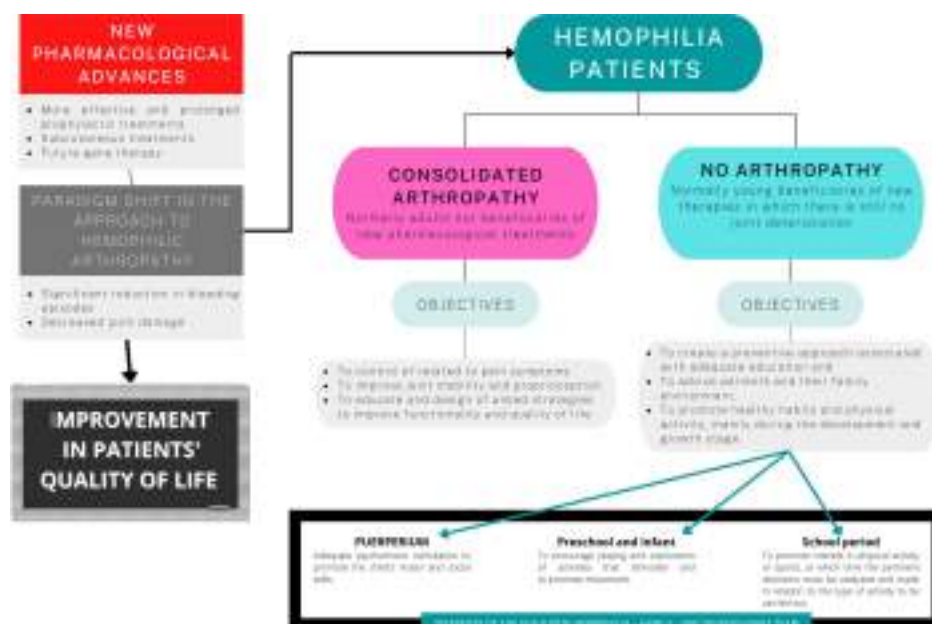


Figure 4 New perspectives in the approach and management of patients with hemophilia.

Conclusions

Progress made in the pharmacological treatment of patients with hemophilia has significantly improved the life prospects of these patients. The interdisciplinary approach should focus on the total prevention of hemarthrosis and of the development of hemophilic arthropathy. In children and adolescents, the chances of success in the prevention of joint damage is favored by recent medical progress. For patients with already established hemophilic arthropathy, it is necessary to change the therapeutic model, with the patients actively participating in their treatment. The adoption of an active role by patients can facilitate a better control of the disability associated with hemophilic arthropathy and their quality of life.

Data Sharing Statement

All data are available within the manuscript and may further be available upon reasonable request to the corresponding author.

Consent for Publication

All Authors approved the present submission.

Author Contributions

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

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A Comparison of the Knowledge, Attitude, Practice and Motivation Towards Blood Donation Among Albanian, Saudi and Turkish Citizens

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Background and Objectives: Blood donation is a remarkably safe medical procedure. However, it is affected by attitudes, beliefs, and knowledge level. A knowledge, attitude, and practice survey was carried out in Albania, KSA (Kingdom of Saudi Arabia) and Turkey to understand and identify: (1) the factors that influence blood donation, and stop people from blood donation, (2) the behavior and approach of the people towards blood donation (3) the knowledge level among donors and non-donors, and (4) the blood donation-related misconceptions.

Materials and Methods: This search was conducted through online surveys using Google Forms, which were shared through social media platforms, ie, Facebook, WhatsApp, Gmail, etc. A self-administered questionnaire form was shared to random people through social media platforms and 1281 responses were received.

Results: A total of 1281 forms were filled and submitted through Google Forms. Among these answers, 858 were from Albania, 273 were from KSA, 90 were from Turkey, and the remaining was from other countries. According to the answers, only 32% people were blood donors in Albania compared to 46% in KSA and Turkey. The factors primarily encouraging the people in Albania, Turkey, and KSA to donate blood included intention to help others in need, save lives, and help their family members. The primary reasons preventing people from blood donation included that they were not asked and some medical reasons. In Albania 88.6% would donate if asked, compared to 86% in KSA and in 77.7% in Turkey 77.7%.

Conclusion: Despite considerable differences in the culture and demographics of the three countries, several common factors emerged from the surveys. Some of these results reflected an encouraging attitude toward blood donation. Further future planning with emphasis on educational programs and more organizations of donor recruitment campaigns can increase blood donations to fulfill the patients' needs in these countries.

Keywords: blood donation, attitude, motivation, blood donation system, blood transfusion

Introduction

On World Blood Donor Day 2017, on the 14th of June, the theme “Give blood, Give now, Give often” emphasized the critical need for more people worldwide to become life-savers by regularly donating blood. According to the World Health Organization (WHO) data from 173 countries, 112.5 million donations are made annually.¹ In a recently published report, Ministry of Health (MoH) in KSA received a collection of 252 thousand units during 2020 (<https://www.statista.com/statistics/1308070/saudi-arabia-amount-of-collected-blood-units/>) which was higher than 180 thousand units donated in Turkey annually (<https://jpma.org.pk/article-details/6877>) and 30,000 units in Albania (<https://www.otpbank.al/en/blood-donation/>). These figures hardly meet the 1–3% donor limit set by WHO. Currently, only 57 countries fulfill their blood requirements from volunteering donors. According to one estimate, if only one percent of the population donated, it would be sufficient for each country to achieve its basic requirements for blood supply. As donated blood can save human lives, blood availability remains a significant concern to health-care institutions and society.

Several questions can help to understand people's approaches toward blood donation. What encourages a person to donate blood voluntarily? What kinds of obstacles are faced by a donor? How can the blood centers convince the donors to donate again and become regular donors? What are the misconceptions about blood donation? Answers to these questions provide information for blood collection agencies in their recruiting campaigns.^{2,3} The knowledge about the psychology and motivation of blood donors is essential to establishing a safe blood supply based on voluntary, non-remunerated donors in many countries worldwide. On the other hand, more research should be conducted on the motivational factors affecting blood donations.

The three countries included in this study, ie, Albania, Turkey, and KSA, differ in their history, cultural background, economy, geography, and many other things. The primary objective of our study was to observe how these changes impact their respective population's attitudes toward blood donation. Previous studies have shown that different countries with differences in culture, economy, and geography face similar problems in recruiting blood donors.² For instance, over the past decades, there has been an increasing shift of reliance on blood from imported blood and paid donations to local volunteering donations in Albania, KSA, and Turkey. It was during the COVID-19 pandemic that this trend changed, and a significant impact was given on the blood supply system worldwide. Anxiety and fear of infections were the most common misconceptions hindering donor's access to donation facilities.⁵ WHO estimated that the COVID-19 pandemic caused a significant 20–30% blood supply reduction in KSA alone. That drop reached up to 39% in some cases.^{4,6} This resulted in a more active campaign to eliminate misconceptions and recruit more donors to fulfill the demands.

Knowledge, Attitude, and Practice (KAP) surveys are routinely used by government institutions and donor recruiting agencies to better understand the behaviors of the general population on blood donation in developing settings. The logic model underlying KAP surveys on blood donation provides a better understanding of the knowledge, attitude, practices, and behaviors surrounding blood donation. They will lead to better actions to promote safe, voluntary blood donation worldwide and form the basis for developing educational material and communication strategies. Moreover, one of the leading implementations of the World Assembly Resolution 28.72 of May 1975 is that the member nations should work to establish a national BTS based on unremunerated blood donation. Hence, this study aimed to find, understand, appraise, compare and summarize the results of KAP surveys on blood donation in Albania, KSA, and Turkey. Such information is crucial for planning the attainment of total voluntary blood donations in accordance with World Assembly Resolution 28.72 of May 1975.

Material and Methods

We prepared a self-administered online [questionnaire form](#) using Google Forms after a review of other similar studies that probed the attitudes toward blood donation in different countries and was shared using social media.

The questionnaire form was distributed randomly among people. It was shared on social media, and receivers were encouraged and reminded to share it with others between March and April 2021. The questionnaire form was available in English, Albanian, Turkish, and Arabic. The participants were of genders, males and females. The questionnaire form analyzed various aspects of the knowledge, beliefs, attitudes, and motivations toward blood donation. Respondents were asked to answer with a "Yes" or "NO" to some questions; on other questions, they were asked to select or even write reasons for donating or not donating. There was a section where questions had to be answered using a "Likert scale". The questionnaire form needed 5–10 minutes to be completed and was automatically collected by Google Forms.

A total of 1222 invitations were sent out in the three countries, 274 in KSA, 90 in Turkey and 858 in Albania.

Results

The study was conducted during March and April 2021. The distribution was random and responders were encouraged to share and encourage others to fill the survey. The total number of respondents was 1281. [Table 1](#) shows the country-wise breakdown of the responders from each country. [Table 1](#) provides a summary of the participants per country and sample selection.

Behavior

[Table 2](#) shows the percentage of those who had previously donated blood among the responders. In Albania, only 32% reported having donated blood; and from them, only 5.2% responders were regular donors. In KSA, 46% of responders

Table 1 Summary of the Participants per Country and Sample Selection

Country	Author	Time Period	Population Surveyed	Sample Selection	Survey Delivery
Albania	Idris Sula	March-April 2021	858 Adults	Random sample	Self-administered online questionnaire
KSA	Idris Sula	March-April 2021	274 Adults	Random sample	Self-administered online questionnaire
Turkey	Idris Sula	March-April 2021	90 adults	Random sample	Self-administered online questionnaire

Table 2 The Percentage of Study Responders Who Previously Donated Blood

Country	Population Surveyed	Total Blood Donors	Regularly 2–3 Times a Year	Once a Year	Every Two or Three Years
Albania	General population	32% (273)	5.2%	38.4%	56.4%
KSA	General population	46% (131)	8%	35.2%	56.8%
Turkey	General population	46% (46)	28.6%	45.2%	26.2%

have previously donated, and only 8% were regular donors. In Turkey, the percentage of donors was the same as in Saudi Arabia, 46%, but the rate of regular donors was significantly higher, up to 28.6%.

Knowledge

Overall, as seen in [Table 3](#) general knowledge of the health benefits, required age, and blood group was high across all countries. On the other hand, specific knowledge of the donation intervals was relatively low. Donors tended to have more general knowledge than non-donors, except in Albania, where non-donors knew more about health benefits, required age, and donation intervals, as shown by in [Table 3](#).

In the survey, some questions evaluated the degree of misconception about the blood donation process. As shown in [Table 4](#), the most common misconceptions regarding blood donation in the three countries are the conditions that “should the donor be fasting” and that “a smoker cannot donate”. Another misconception was that the donated blood was being sold to patients which significantly discouraged the volunteers.

Attitude and Motivation Toward Blood Donation

The current report found an overall positive attitude towards blood donation among people in the three countries. [Table 5](#) shows the percentages of the three countries reporting a positive attitude toward different scenarios. No negative attitude was observed towards blood donation among the responders from the three countries. In addition to these results, more than 80% of responders agreed or strongly agreed that every healthy person should donate blood. Almost 90% of all responders agreed or strongly agreed that their donation would encourage others to donate. More than 95% believed that

Table 3 General Knowledge of the Health Benefits, Required Age, and Blood Group Amongst the Responders

Country	Knowledge on Health Benefits of Blood Donation		Knowledge of Age Requirement		Knowledge of Their Blood Group	
	Donors	Non-Donors	Donors	Non-Donors	Donors	Non-Donors
Albania	92.4%	95.2%	69%	75.3%	90.6%	53.8%
KSA	87.3%	67.3%	63.4%	72.1%	98.4%	90.4%
Turkey	96%	85%	76%	75%	100%	40%

Table 4 The Most Common Misconceptions Regarding Blood Donation Amongst the Responders in Three Countries

Country	A Smoker Cannot Donate		Blood is Sold to Patients by Hospitals		In Order to Donate Blood, the Donor Should be Fasting	
	Donors	Non-Donors	Donors	Non-Donors	Donors	Non-Donors
Albania	38%	60.5%	15.8%	21.5%	33.8%	60.8%
KSA	51.6%	55.8%	16.7%	23.8%	22.2%	42.8%
Turkey	20%	62.5%	2%	7.5%	30%	47.5%

Table 5 The Percentages of the Three Countries Reporting a Positive Attitude Toward Different Scenarios

Country	I Would Encourage Others to Donate	I Think Blood Donation is a Duty of Citizens	I Will Donate if Called Upon	I Would Donate to an Unknown Person	I Think I am Fit to Donate?
Albania	83.9%	74.5%	88.6%	87.2%	65.7%
KSA	82.7%	74.7%	86%	78.3%	71%
Turkey	78.8%	70%	77.7%	81%	70%

blood donation could save lives. More than 75% of the responders believed that blood donation was a duty of citizenship. More than 80% responders would not accept monetary compensation for their donation.

Reasons Preventing Non-Donors from Donating

As summarized in Table 6, the top reasons for not donating in Albania were fear, nobody had ever asked for donation, and some serious medical conditions. Fears of anemia, sight of blood, and injection were the most common deterrent fears among non-donors in Albania. Medical reasons included anemia, heart diseases, underweight, blood infection, cancer, etc. In KSA, the top reasons for not donating were that they never thought of it, and nobody ever asked for blood donation, and medical reasons. It was indicated in a previous study that the primary reason for not donating blood was that “nobody had ever asked for it”.⁷ In Turkey, 34% non-donors reported not having a specific reason for not donating. Other reasons included medical reasons and the fact that they had never thought of it.

The main reason for donation in the three countries was altruism (the desire to help those in need and save lives). In Albania and KSA, the second most common reason for blood donation was to support their family members, whereas in Turkey, the second most common reason was encouragement from family members or friends. The consciousness to donate due to a rare blood group was observed among donors from all three countries, as shown in Table 7.

Table 6 The Primary Reasons for Not Donating Blood in Three Countries

Reason Country	Fear	I Have Never Thought of it	Nobody Asked for	Objection from Family	Medical Reasons	No Specific Reason	I Would Rather Not Say
Albania	33.1%	20.3%	32.2%	1%	23.4%	16.5%	3.6%
KSA	16%	44.3%	24.6%	6.3%	24.6%	0%	1.4%
Turkey	16.3%	18.3%	10.2%	0%	28%	34%	2%

Table 7 The Main Reason for Donation Among the Three Countries Was Altruism (the Desire to Help Those in Need and Save Lives)

Reason Country	I Want to Help Others in Need and Save Lives	My Family/Friends Donate and Encouraged Me to Contribute	I Donated for My Family Members	I Have a Rare Blood Group
Albania	81.3%	10.2%	34%	5.8%
KSA	59.8%	16%	17.9%	4.9%
Turkey	74%	14%	4%	4%

Blood Group Distribution Among Responders

As shown in Table 8, the blood group distribution among people in the three countries is similar, and these values are close to the ones reported prior studies.^{8,9}

Discussion

KAP surveys provide a contextual evidence base for developing communication strategies and interventions to encourage voluntary, non-remunerated blood donors, a crucial component of a safe and continuous blood supply in any health-care setting. Undoubtedly, donor recruitment problems diverge in different countries and are primarily determined by cultural, social, educational, and other factors. Thus, in many developed countries where donations are mainly voluntary and unremunerated, the main concern is a decline in blood supply. On the other hand, in most developing countries where they are facing a severe shortage of blood, blood donation is still predominantly involuntary in which family members, relatives, and friends of patients give a significant contribution in fulfilling the demands for blood, leaving a small proportion to voluntary unremunerated donors. In both situations, donor behavior and attitude must be analyzed to ensure that the blood supply is sustained by recruiting new donors and maintaining those who have already donated, mainly the voluntary unremunerated donors.

While there is a considerable difference in the demographics, culture, and social life of these countries, several common themes emerge, such as fear of blood donation, misinformation, the potentially positive effect of altruism, the fact of not being asked to donate, and willingness to donate even for unknown people. This is evident from the responses summarized in Tables 5 and 6. For example, more than 24% respondents in KSA stated that they have never been approached for blood donation while more than 44% respondents have never thought of blood donation. These data point to an opportunity here that proper campaigning can help to bring these more 68% people to donate. Additionally, the 16% population who is scared can be convinced by educating them regarding the benefits of blood donation. Such opportunity

Table 8 the Blood Group Distribution Among People in the Three Countries Included in the Study

	Albania	Saudi Arabia	Turkey
A Positive (A+)	34%	30.5%	31.7%
A Negative (A-)	4.2%	2.7%	6%
B Positive (B+)	13%	12.5%	10.9%
B Negative (B-)	1.4%	1.1%	1.2%
AB Positive (AB+)	7%	7.8%	6.9%
AB Negative (AB-)	1.2%	1.5%	0%
O Positive (O+)	31.9%	36.8%	34.1%
O Negative (O-)	6.5%	6.7%	9.7%

to convince this huge section of society is supported by the observation in Table 8 that family and friends encouraged the non-donors to become donors. Hence, there is an urgent need for a systematic drive from relevant authorities in this regard. Table 5 clearly supports this opportunity as between 77% and 86% respondents are willing to respond positively if they are asked to donate blood even if the recipient is unrelated to them (Table 5).

Money compensation has remained out of favor in many studies. According to our data, nearly 85% of the responders objected to money as compensation for their donations. All three countries tried to move from paid donations to volunteering donations. Presently, paid blood donations are being used widely for plasma donations in developed countries. For example, paid plasma donations account for almost 70% of total donations in Canada.¹⁰ According to the reports published by the Albanian ministry of health, nearly 70% of the donations were paid donations, 25% were donations for family members, and only 5% accounted for voluntary donations. In 2019 family replacement donations accounted for 73% of total donations and paid donations dropped as low as 1%.¹¹

Recruiting a sufficient number of safe blood donors is an emerging challenge worldwide, especially during COVID-19 pandemics, which have reduced blood-related activities, putting the safe blood supply and demand at risk.⁵ The COVID-19 pandemic has negatively impacted donor attendance.⁴ WHO estimated that the COVID-19 pandemic caused a 20% to 30% reduction in blood supply in KSA alone, dropping to 39%.^{4,6} According to the Turkish Red Crescent, 2020 observed a decrease of 18% in donations in Turkey.

It is possible that with the decline in the number of voluntary donors in the surveyed countries, monetary compensation may be reconsidered as a way to attract more donors. However, there are potential serious disadvantages of paid donations, ie, attracting risky donors, especially drug users. Also, monetary rewards tie donors' interests with their financial needs rather than regular donations toward establishing regular unremunerated donations. Besides this, a paid donation cannot be a wholly reliable option by taking into consideration that many developing countries, including Albania, might not be able to have a sufficient budget to afford and fulfill the needs of the country.

The current study shows that in KSA and Turkey nearly half (46%) of the responders are blood donors, whereas, in Albania just one-third (32%) of the responders were blood donors. However, in recent years, Albania, Saudi Arabia, and Turkey have seen an increasing trend in blood donations. In 2004, local blood donations met only 11% of the country's needs; in 2017, that number increased to 45%.¹¹ In KSA and Turkey according to some studies and reports from World Health Organization blood banks can almost fulfill their requirements from volunteer donating, and there is an increasingly positive trend toward donation.^{7,12} In KSA, 71% of blood in blood banks is from voluntary donors.⁷ According to the Turkish Red Crescent, one million 937 thousand 932 donations were made during 2015, which increased to two million 809 thousand 237 blood units across the country that is incidentally the highest number in the past five years.¹³ The level of knowledge among responders was good, where donors scored higher than non-donors. More than 67% of the responders knew health benefits of blood donation. Interestingly, in Albania, there is no significant difference between donors and non-donors, but non-donors scored higher, 95.2%, versus donors 92.4%. Even though the level of knowledge on health benefits of blood donation is high among Albanians only 32% of responders were actual donors. Considering that the primary reasons for not donating in Albania were fear and that nobody had ever been asked, it means more work needs to be done in recruiting volunteering-donors. In KSA, there is a clear gap between donors and non-donors (87.3% vs 67.3% respectively). Even though the knowledge level among Saudis compared to Albanians is lower, their awareness and practice are better as 46% of the responders from KSA were donors. In Turkey, knowledge of the health benefits of blood donation was good as the donors scored 96% and non-donors scored 85%. Besides this, their awareness and practice are good, as 46% of responders were donors.

The knowledge of the required age was significantly good as more than 63% of responders answered: "NO" when asked if someone younger than 16 years old could donate blood.¹⁴ There was a clear knowledge gap regarding the blood groups. More than 90% of the Saudi donors knew their blood group as compared to 50% of non-donors in Albania and Turkey knew their blood group. This number was much higher in Saudi Arabia, where 90% of non-donors knew their blood group.

Some misconceptions might have hindered blood donation. For example, the most common misconceptions among all responders were that a donor should be in a fasting state at the time of blood donation, and a smoker cannot donate. These misconceptions were almost twice elevated amongst the non-donors compared to donors.

Among all surveys, the most important motivating factor for blood donation was altruism, which was “the desire to help others in need and save lives”. This can be related to the religion as these countries are of Muslim majority and according to many Muslim scholars, donating blood is a duty for every Muslim. For example, the late Sheikh Abdul Aziz bin Baz urged Muslims to donate blood in the local hospitals to save the lives of needy patients. This trend is consistently overcoming the paid donation and increasing the donation rate in these countries.^{7,12} Donation for family members is the second leading and motivating reason for blood donation. According to WHO, the strong willingness to donate blood for friends or family is a way for blood banks to recruit more donors. In Nigeria, around 67% of blood donations in Nigeria are made for family members; this is due to the policies of many hospitals, which require the donation of a blood unit as a condition for a family member to receive antenatal care¹⁵ because of the fragmented blood supply system and an inadequate supply in the country. Social encouragement plays a minor role in recruiting donors, where just 10–16% of responders are encouraged by their relatives and friends. Only 4–6% of donations were made due to the awareness of the rarity of their blood group.

Although having many commonalities, the three countries differed in the reasons preventing people from donating. For example, in Albania, the most common deterrent to blood donation was fear and the fact of not being asked to donate. Several specific fears were reported, ie, fears of anemia, the sight of blood, and injection. In Saudi Arabia, the top reasons for not donating were that they never thought of it, that nobody asked for it, and for medical reasons, which included anemia, heart diseases, underweight, blood infection, cancer, etc. It was indicated in a previous study in Saudi Arabia that the primary reason that they did not donate blood was that nobody ever asked them to donate.² In Turkey, 34% of non-donors reported no specific reason for not donating. Other reasons included medical reasons and the fact that they had never thought of it.

A common theme that emerged from survey results in the three countries is the need for more work to convert positive attitudes towards blood donation into the practice of volunteering donations. In Albania, nearly 90% of responders were willing to donate even to someone they did not know, while 65% believed that they were fit enough to donate, and only 32% had donated at least once. In Saudi Arabia and Turkey, the positive attitude was better translated into voluntary donations where around 80% of responders were willing to donate even to someone they did not know, and about 70% believed they were fit enough to donate, while 46% of the responders were blood donors.

There are different ways to recruit repeated donors, and diverse reasons affect their willingness to donate again. Donating for the first time significantly contributes to the willingness to donate again. Thus, future KAP studies should focus more on strategies to convert first-time donors into repeat donors by trying to identify and eliminate anything preventing them from donating again. Proper knowledge of blood donation plays a crucial role in encouraging blood donation. The present study found that more knowledgeable subjects tended to donate blood more than those with limited or scanty knowledge.

Conclusions

The collection of blood donation surveys undertaken in the three countries confirms that KAP surveys provide an essential source of information about blood donation trends that can be used to develop targeted strategies for encouraging voluntary unremunerated donors and building a safe blood donation system. Although knowledge, beliefs, attitudes, and behaviors toward blood donation are similar among these countries, our data show that new strategies are warranted to recruit non-donors. Fear of donating and the fact that “nobody has ever asked me to donate” were the two most common deterrents to blood donation; altruism was the primary motivating factor. There is an apparent gap between the positive attitude toward blood donation and the number of donors in the Albanian, Saudi, and Turkish populations. New communication strategies are warranted to overcome these issues, establishing a safe and optimum blood supply from voluntary, non-remunerated donors.

Ethical Approval

This study was approved from Qassim Ethical Committee, Ministry of Health, KSA, with written consent form all participants and complies with the Declaration of Helsinki.

Disclosure

The authors report no conflicts of interest in this work.

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Five Challenging Cases of Hereditary Antithrombin Deficiency Characterized by Thrombosis or Complicated Pregnancy

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Five Challenging Cases of Hereditary Antithrombin Deficiency Characterized by Thrombosis or Complicated Pregnancy

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Abstract: Hereditary antithrombin deficiency (ATD) is a rare autosomal dominant condition (estimated prevalence 1:500–1:5000). Most ATD patients have AT activity levels 40–60% of normal. We present treatments for venous thromboembolism (VTE) in five cases of hereditary ATD. Four patients had a family history of ATD, and one had a *de novo* mutation. The majority of patients had a VTE while on prophylactic anticoagulation. AT concentrate augmentation was added in these cases to treat the VTE and for prophylaxis against further episodes. Two patients had significant bleeding events, one had permanent physical sequelae. Two of the patients were pregnant. VTE is a common cause of morbidity and mortality during pregnancy. Although low molecular weight heparins are the drugs of choice during pregnancy, this treatment was inadequate in one patient (developed VTE on therapy). These cases emphasize the need to screen for ATD in young patients (<55 years) presenting with VTE. AT augmentation therapy may be necessary in patients inadequately treated with conventional anticoagulants. Careful monitoring and individualized care are needed in ATD patients, especially those with demonstrated bleeding tendencies.

Keywords: clotting disorders, venous thromboembolism, anticoagulation, bleeding tendencies

Introduction

Antithrombin (AT) is a serine protease inhibitor and natural anticoagulant that acts primarily through inactivation of the clotting factors thrombin and factor Xa and less effectively through factors IXa and XIa.^{1,2} In addition, the anticoagulant, heparin, depends on AT for its activity. Heparin binds to and induces a thousand-fold enhancement of the anticoagulant effect of AT.³ In addition, AT inhibits the tissue factor (TF)-factor VIIa (FVIIa) complex (as does TF pathway inhibitor: TFPI) preventing FVIIa activation of factor IX and X.^{4,5}

Inherited AT deficiency is a rare autosomal dominant condition that was first described in 1965.⁶ AT deficiency makes affected individuals susceptible to the development of thrombosis. It has been estimated that 50% of individuals with congenital AT deficiency will have experienced a venous thromboembolism (VTE) by age 50.^{7–9}

AT deficiency is classified as either a quantitative (Type I) or qualitative deficiency (Type II).¹⁰ Type I deficiency presents with reduced AT antigen and activity levels. Type II presents as normal AT antigen levels but reduced AT activity. Type I deficiency, commonly associated with heterozygous *SERPINC1* gene variants (generally nonsense or frameshift mutations), is usually associated with a severe phenotype.¹¹ Homozygous type I ATD has been found to be lethal in mice.¹² Type II ATD is usually due to missense mutations and is generally associated with milder protein dysfunction.¹³ Type II is subdivided based on the site of the mutation: reactive site, heparin-binding site or pleiotropic.^{14–16} Treatment and prevention of VTEs in patients with hereditary AT deficiency can be difficult due to heparin resistance. Treatment can also include administration of exogenous concentrated AT (either plasma-derived or recombinant). In this case series, the treatment of

Table 1 Clinical Characteristics of Five Antithrombin Deficiency Cases

Case No.	1	2	3	4	5
Sex	Male	Female	Male	Male	Female
Age (yrs)	32	21	49	27	22
Initial AT Activity Level (%)	42–45	58	42	30	57
Initial AT Antigen Level (%)	18	NA	40	51	56
AT Deficiency Type	I	I	I	I	I
Positive Family History of AT Deficiency	No	Yes	Yes	Yes	Yes
VTE on Prophylaxis	Yes	No	Yes	No	Yes
AT Therapy	Yes	Yes	Yes	Yes	Yes
Age at Diagnosis (yrs)	17	21	49?	23	22
Age at 1 st VTE (yrs)	12	NA	43	23	23
Comments	<i>De novo</i> mutation. No new VTE post treatment. Expired at age 37 years due to PH and CHF.	Pregnancy w normal delivery, AT & LMWH prophylaxis	GI varices and bleeding → cardiac arrest → anoxic brain injury. Recovery with residual deficits; No new VTE or bleeding events.	Necrotizing pancreatitis and septic shock. Recovered. No new VTE.	VTE during pregnancy, Treated → unremarkable delivery. No new VTE.

Abbreviations: ATD, antithrombin deficiency; VTE, venous thromboembolism; PH, pulmonary hypertension; CHF, congestive heart failure; AT, antithrombin; LMWH, low molecular weight heparin; GI, gastrointestinal; NA, not available.

five cases of congenital AT deficiency with severe clotting tendencies will be described. A brief summary of patient and case characteristics is shown in Table 1.

Clinical Presentations

Case 1

A 32-year-old male had a known AT deficiency due to a *de novo* non-sense mutation 7522G>T, causing Q271X (GAG to TAG at codon 271 producing a stop codon) resulting in AT activity of 42–58% of normal and AT antigen of 18% of normal.¹⁷ The patient had his first thrombotic episode at age 12 years – a provoked DVT in a lower extremity that was treated with warfarin for three months (treatment goal prothrombin time two times baseline). At age 17 years, the patient had a recurrent lower extremity deep vein thrombosis (DVT) with a simultaneous retinal artery thrombosis. The latter resulted in a permanent reduction of the patient's visual field.

Antithrombin deficiency was discovered at this second episode. Family history was negative for VTE and AT antigenic and functional tests were normal for both parents. Genetic studies, including linkage studies to confirm parentage, revealed a *de novo* nonsense mutation, and other markers of thrombophilia were unremarkable.¹⁷

The patient had a third DVT episode despite warfarin therapy with therapeutic prothrombin times. At that time, the patient received a permanent inferior vena cava filter and was started on AT therapy (Thrombate III, antithrombin III [human], Grifols, Research Triangle Park, NC). AT was administered daily initially (treatment goal = peak 120% of normal value based on the package insert formula for calculating dosage), then decreased to twice weekly with warfarin for VTE prophylaxis.

Approximately one year later, the patient voluntarily discontinued AT treatment and was lost to follow-up. The patient returned to the clinic at age 32 with a history of multiple VTE episodes including at least two pulmonary embolisms (PE) in the interim and an open-heart embolectomy at age 21. The patient also had a superior mesenteric vein thrombus treated by open laparotomy and small bowel resection at age 22 and pulmonary thromboendarterectomy at age 26 years. In the patient's hiatus from the clinic, he continued to receive anticoagulant treatment with warfarin (target International Normalized Ratio (INR) = 2.3–3.5).

The sequelae of the multiple thromboembolic episodes included congestive heart failure, chronic pulmonary hypertension, atrial fibrillation, tachycardia-induced cardiomyopathy, hepatic cirrhosis and chronic severe venous stasis and post-thrombotic syndrome.

Upon his return to the clinic, due to the perceived high risk of VTE, the patient was restarted on prophylactic treatment with AT (twice weekly with the dose calculated to achieve trough AT activity of >75% of normal value). Central venous access to administer AT was extremely difficult due to radiographic and ultrasound evidence of occlusive thrombi in virtually every deep vein along with extensive, tortuous collateral venous circulation.

Over the following five years, the patient received either warfarin or rivaroxaban (Xarelto, Janssen Pharmaceuticals, Titusville, NJ, USA, 20mg daily: INR goal 2.5–3.5 measured monthly) along with twice weekly AT concentrate (peak activity level goal 120%). No new thromboembolic events occurred during this period. However, despite aggressive treatment, the patient died at age 37 years from complications of congestive heart failure and pulmonary hypertension.

Case 2

A 21-year-old female was evaluated for AT deficiency during family screening due to a strong family history of VTE. Her baseline AT activity was 58% (range of normal values 73–125% of the mean). The patient's great-grandfather died of a VTE and her grandmother, father and aunt have all experienced VTE. Several females in the family also experienced pregnancy complications. At diagnosis, the patient had no history of VTE and had not been on any hormonal therapy.

The patient was tested monthly for pregnancy (beta-human chorionic gonadotropin (HCG)). After two months, she was found to be pregnant (six weeks, 1 day gestation). At that time, the patient was started on AT replacement therapy (Thrombate III, Grifols) and a low molecular weight heparin, enoxaparin (40 mg subcutaneously daily). AT was administered as a 50 units/kg loading dose followed (after 24 hr) by a daily maintenance dose of 30 units/kg. AT levels were monitored and the daily dose of AT adjusted to maintain AT activity levels in the range of 76–128% of normal. After 2 weeks of daily AT therapy, every other day infusions were started. During her pregnancy, the patient had the following adverse events: contact dermatitis in response to adhesive dressings, leg cramps (VTE excluded by ultrasound), two vessel umbilical cord with normal ultrasound, fetal growth restriction (<10th percentile), and mild hypertension and proteinuria at 37 weeks, 1 day.

The patient delivered at 37 weeks and 2 days by Caesarean section after forceps delivery failed. Epidural anesthesia was administered more than 24 hours after the last enoxaparin dose. The newborn received a “moderately abnormal” APGAR score of 5 at one min and a “reassuring” score of 7 at 5 min.¹⁸ AT infusions were continued every other day for six weeks post-partum and enoxaparin (40 mg subcutaneously daily) was resumed post-partum.

Case 3

A 49-year-old male with AT activity of 42% of normal at diagnosis (AT antigen levels were similar to activity levels with a nadir of 40% (reference range for this laboratory was 82–136%)), and several low repeat levels thereafter (~40% on warfarin) had extensive thromboses including the hepatic portal system which resulted in portal hypertension and gastroesophageal varices. His first occurrence of a distal VTE was at age 43 and was treated with warfarin.

While on warfarin prophylaxis, the patient developed multiple recurrent DVTs in his femoral and popliteal veins as well as thromboses in his superior mesenteric, portal, and splenic veins. While the patient was on warfarin, his INR values fluctuated but was subtherapeutic (1.1) at this admission. The patient reported that one of his brothers and his mother died in their twenties and sixties, respectively, due to “abnormal blood clotting”, while another brother suffered from DVT in his youth.

The portal vein thromboses resulted in gastroesophageal varices and predisposed the patient to gastrointestinal bleeding. The patient suffered a devastating variceal bleeding episode in 2013 resulting in severe hemorrhagic shock with cardiac arrest, anoxic brain injury and a spinal cord infarction. Records regarding warfarin compliance and actual INRs were unavailable. Anticoagulant therapy was discontinued, and an inferior vena cava (IVC) filter was placed.

Subsequently, the patient was transferred to a tertiary care facility for further treatment. AT replacement was initiated with plasma-derived AT (Thrombate III, Grifols). The patient developed thrombus extension to the IVC filter on standard AT replacement dosing. AT treatment was intensified by increasing the dose and frequency of administration from 2100 units three times a week to 2200 units every other day with the goal to maintain AT trough activity levels as close to 80% as possible.

Over the past eight years, the patient gradually recovered from his neurological deficits but will likely remain wheelchair-bound with residual mentation deficits. However, there have been no new thrombotic or bleeding events since the initiation of the intensified AT regimen. Lifelong AT concentrate therapy will likely be necessary to prevent VTEs and anticoagulation should be avoided due to the patient's history of catastrophic bleeding episodes.

Case 4

A 27-year-old male presented to the hospital with necrotizing pancreatitis and abdominal compartment syndrome. Hematology was consulted due to the patient's personal and family history of type 1 AT deficiency. The patient's brother was reported to have type 1 AT deficiency, diagnosed at 28 years of age subsequent to a cerebral vascular accident with a baseline AT activity 55% and AT antigen 51%.

The patient was diagnosed with type 1 AT deficiency by his primary care physician four years prior when he suffered bilateral lower extremity DVTs with a subsequent PE developing a few months after the initial thrombotic event. A permanent IVC filter was placed approximately five months prior to the presenting episode when he was being treated for acute pancreatitis. The patient had an elective cholecystectomy one month prior to admission. Subsequent to this surgery, he developed necrotizing pancreatitis with abdominal compartment syndrome.

The patient developed a duodenal hemorrhage after esophagogastroduodenoscopy and was subsequently intubated with central line placement. The patient developed septic shock secondary to necrotizing pancreatitis which was treated with broad spectrum antibiotics. An exploratory laparotomy was performed with placement of a wound vacuum. Abdominal CT revealed that the necrotizing pancreatitis involved 50% of the parenchyma.

At the initial hematology consult, the patient's AT activity was 30%. Exogenous AT concentrate (Thrombate III, Grifols) was administered (5000 units) with a post-infusion peak of 111%. AT activity was then monitored daily with a target range of 80–120%. Unfractionated heparin was also administered with a PTT target of 50–70 seconds (institutional therapeutic PTT range is 70–100 sec), due to recent hemorrhage and temporal proximity to surgery. While an outpatient, he had been treated with warfarin with an INR target of 2.0–3, and no other family history of thrombophilia or bleeding disorders was reported.

During the patient's hospitalization, he developed a vancomycin-resistance enterococcal infection in his abdominal compartment. Treatment included seven surgeries to drain and clean abdominal abscesses. The patient was placed on a continuous AT infusion during hospitalization to maintain AT activity in the target range. Other complications during his hospital course included superficial cephalic venous thrombosis during the first month and right popliteal vein thrombosis in July 2015 during the second month. After peripheral placement of a central catheter and continued vacuum wound management, the anticoagulation was transitioned to warfarin (goal INR 2.5–3.5) and the patient was discharged with completion of intravenous antibiotic therapy as an outpatient.

The patient was readmitted for abdominal wall reconstruction five months after the initial admission. During the perioperative period, he was administered AT infusions (goal 80–120%). The patient was again transitioned back to warfarin (INR goal 2.5–3.5). The patient has had no additional thrombotic complications since that time.

Case 5

A 22-year-old patient with AT deficiency (baseline AT activity 57%; antigen level 56%) presented with an intrauterine pregnancy. Her family history was remarkable for type 1 AT deficiency in her father and sister. Her father had a history of

multiple thrombotic events beginning at the age of 23. At 31 years old, he died from refractory progressive VTE related to small cell carcinoma of the lung.

The patient's first pregnancy was uncomplicated. She received enoxaparin (40 mg subcutaneously daily) during her pregnancy and post-partum. Prior to her scheduled induction of labor, the patient received AT concentrate (Thrombate III, Grifols) dosed to maintain AT activity at 80–120% of normal levels. After delivery, her AT activity was 63%, so she received an additional dose of AT. The patient was scheduled to receive prophylactic anticoagulation for 12 weeks post-partum, but she voluntarily discontinued therapy at six weeks. Despite her non-compliance, the patient did not experience any thrombotic events.

This patient became pregnant again less than one year later. During this second pregnancy, she was prescribed prophylactic enoxaparin (40 mg subcutaneously daily). Despite receiving enoxaparin, the patient developed a DVT in her right lower extremity at gestational week 13. The DVT extended from the common femoral vein to the popliteal vein. Her enoxaparin dose was increased to therapeutic levels (1 mg/kg, subcutaneously, every 12 hr) and she received AT concentrate, targeting a peak AT activity of 120%. Despite the increased dose of enoxaparin, she experienced progression of the DVT in her right lower extremity to involve the common femoral vein, popliteal vein, and calf veins. The patient underwent pharmaco-mechanical thrombolysis and was given ticagrelor, 180 mg $\times$ 1 day, 90 mg on the second day, then discontinued. Anticoagulation management was escalated by the addition of AT concentrate to therapeutic dosing of enoxaparin. AT was administered three times a week with a peak AT activity target of 120%. Trough AT activity was measured at least weekly and low-molecular weight heparin anti-factor Xa activity was monitored monthly. Enoxaparin was discontinued 24 hrs prior to delivery and resumed 12 hrs after delivery. There was no further progression of the DVT, and the baby's vaginal delivery was unremarkable.

Discussion

Hereditary AT deficiency is a rare autosomal dominant disorder. The prevalence is estimated to be between 1 in 500 to 1 in 5000.^{19,20} Normal plasma levels of AT range from 112 to 140 μ g/mL. Due to variability between laboratories, AT activity levels are usually expressed as a percentage of the normal level for that laboratory with the usual normal range being 80–120%.²¹ Most patients with AT deficiency have AT activity levels in the range of 40–60%.²² The five patients included in this paper were mostly in this range: 30–58%.

The five case summaries presented here represent the more clinically complicated and severe phenotype of HATD and illustrate important commonalities that could influence the approach to diagnosis and management of AT deficiency. Four of the five patients presented had a positive family history for AT deficiency. This emphasizes the need for obtaining a thorough family history regarding the occurrence of DVTs or diagnosed AT deficiency. This is especially true of DVT patients younger than 55 years. In the general population, the vast majority of thromboembolic events (DVT or PE) occur in patients over 55.^{23–25}

In addition, most of these patients experienced a DVT episode while on prophylactic therapy with anti-coagulants. This observation demonstrates the need for careful monitoring of AT deficiency patients to determine their thrombosis risk and assess their compliance with therapy. Due to the occurrence of DVTs in these patients while on prophylactic anticoagulation, AT supplementation therapy was added to help prevent these episodes.

One patient (Case 1) was treated with rivaroxaban, a direct oral anticoagulant (DOAC) in the course of his treatment for AT deficiency. Although studies directly evaluating the use of DOACs in AT deficiency are limited. Two recent review articles on the use of DOACs in inherited thrombophilic disorders (including AT deficiency) concluded that, although there was some evidence of efficacy, current data were insufficient to recommend the use of DOACs in inherited thrombophilic conditions.^{26,27}

Two of the cases presented were in pregnant women. Pregnancy is a documented risk factor for development of DVT and PE. The risk of DVT in pregnant women has been estimated to be four times greater than non-pregnant women of the same age.²⁸ In fact, thromboembolism is a leading cause of death in pregnant women in developed countries.^{29,30}

Both of these patients were treated with a low molecular weight heparin (enoxaparin) and AT concentrate augmentation therapy. Low molecular weight heparins are the drugs of choice in pregnancy as they do not cross the placenta and are not excreted in breast milk.³¹ It is unknown whether pregnant women with AT deficiency have heparin resistance and

whether a low molecular weight heparin will be adequate for prophylaxis of thromboembolism. In one of the cases presented here, prophylaxis with a low molecular weight heparin was clearly inadequate as a thrombotic event occurred while on this therapy. Both pregnant women presented here received successful treatment or prophylaxis from a combination of AT and a low molecular weight heparin. These cases illustrate that ante- and/or post-partum prophylaxis as now recommended by guidelines of the American Society of Hematology³² may not always be sufficient to prevent pregnancy-related VTE in HATD.

One of the pregnant patients was treated with ticagrelor per her physician prior to hematology's involvement in her care. Her obstetrician preferred ticagrelor over alternative therapy based on their teratogenic risk versus benefit.

In both cases, enoxaparin was discontinued 24 hrs prior to delivery and resumed post-partum. Despite an extensive DVT during pregnancy in one of the patients, both of the deliveries (one Cesarean and one vaginal) were uneventful and resulted in healthy babies.

A recent retrospective study analyzed 64 pregnancies in 28 women with AT deficiency.³³ This study showed that risk of pregnancy-related VTE was similar between patients with type I and type II AT deficiency. However, risk of an adverse pregnancy outcome was related only to type II deficiency especially the homozygous AT Budapest 3 variant.

Another retrospective study investigated 219 pregnancies in 87 women with type I AT deficiency.³⁴ This study showed a lower risk of VTE in the women treated with LMWH (3 events/43 pregnancies; 7.0%) compared to women not treated with LMWH (17 events/146 pregnancies; 11.6%), but the difference was not statistically significant ($p = 0.57$). However, the incidence of late complications was increased in the LMWH-treated group (24%) compared to the untreated group (6% $p = 0.0006$). This study also showed that the risk of pregnancy-related VTE was highest among women with a positive family history of VTE suggesting LMWH therapy should be considered in these patients.

A study of the clinical characteristics of AT deficiencies found that the type II heparin-binding site mutation, AT Padua I, was more frequently associated with pregnancy complications than type I or other type II mutations.³⁵ Taken together, these studies indicate that genetic information on the mutation underlying AT deficiency, if available, can help guide the course of prophylaxis and treatment.

There are some distinct differences among the cases as well. One patient had a *de novo* mutation resulting in significant AT deficiency (42–45%),¹⁷ suggesting measurement of AT activity may be called for in patients with DVTs without a family history of AT deficiency especially patients younger than 55 years old. VTE is a relatively rare occurrence in people under 55 years old.^{23–25} For patients under 55 without known risk factors for VTE (eg, cancer, surgery, chronic obstructive pulmonary disease, some cardiovascular diseases), AT deficiency is certainly worth considering as the cause of their thrombotic event.

Two of these patients suffered significant bleeding events – one was catastrophic with significant clinical sequelae. These cases emphasize the need for careful monitoring of coagulation parameters especially in patients with a history of bleeding episodes. Both of these patients were initially managed with AT and without anticoagulants (warfarin or low molecular weight heparins). The patient with less severe bleeding was eventually transitioned to warfarin monotherapy. The patient with the severe bleeding episode has been maintained on AT therapy without the use of anticoagulants to avoid another potentially catastrophic bleeding event. Management of patients with AT deficiency and bleeding tendencies can be especially challenging.

Conclusion

These cases of AT deficiency demonstrate a wide range of manifestations, some commonalities, but also important differences necessitating an individualized approach to management. All of these patients received AT augmentation and, in most cases, after anticoagulant therapy proved inadequate. This emphasizes that in some instances AT replacement therapy, although costly, is a necessary component of successful treatment. Treatment of patients with AT deficiency in combination with bleeding tendencies can be particularly difficult.

Abbreviations

AT, antithrombin; ATD, antithrombin deficiency; DVT, deep vein thrombosis; HCG, human chorionic gonadotropin; INR, international normalized ratio; IVC, inferior vena cava; PE, pulmonary embolism; VTE, venous thromboembolism.

Data Sharing Statement

All the relevant data for this study are available within the article. Complementary data can be made available by the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

Ethics committee approval was not sought for this study because the institutions involved do not require approval for reporting individual cases. This study was completed in accordance with the Helsinki Declaration as revised in 2013. Written informed consent was obtained from the patients described below or their authorized representatives for their anonymized information to be published in this article.

Consent for Publication

There are no images, videos, recordings, or other materials requiring consent to be published other than the informed consent described above.

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Author Contributions

All authors made significant contributions to the work including conception, execution, acquisition of data, and analysis. All authors participated in the drafting and revision of the manuscript and have agreed to submission to this journal. All authors have agreed to revisions and final form of the manuscript for submission and take responsibility and accountability for the contents therein.

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Risk Assessment for Newly Diagnosed Fit Young Patients with Multiple Myeloma in the Era of Novel Treatment Modalities: Should There Be Additional Factors Taken into Consideration??

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Introduction: The optimal treatment for young patients with high-risk newly diagnosed multiple myeloma (NDMM) remains a challenge.

Methods: We retrospectively evaluated 58 NDMM patients younger than 55 years treated in our center from 2010 to 2021 with the current recommended protocols.

Results: After a median follow-up of 48 months, median overall survival (OS) was not reached; however, approximately 25% of them died within 4 years after diagnosis. Advanced disease stage, presence of extramedullary disease, elevated LDH, and less than very good remission before autologous hematopoietic stem-cell transplantation adversely affected patient survival. Based on these factors, we created a risk-assessment scoring system that sufficiently discriminated young NDMM patients at risk of poor outcome. The 4-year OS was superior for patients with zero to two factors to those with three to five factors (86% vs 44%, $p < 0.001$).

Conclusion: The proposed scoring system could be reliably used at diagnosis and at interim disease evaluation in aiming for personalized treatment for young NDMM patients.

Keywords: multiple myeloma, high-risk multiple myeloma, multiple myeloma risk assessment, young newly diagnosed multiple myeloma patients

Introduction

Multiple myeloma (MM) is the second-most common hematological malignancy, accounting for approximately 13% of all malignant hematological disorders.¹ The current therapeutic protocols using combinations of novel agents (proteasome inhibitors, immunomodulatory drugs, monoclonal antibodies) along with dexamethasone plus or without autologous stem-cell transplantation (ASCT) indisputably result in prolonged survival rates and better quality of life; however, the disease remains incurable.^{2–4} The truth is that clinical trials with the innovative agents have been designed with a focus on the median age of newly diagnosed MM (NDMM) patients, which is approximately 65–70 years.^{5–9} However, 40% of NDMM patients are aged <65 years and around 15%–17% <55 years, which raises the question of whether young NDMM (Y-NDMM) patients, especially those with high-risk disease, should be treated with the same rationale as older patients or those with standard-risk MM. Also, how can Y-NDMM patients, who may need more intensive treatment approaches, be easily and early identified?^{10–19}

In this retrospective study, we reviewed outcomes of NDMM patients who had been treated in our center with the current recommended treatment protocols.^{19–21} Focusing on the clinical course and ultimate outcome in younger patients (≤ 55 years),

we created a risk-assessment scoring system (MM-RASS) based on just five easily assessable parameters (sex, serum lactate dehydrogenase [LDH] levels, stage as per the International Staging System [ISS], presence of extramedullary disease [EMD], and response to initial treatment), which were found to have a strong impact on survival rates. This novel MM-RASS was validated in Y-NDMM patients (≤ 55 years) and proved to be efficient and reliable in discriminating patients with poor outcomes who might have benefited from more intensive treatment approaches.

Methods

Study Design

Following the Declaration of Helsinki for ethical principles and after King Fahad Specialist Hospital's Institutional Review Board approval, we retrospectively evaluated the records of 116 NDMM patients who had been treated in our department during the last decade and had had a minimum of 6 months follow-up. All had signed informed-consent forms regarding the diagnostic procedures, treatment approaches, and use of their clinical data for research purposes. Patients with asymptomatic/smoldering MM, plasma-cell leukemia, or inadequate data regarding disease characteristics or disease response after the initial treatment were excluded from the study. Patients considered too frail for treatment as per primary physician assessment (based on age, comorbidities, and inability to tolerate duplet or triplet therapies) were also excluded from the study. Because a considerable number of patients had missing cytogenetic/molecular data, for statistical analysis purposes, we used the International Staging System (ISS) instead of the revised ISS (R-ISS).^{10,11} All patients received initial treatment with combinations of a proteasome inhibitor (bortezomib) plus immunomodulatory drugs (lenalidomide or thalidomide) or cyclophosphamide with dexamethasone with or without AHSCT. Disease response was evaluated after three or four cycles of treatment, and remission status was defined as per International Myeloma Working Group (IMWG) criteria.²²

Statistical Analysis

SPSS version 22 was used for the statistical analysis. The Kaplan–Meier method with log-rank test and Cox regression analysis, crosstabs, and Student's independent *t*-test were used to estimate survival rates and to identify potential risk factors for patient outcomes. Overall survival (OS) was defined as the duration from the day of diagnosis until death or the date of last follow-up for surviving patients. Relapse/progression was defined as the period from treatment initiation till the first documentation of disease recurrence, while progression-free survival was defined as the period from treatment initiation till the first documentation of disease recurrence or the last follow-up for non-relapsed/progressed patients. For the purposes of this study, disease reappearance within 18 months post-treatment initiation was defined as early relapse/progression. Non-disease-related mortality (NDRM) was defined as any cause of death other than related to refractory MM. Patients' sex, age, LDH levels, ISS stage, cytogenetic profile, presence of EMD at diagnosis, response to initial treatment, and early relapse were analyzed as risk factors affecting the survival rates. Factors with missing data for $>20\%$ of patients were not incorporated in the statistical analysis.

Patient Characteristics

In all, 67 male and 49 female NDMM patients who fulfilled the aforementioned criteria were included in the analysis. Median age at diagnosis was 55 (30–85) years. The male:female ratio was 1.3:1. A total of 25 patients had elevated LDH levels, eleven poor cytogenetic characteristics at diagnosis (del[17p] four, del[13q] five, t[4;14] two), whereas for 23 (20%) karyotypic analysis either failed or was not available. According to the ISS, 51 were assessed as stage III, 47 as stage II, and 17 as stage I. Among stage III patients, 13 had LDH in upper to normal ranges (reference lab values 125–220 IU/L), two had poor cytogenetic characteristics (17p[del]), while for five, karyotypic analysis had failed. Based on the R-ISS, eleven patients had stage I, 78 stage II and 16 stage III, while for eleven patients, staging was not able to be calculated as per the R-ISS due to unavailable data. Twelve (10%) patients presented with EMD at diagnosis, which was confirmed by imaging tests (CT, MRI, or PET/CT) and from tissue biopsy if feasible. IgG isotype (83/116) and k light chain (78/116) were the dominant MM subtypes. We did not find any statistical difference between younger and older (than 55 years) NDMM patients regarding sex, disease stage, EMD presence, high LDH levels, high β_2 microglobulin (B2M) levels or high-risk cytogenetics. However, in the group of patients >55 years old, a significantly higher number had albumin levels <3.5 g/dL at disease diagnosis (Table 1).

Table I Patient and disease characteristics, treatment approaches, and response to treatment for all patients (n=116) patients and those aged ≤55 years and >55 years

	Total (n=116)	Patients aged ≤55 years (n=58)	Patients aged >55 years (n=58)	p
Age (median)	55 years	46.5 years	65.5 years	
Sex				
Male	67	30	37	ns
Female	49	28	21	
LDH				
Upper normal	25	14	11	ns
Within normal	88	43	45	
High-risk cytogenetics	11	7	4	ns
Albumin				
>3.5 g/dL	34	26	8	<0.0001
≤3.5 g/dL	80	32	48	
EMD				
Yes	19	9	10	ns
No	96	48	48	
B2M				
≥3.5	76	41	35	ns
<5.5	39	17	22	
Stage (ISS)				
I–II	64	34	30	ns
III	51	24	27	
MM subtype				
IgG	83	41	42	ns
IgA	14	5	9	
FLC	16	10	6	
k-LC	78	39	39	
λ-LC	35	17	18	
Nonsecretory	2	1	1	
CCT				
VRD	53	26	27	ns
VCD	31	17	14	
VTD	7	5	2	
VD	16	10	6	
RD	5	—	5	
Other	4	—	4	
ORR				
Yes	96	51	45	ns
No	20	7	13	
CR + sCR	43	22	21	ns
≥VGPR	71	36	35	
<VGPR	46	23	23	
AHSCT (conditioning regimen: high-dose melphalan)				
Yes	74	53	21	<0.001
No	42	5	37	

Abbreviations: EMD, extramedullary disease; B2M, β_2 microglobulin; ISS, International Staging System; ORR, overall response rate; FLC, free light chain; CCT, conventional chemotherapy; VRD, bortezomib–lenalidomide–dexamethasone; VCD, bortezomib–cyclophosphamide–dexamethasone; VTD, bortezomib–thalidomide–dexamethasone; BD, bortezomib–dexamethasone; RD, lenalidomide–dexamethasone; VGPR, very good partial remission; AHSCT, autologous stem-cell transplantation.

Treatment Approaches

The majority of patients received a median four (three to 12) cycles of combined therapy with bortezomib, lenalidomide, and dexamethasone (VRD; n=53), bortezomib, cyclophosphamide, and dexamethasone (VCD, n=31), bortezomib, thalidomide, and dexamethasone (VDT, n=7), or bortezomib or lenalidomide plus dexamethasone (n=21). As per the treatment plan, early AHSCT was offered to all eligible and fit-enough individuals (shown in Table 1).

Results

At data-collection cutoff, median follow-up for the surviving patients was 48 (6.5–132) months. The overall response rate (ORR) after frontline treatment was 83%: 43 (37%) achieved stringent complete remission (sCR) or CR, 28 (24%) very good partial remission (VGPR), 26 (22%) PR, and 20 (17%) were assessed as having stable or progressive disease. Eventually, 74 of 116 (65%) patients underwent early ASCHT. The reasons for not performing ASCHT were age >70 years (15%, n=17), disease refractoriness (9%, n=10), severe comorbidities, or patient refusal (13%, n=15; Table 1).

Median OS was not reached, and projected 4- and 8-year OS was 67% and 51%, respectively. Median time to disease relapse/progression was 46 months, while the 4- and 8-year probability of disease recurrence was 52% and 75%, respectively (Figure 1A and B). For the whole cohort (n=116), on univariate analysis, advanced stage (ISS stage III), elevated LDH levels, response of less than VGPR pre-AHSCT, presence of EMD at diagnosis, and early disease relapse/progression had an adverse impact on survival rates, while disease recurrence was negatively affected only by less than VGPR achievement pre-AHSCT (shown in Table 2A). On multivariate analysis, only LDH level upper to normal was found to be an independent factor for inferior long-term OS, whereas age >55 years and achievement of less than VGPR pre-AHSCT adversely influenced disease relapse/progression. Finally, 38 patients died. A majority of them (31 of 38, 82%) succumbed to uncontrolled disease and seven died from NDR causes. The 10-year NDRM rate was estimated to be 6%.

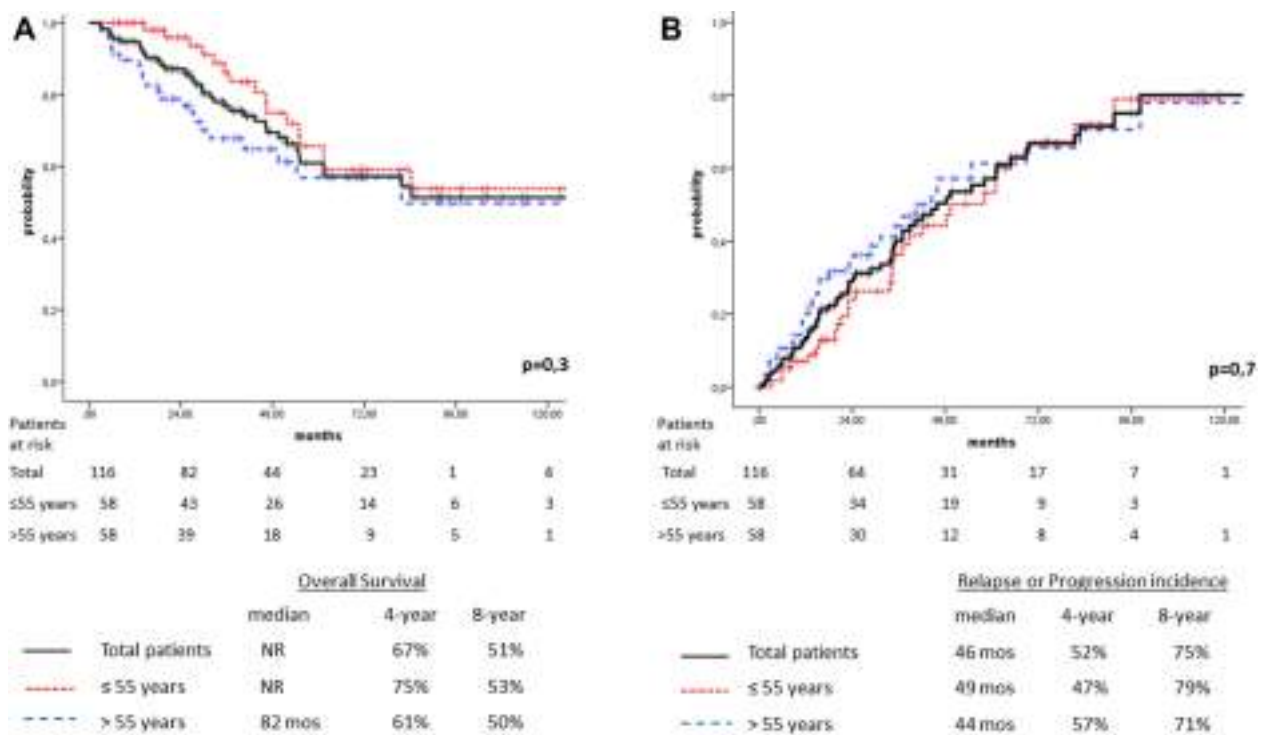


Figure 1 (A) Overall survival for the total cohort of patients (n=116, solid line) and those aged ≤55 years (n=58, dotted line), and >55 years (n=58, dashed line). (B) Relapse/progression of disease for the total cohort of patients (n=116, solid line), and those aged ≤55 years (n=58, dotted line) and >55 years (n=58, dashed line). **Abbreviations:** NR, not reached; mos, months.

Table 2 Univariate and multivariate analysis of factors affecting survival and disease/progression incidence in multiple myeloma patients

A. Univariate analysis	Whole cohort (n=116)			
	Survival		Relapse/progression	
	Relative risk (95% CI)	p	Relative risk (95% CI)	p
Age >55 years	1.10 (0.85–1.4)	0.2	1.03 (0.70–1.50)	0.5
Female sex	1.17 (0.89–1.53)	0.16	1.13 (0.76–1.68)	0.32
Advanced stage (ISS:3)	1.31 (0.99–1.79)	0.03	1.38 (0.92–2.08)	0.07
LDH upper normal	2.14 (1.30–3.51)	0.006	1.26 (0.86–1.84)	0.18
EMD at diagnosis	1.80 (1.06–3.06)	0.04	1.15 (0.75–1.78)	0.35
<VGPR achievement pre-AHSCT	1.31 (0.98–1.7)	0.03	1.64 (1.05–2.50)	0.014
Early relapse	3.44 (1.9–6.12)	<0.001		
No AHSCT	1.38 (0.78–2.4)	0.2	0.97 (0.6–1.6)	0.6
B. Univariate analysis	Patients ≤55 years of age (n=58)			
	Survival		Relapse/progression	
	Relative risk (95% CI)	p	Relative risk (95% CI)	p
Female sex	1.45 (1.01–2.08)	0.05	1.6 (0.91–2.80)	0.045
Advanced stage (ISS III)	1.51 (1.01–2.21)	0.021	1.30 (0.74–2.3)	0.24
LDH upper normal	2.38 (1.09–5.22)	0.04	1.83 (1.17–2.85)	0.02
EMD at diagnosis	1.64 (0.69–3.80)	0.01	1.10 (0.65–2.10)	0.03
<VGPR achievement pre-AHSCT	0.99 (0.66–1.29)	0.44	1.04 (0.65–1.80)	0.54
Early relapse	1.51 (0.69–3.31)	0.23		
No AHSCT	0.87 (0.54–1.40)	0.53	1.25 (0.41–3.78)	0.51
MM-RASS	1.85 (1.0803.1)	<0.001	1.78 (0.83–3.41)	0.02
C. Multivariate analysis	Patients ≤55 years of age (n=58)			
	Survival		Relapse/progression	
	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p
MM-RASS	4.59 (1.58–13.3)	0.004	2.46 (1.11–5.43)	0.031
Female sex	7.10 (1.6–30.05)	0.002	2.3 (0.88–6.27)	0.086
Early relapse	0.032 (0.005–0.17)	< 0.001		
Advanced stage (ISS III)	2.6 (0.41–15.41)	0.28	1.88 (0.62–5.69)	0.26
LDH upper normal	0.40 (0.039–4.20)	0.45	0.43 (0.13–3.89)	0.16
EMD at diagnosis	0.1 (0.014–2.30)	0.1	1.10 (0.65–2.10)	0.13
<VGPR achievement pre-AHSCT	1.2 (0.17–8.86)	0.83	1.00 (0.37–2.68)	0.54
No AHSCT	0.64 (0.27–15.5)	0.80	8.5 (1.80–40.03)	0.007

Notes: A: Whole cohort. B: Patients aged ≤55 years. C: Patients aged ≤55 years. MM-RASS demonstrated as an independent significant factor for both survival rates (along with sex and relapse/progression within 18 months postdiagnosis) and relapse/progression incidence (along with AHSCT).

Abbreviations: ISS, International Staging system; LDH, lactic acid dehydrogenase; EMD, extramedullary disease; MM-RASS, multiple myeloma risk-assessment scoring system; VGPR, very good partial remission; AHSCT, autologous stem-cell transplantation.

Patients ≤55 Years Old

A total of 58 patients were aged ≤55 years at the time of disease diagnosis: 48 (83%) received VRD (n=26), VCD (n=17), or VTD (n=5), and ten (17%) received only the VD combination (Table 1). The ORR was superior for patients aged ≤55 years (88% vs 78%); however, this difference was not statistically significant ($p=0.15$), while sCR and CR rates were similar (38% vs 36%; Table 1). Given their fitness and the lower incidence of comorbidities, it was expected that the vast majority of patients ≤55 years (n=53, 91%) underwent AHSCT.

All in all, 29 patients found to have disease progression/recurrence (either biochemically only or along with clinical symptoms) after a median of 49 months. The 4- and 8-year probability of disease relapse/progression was 47% and 79%, respectively (Figure 1B). Patients with clinical symptoms or who had only biochemical relapse/progression but experienced fast disease turnover were salvaged with combinations of carfilzomib plus pomalidomide, while lenalidomide was used for those patients who did not receive lenalidomide as frontline treatment. Only a minority of relapsed/progressed patients received daratumumab-based regimens, since this agent has been available for only the last 3 years in our center.

Median OS for the subgroup of patients aged ≤55 years was not reached, while 4- and 8-year OS was only marginally superior than those >55 years (75% vs 61% and 53% vs 50%, respectively, $p=0.3$; Figure 1A). However, based on the OS analysis, it was also conspicuous that a considerable number of the younger patients (eleven of 58) died within 4 years after diagnosis. The 4-year probability of death was estimated to be 25%, and in nine of eleven, death was attributed to uncontrolled MM. Median time from disease recurrence to death was 18.5 (3–31) months, despite the fact that they were salvaged with worldwide recommended therapeutic protocols. Two patients died from NDR causes (SARS-CoV-2 and car accident).

Contributing Factors in Survival of NDMM Patients ≤55 Years Old and Risk-Assessment System

Similarly to previously published studies, in our group of Y-NDMM patients, advanced disease stage (ISS III), LDH levels higher than the upper normal limit, and EMD at diagnosis were identified as negatively contributing factors to prolonged survival.^{10,11,22–28} Although the male preponderance in MM patients is a well-known phenomenon, in patient aged ≤55 years, the male:female ratio was similar (30:28).¹⁷ However, an interesting finding was that young female patients demonstrated significantly inferior survival rates to male patients, with projected 4- and 8-year OS of 65% vs 76% and 35% vs 76%, respectively ($p=0.05$). Karyotypic analysis was available for almost 90% of patients ≤55 years old. Only seven (12%) had high-risk karyotypes, and presumably for this reason high-risk cytogenetics unexpectedly showed no impact on OS in this group of patients. Nevertheless, five of seven with high-risk cytogenetics relapsed in a median of 20 (3–33) months. Based on these clinical findings, we created a novel risk-assessment system for Y-NDMM patients (MM-RASS), using five risk factors: female sex, LDH levels higher than the normal limit, EMD at presentation, ISS stage III, and less than VGPR achievement prior to AHSCT. Although in patients aged <55 years, achievement of VGPR prior to AHSCT showed no statistically significant impact on survival rates, we chose to incorporate the response rates after frontline treatment in the proposed MM-RASS as an indicator of disease kinetics.^{30–33} With regard to OS, no significant differences were found for patients with zero, one, or two factors; however, for those with more than two factors, the outcome was extremely dismal. Therefore, we separated patients in two groups: those with zero to two factors (n=40, favorable group) and those with three or more factors (n=18, unfavorable group). The favorable group achieved significant better 4- and 8-year OS of 86% and 70%, respectively, and median OS was not reached, while for the unfavorable group 4- and 8-year OS was 44% and 18%, respectively, and median OS estimated to be 46 months ($p<0.001$, Figure 2A). More importantly, in the Cox proportional hazard model, the MM-RASS emerged as an independent predictive factor for prolonged survival ($p=0.004$, Table 2C). The MM-RASS was also studied as a predictive factor for disease recurrence. On univariate analysis, median time to relapse/progression was 2.5-fold shorter for the unfavorable group than the favorable group (25 vs 60 months, $p=0.02$), while on multivariate analysis the MM-RASS proved also to be an independent prognostic factor for disease relapse/progression ($p=0.031$, Figure 2B and Table 2C).

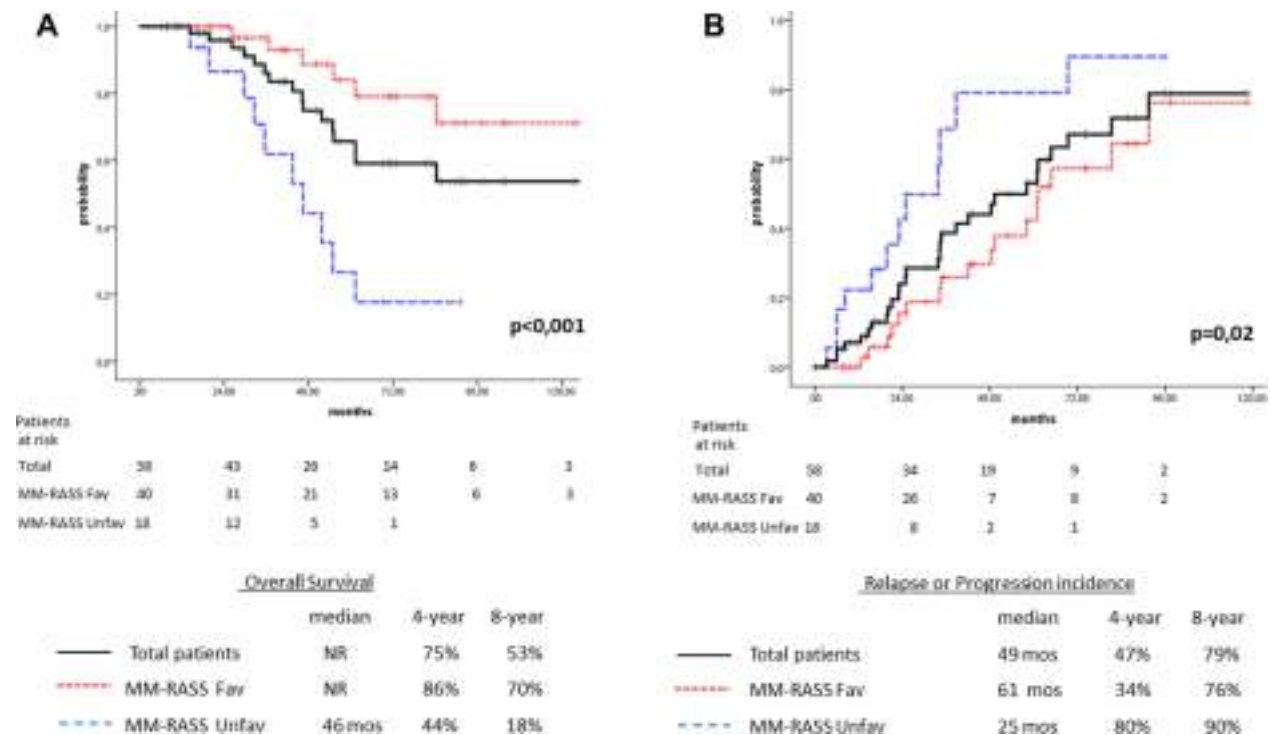


Figure 2 (A) Overall survival for patients aged ≤ 55 years according to the MM-RASS: total cohort ($n=58$, solid line), favorable score ($n=40$, dotted line), and unfavorable score ($n=18$, dashed line). **(B)** Relapse/progression of disease for patients aged ≤ 55 years according to the MM-RASS: total cohort of patients ($n=58$, solid line), favorable score ($n=40$, dotted line), and unfavorable score ($n=18$, dashed line).

Abbreviations: NR, not reached; mos, months; MM-RASS, multiple myeloma risk-assessment scoring system.

Comparison Between ISS and MM-RASS

Five patients who had been assessed with non-advanced disease (ISS stage I or II) were escalated to the unfavorable group as per the proposed MM-RASS: three of five (60%) died early due to disease relapse/progression at 20, 30, and 32 months postdiagnosis. On the other hand, eleven patients with advanced disease (ISS III) were de-escalated to the favorable group as per the MM-RASS, and interestingly only three of eleven (27%) succumbed to relapse/progression at 46, 55, and 84 months after disease diagnosis. Survival rates were similar for the non-high-risk patients (ISS I–II or favorable MM-RASS). Median OS was not reached for either system, 4-year OS was 80% vs 86% and 8-year OS was 75% vs 70% for the ISS and MM-RASS, respectively. For high-risk patients (ISS III or MM-RASS unfavorable), median OS was 55 vs 44 months, 4-year OS 56% vs 44%, and 8-year OS 26% vs 18% for the ISS and MM-RASS, respectively ($p=0.25$, Figure 3A). Although the MM-RASS discriminated patients at high-risk for relapse/progression better than the ISS (4- and 8-year relapse/progression incidence 68% vs 80% and 84% vs 90% and median time for disease relapse/progression 34 months vs 25 months for ISS III and MM-RASS unfavorable, respectively), this superiority was not statistically significant ($p=0.3$, Figure 3B).

Discussion

The advent of novel agents has completely altered the clinical course of MM, improving patients' quality of life and survival rates; however, they have failed to put an end to the typical course of repeated relapses, which inevitably lead to death due to uncontrolled disease. Since MM is typically a disease of elderly people, scant data exist regarding the long-term efficacy of the currently used treatment protocols for Y-NDMM patients, especially for those with high-risk characteristics. In our study group, the median age of 55 years was lower than the reported median age in other published studies from the US and Europe, apparently because $<5\%$ of Saudis are aged >65 years, while the vast majority of patients had been treated with the current recommended protocols.^{19–21,34}

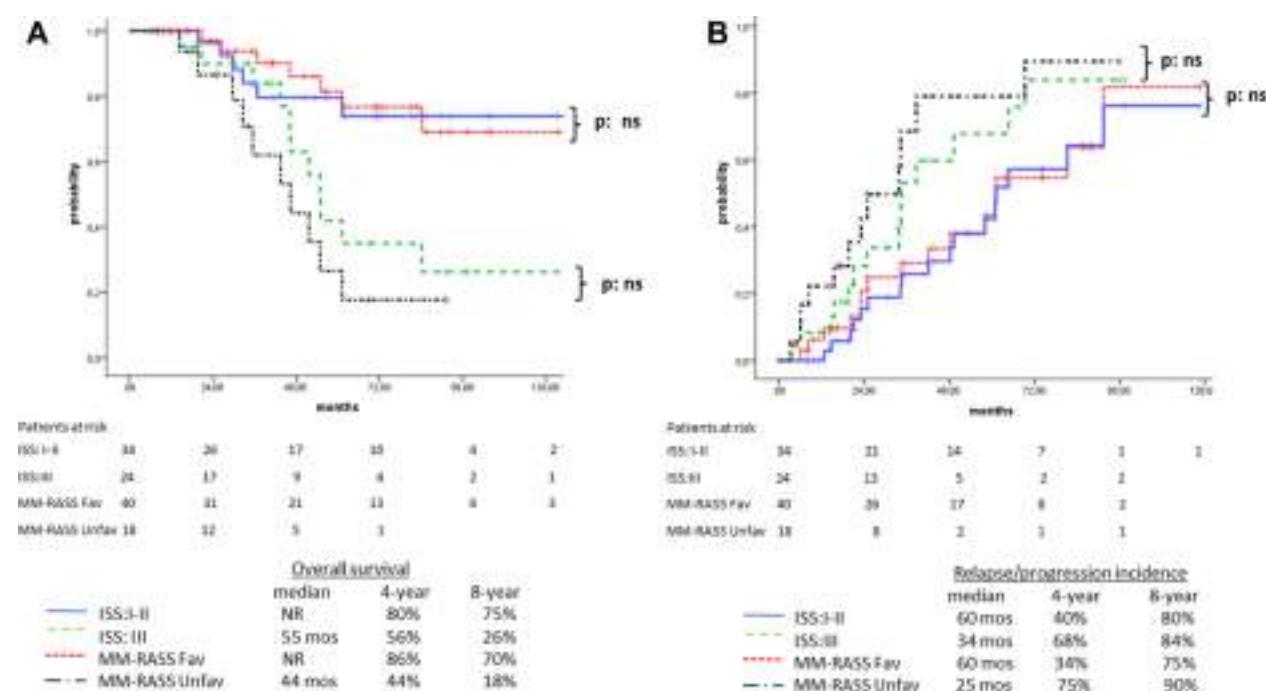


Figure 3 (A) Overall survival for patients with ISS I–II ($n=34$, solid line) and ISS III ($n=24$, dashed line) versus patients with favorable MM-RASS score ($n=40$, dotted line) and unfavorable MM-RASS score ($n=18$, dot/dashed line). The differences were not statistically significant. **(B)** Relapse/progression of disease for patients with ISS I–II ($n=34$, solid line) and ISS III ($n=24$, dashed line) versus patients with favorable MM-RASS score ($n=40$, dotted line) and unfavorable MM-RASS score ($n=18$, dotted/dashed line). The differences were not statistically significant.

Abbreviations: NR, not reached; mos, months; ISS, International Staging System; MM-RASS, multiple myeloma risk-assessment scoring system; Fav, favorable; Unfav, unfavorable.

It is ambiguous whether Y-NDMM patients have different disease characteristics from older patients. In the largest published study, which evaluated more than 10,000 NDMM patients, female sex, advanced ISS stage, elevated B2M, and low albumin were more frequent in patients aged >50 years, while LDH levels did not differ significantly between the two groups.³⁵ In our study, though with a smaller patient series, we did not observe any significant differences between patients younger or older than 55 years in terms of sex, advanced stage, B2M, levels of LDH, or presence of EMD at diagnosis, and we only observed low albumin levels more often in patients aged >55 years.

In disagreement with other studies, we observed only slightly better OS for patients aged ≤ 55 years than those aged >55 years.^{35,36} Possible explanations for this marginal OS superiority could be that the treatment protocols used were effective enough to get over the adverse impact of older age and that only 25% of our patients were older than 65 years. In the few studies designed for young patients, the only available data regarding the impact of novel agents on the outcome of younger patients come from studies with AHSCT-eligible patients.¹⁹ Undoubtedly, over the last few years OS has been prolonged to 7–10 years for transplant-eligible individuals. However, looking at the opposite edge of the survival curves, the indisputable fact is that even for this favorable age-group, the probability of death within 4–5 years post-MM diagnosis is approximately 20%–30%.^{35–40} In Ludwig et al study, autografted patients with aged <50 years demonstrated 4-year probability of death of approximately 30%. Nevertheless, it should be noted that patients were treated between 1981 and 2002 and thus novel triplet or quadruplet combinations were not in use.³⁵ Similarly, in a more recent multicenter study on NDMM up to 30 years, the 5-year risk of death was approximately 23%, but the evaluation period was 1989–2016, so the currently used treatment protocols were not available for a considerable number of patients.⁴⁰ In another study from Israel on a small series of Y-NDMM patients who received bortezomib-based therapy, eventually all patients relapsed shortly after treatment in a median 12 months. No deaths were reported, but it should be kept in mind that follow-up of the surviving patients was not mentioned in the study.⁴¹

In our study with 58 patients aged ≤ 55 years treated with widely accepted triplet therapies and salvaged with novel agents and with quite prolonged follow-up, we found that the probability of death within 4 years post-diagnosis was 25%.

This result confirms that the heterogeneity of disease outcome is not only a characteristic of older but also of younger patients. On uni- and multivariate analyses, we found that female sex, elevated LDH, presence of EMD at diagnosis, and ISS stage III after frontline treatment were strongly associated with poor outcomes. Disease response of less than VGPR after initial treatment was also associated with inferior survival rates, though with no statistical significance, probably due to the number of patients and/or because of therapies given to control relapsed disease post-AHSCT.

Data on the impact of patient sex on outcomes in MM are conflicting. A survey from the UK and an analysis from the International Myeloma Working Group reported similar survival rates for male and female MM patients.^{18,35} However, in the MRC Myeloma IX phase III clinical trial that evaluated 1,960 patients, significantly better median OS for male patients was observed (49.9 vs 44.8 months, $p=0.02$). The inferior survival of female patients was correlated with higher prevalence of poor prognosis and cytogenetic abnormalities.⁴² In our whole cohort ($n=116$), patient sex had no significant impact on survival or relapse/progression (Table 2A), but in Y-NDMM patients, similarly to the MRC Myeloma IX trial, female patients had poorer outcomes (Table 2B and C).

Elevated LDH levels suggest aggressive disease and usually indicate extramedullary and/or extraosseous MM, which is associated with shorter OS.^{11,23,24} In our Y-NDMM group, high LDH was correlated with a strong trend for inferior outcomes (Table 2A and B). There is increasing evidence that EMD represents a poor prognostic factor in MM. Although satisfying responses can be achieved with novel agent combinations, they are not sustainable and relapses are common, even after consolidation with AHSCT.^{25–27} In our patient series, in agreement with previous reports, the presence of EMD at diagnosis was associated with poor prognosis. It is generally accepted that achievement of CR or VGPR before AHSCT is not a mandatory objective for prolonged OS, but it has also been well documented that the deeper the response prior AHSCT, the better the outcome.^{30–33,43} We found that the achievement of at least VGPR was a favorable predictor of prolonged survival in both the whole group and <55-year-old patients, though it was not statistically significant (Table 2A–C).

An advantage of our proposed MM-RASS is that all the factors are easily assessable in any hematology center, not requiring special or laborious processing. A limitation could be the nonincorporation of cytogenetics or molecular characteristics in the proposed system. The high number of patients (20% of the whole cohort) lacking cytogenetic profiles did not allow us to incorporate cytogenetics in the proposed MM-RASS.¹⁶ This fact, at least partially, reflects the difficulty of analyzing cytogenetically NDMM patients because of the low proliferation rate of the malignant clone.⁴⁴ The sensitivity of interphase fluorescence in situ hybridization analysis does not require cell proliferation, but is sometimes affected by the low percentage of malignant plasma cells in whole-bone marrow samples. Therefore, for accurate cytogenetic/molecular results, more specific, laborious, and costly methods are required, which are eventually infeasible for several centers lacking specialized laboratory and experienced staff.^{45,46}

The MM-RASS showed better predictability of OS and relapse/progression incidence than the ISS, though the differences were not statistically significant. A plausible explanation for the better discrimination with the MM-RASS could be that two more factors with well-documented impact on MM outcome are additionally incorporated in the MM-RASS: EMD presence at diagnosis and response after frontline treatment, which reflects disease kinetics.^{25–33,43} Moreover, since the proposed MM-RASS takes into account disease response prior to AHCT (three to four cycles following initial treatment), it could be used as a reliable interim risk-assessment system during the treatment course.

Conclusion

This retrospective analysis of 58 patients aged ≤ 55 years represents one of the few studies of Y-NDMM patients who have been treated with the currently recommended triplet or doublet therapies. Our results confirm that even in the contemporary era of MM treatment, the appropriate therapy for a considerable number of Y-NDMM patients remains a major challenge. The MM-RASS we propose herein, which is exclusively targeted at Y-NDMM patients, shows remarkable strength in discriminating younger patients at higher risk of dismal outcomes. The MM-RASS merits further investigation and validation in larger series of patients and could potentially serve as an additional tool not only for initial but also for interim risk assessment, thus increasing the capability of early identification of high-risk Y-NDMM patients who are candidates for more intensive treatment approaches.

Data Sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

All authors made a significant contribution to the work reported, whether is in conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas, took part in drafting, revising, or critically reviewing the article, gave final approval to 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.

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Disclosure

The authors declare no competing interests.

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Hematological Profiles and Clinical Outcome of COVID-19 Among Patients Admitted at Debre Markos Isolation and Treatment Center, 2020: A Prospective Cohort Study

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Background: Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is coronavirus isolated from SARS patients. As far as the researchers' knowledge, there was paucity of studies conducted in Ethiopia, particularly in the study area. As immune protection is arisen from our blood cells, assessing their level will provide a clue for controlling the disease and monitoring the prognosis. This study will also provide additional information for clinical intervention and patient management.

Purpose: This study aimed to investigate the hematological profile and clinical outcome of coronavirus disease-19 (COVID-19) among patients admitted to the Debre Markos Isolation and Treatment Center (DMITC).

Material and Methods: A prospective cohort study was conducted among 136 COVID-19 adult patients at DMITC from January 1, 2020 to March 30, 2021. Data related to clinical, hematological profiles and socio-demographic factors were collected, entered into Epi data, and analyzed using STATA 14.2 software. Multivariable logistic regression was applied to determine the predictor variable and a p-value <0.05 was considered significant.

Results: Of 136 COVID-19 patients, 28.68% had died. The mean age of patients was 47.21±1.29 years. The hematological profile of the patients revealed that 28% had abnormal leukocyte, 23% abnormal lymphocyte, 44.85% abnormal granulocyte, 22.06% abnormal monocyte, 30.15% abnormal RBC and 87% abnormal platelet counts. The prevalence of anemia was 13.24%.

Conclusion: Leukocytosis (mainly granulocytosis and monocytosis) and lymphopenia, were the predominant abnormal findings of complete blood cell count (CBC) analysis of the patient's blood. Most of the patients had abnormally low platelet counts. RBC count and hematocrit determination were the only significant predictors of death. The clinician could manage cases according to the hematological findings of the patients. Further experimental studies should be conducted to determine hematological parameter changes and the clinical outcome of the disease.

Keywords: leukocytosis, hematological profile, COVID-19, clinical outcome, Debre Markos

Introduction

Background

The novel coronavirus was isolated from pneumonia cases in Wuhan city, China, 2019.¹ The disease caused by the novel coronavirus is called Coronavirus disease of 2019 (COVID-19), given by WHO² and the virus causing the disease called Severe acute respiratory syndrome coronaviruses-2 (SARS-CoV-2), given by the Coronavirus Study Group of the International Committee on Taxonomy of Viruses. SARS-CoV-2 is classified under the family *Coronaviridae*, genus *Betacoronavirus*, subgenus *Sarbecovirus*.³ A study⁴ suggested that the human receptor for SARS-COV-2 could be

angiotensin-converting enzyme 2 (ACE2), similar to other SARS-CoV viruses' portal of entry into human cells are through ACE2.⁵ The 5'-terminal two-thirds of the genome encodes a polyprotein (non-structural proteins) that are involved in genome transcription and replication whereas the 3' end encodes structural proteins, including envelope glycoproteins spike (S), envelope (E), membrane (M) and nucleocapsid (N).⁶ The rate of its transmission is higher than SRAS-CoV and the reason could be the occurrence of genetic recombination at S protein in the RBD region of SARS-CoV-2.⁷ The human-to-human spreading of the virus occurs due to close contact with an infected person, exposure to coughing, sneezing, respiratory droplets, or aerosols. These aerosols can penetrate the human body (lungs) via inhalation through the nose or mouth.⁸⁻¹⁰

The exact pathogenesis of SARS-CoV-2 infection in humans is poorly understood. However, some studies stated that the pathogenesis is related to dysregulation of inflammatory mediators and presence of different strain of the virus.¹¹ Most patients have mild symptoms and good prognosis after infection, but some patients developed severe and die from multiple organ complications.¹² The virus can affect the respiratory system, gastrointestinal system, renal system, circulatory system. The pathological changes in these organs may be caused directly by the cytopathic effect mediated by local replication of the SARS-CoV-2; or indirectly as a result of systemic responses to respiratory failure or the harmful immune response induced by viral infection.¹³

There is no specific antiviral drug, but there are vaccines against the virus which are approved to alter the immune function that helps the patient to recover from illness.¹²

The complications of COVID-19 are pulmonary embolism, venous thromboembolism, and arterial thrombotic events.¹⁴ Patients with COVID-19 infection can also present with acute encephalopathy and changes in their level of consciousness.¹⁵ Complications were more common in patients with cardiac injury, acute respiratory distress syndrome (SARS), acute kidney injury, electrolyte disturbances, hypoproteinemia and coagulation disorders.¹⁶

Studies had found increased white blood cell and neutrophil counts¹⁷ but decreased lymphocyte counts. In addition, there was decreased T cells (both regulatory and helper cells) and NK cells.¹¹

However, as far as the researchers' knowledge, there were few studies done in Ethiopia. As a result, we investigated the hematological profile and clinical outcome of COVID-19 among patients admitted at in Debre Markos Isolation and treatment center (DMITC). This study will provide the hematological profile and clinical outcome of COVID-19 patients which will be important to clinicians for management of patients.

Materials and Methods

Study Design, Area and Period

A prospective cohort study was conducted among COVID-19 patients at DMITC from January 1, 2020 to March 30, 2021. DMITC was established at the end of April 2020 in the new Campus of Health Science in Debre Markos town. Debre Markos town, the capital city of East Gojjam zone, is found 299km from Addis Ababa and 264 km from Bahir Dar.

Population

COVID-19 confirmed adult patients admitted to the treatment center during the study period at DMITC site were the study population.

Sample Size Determination and Sampling Technique

In Ethiopia, there were few studies conducted on hematological profile and clinical outcome of COVID-19 patients. As a result, the sample size was determined using single population proportion formula but not calculated for associated factors. It was calculated using the assumption of 50% clinical outcomes, 95% confidence level, and a 5% margin of error. The sample size was determined using 50% proportion clinical outcome (death/recovery=0.5) 95% confidence level ($Z_{\alpha/2}$) =1.96 and margin of error 0.05. The total sample size calculated was 384, but the number of COVID-19 confirmed cases in the last 3 months in the COVID 19 treatment center were 180, which is less than 10,000 and needs population correction formula.

As a result, the corrected sample size is

$$nf = \frac{384}{1 + 384/180} = 123$$

About 10% of non-response rate was added to the calculated sample size (123) and giving 136, that is $123 + (123/10) = 136$.

Systematic random sampling technique was used to obtain 136 study participants. The k value was determined by $135/180 = 0.75$, 1. Therefore, we recruited study participants in every other order of registration sequence.

Data Collection Tools and Procedures

Data were collected from COVID-19 positive patients using a predesigned structured questionnaire. The researchers trained five data collectors; two general practitioners, two nurses, and two laboratory technologists on the aim and objectives of the research, blood sample collection and processing procedures, and infection prevention mechanism to prevent COVID-19 infection at DMITC. The trained data collectors explained the aim of the study to patients or their clinician in order to obtain consents and samples. Socio-demographic variables and COVID-19 related clinical data were collected by trained health professionals working in DMITC. Data were filled on questionnaire and kept confidential, and the consistency of data filled was rechecked every day.

Measurement of Variables

Hematological profile and clinical outcome of COVID-19 patients were determined using independent factors; socio-demographic variables (age, sex, residence, etc) and factors related to change in hematological profile of COVID-19.

Hematological Profile

About 3mL of blood samples were collected from COVID-19 patients during admission and discharge. These samples were analyzed by Mindray BC-3000 Plus CBC hematology analyzer (MINDRAY, China) for complete blood cell count (CBC) determinations and altitude values have been adjusted using WHO guidelines for red cell count and hemoglobin measurements. Mean value of each measurement of the parameter for a patient was used. Mean absolute counts of white blood cells, granulocytes, lymphocytes, and Monocytes were used to categorize abnormal cell counts as “low” and “high”. We used CBC reference range of Debre Markos Comprehensive and Specialized Hospital to categorize the cell count as normal and abnormal.

Data Management, Analysis and Interpretation

Data were entered into the Epi-Data version 4.2 software and analyzed with STATA 14.2 software. Data cleaning was carried out to check the frequency, accuracy, consistency and missed values; any identified errors were corrected. Frequencies and proportion were used to describe the socio-demographic characteristics of the patients. Bivariate logistic regression was done using independent variables in order to identify candidate variables (p -value < 0.25) for the multivariable logistic regression. Multivariate logistic regression model was made and predictors of abnormal hematological profiles were determined among the selected candidate variables fitted to multivariable logistic regression model. The statistical significance was determined in the multivariate analysis at a confidence interval of 95% at a p -value < 0.05 .

Results

Socio-Demographic Characteristics

A total of 136 study participants were involved in the study. From these participants, 85 (62.5%) were males and 51 (31.5%) females. The mean age of the patients was about 47 ± 1.29 (SD) years.

Majority (30.88%) of the patients were in the age range 40–49 years followed by 60 years and above. The least number of patients were found in the age range ≤ 19 years. Most of them (85.29%) had no history of smoking but 97.04% drank alcohol. From the total patients, nearly one-fourth of them (27.94%) used traditional medicine for the treatment of COVID-19 disease (Table 1).

Table 1 Socio-Demographic Characteristics of COVID-19 Patients, 2020

Variables	Category	N (%)
Sex	Male	85 (62.50)
	Female	51 (37.50)
Age	Mean ($\pm$ SD age)	47.21 $\pm$ 1.29
	≤ 19	2 (1.47)
	20–29	25 (18.38)
	30–39	11 (8.09)
	40–49	42 (30.88)
	50–59	25 (18.38)
	≥ 60	31 (22.79)
History of smoking	Yes	20 (14.71)
	No	116 (85.29)
Alcohol	Yes	132 (97.06)
	No	4 (2.94)
Traditional medicine use	Yes	38 (27.94)
	No	98 (72.06)

Abbreviations: SD, standard deviation; N, number; %, percent.

Clinical Profile and Outcome of COVID-19 Patients

Of 136 COVID-19 patients, most (97.79%) of them had symptomatic clinical manifestation. From the clinical manifestations, joint pain (95.59%), cough (94.12%), headache and vomiting (93.38%), loss of appetite (93.38%), respiratory distress syndrome (73.53%) and fever (68.38%) were the predominant symptoms, respectively. The mean respiratory rate, temperature, pulse rate, systolic blood pressure, diastolic blood pressure, and oxygen saturation of the patients was 28.87 $\pm$ 0.56, 37.33 $\pm$ 0.1104.8 $\pm$ 1.86, 109.98 $\pm$ 1.73, 68.04 $\pm$ 0.74, 89.75 $\pm$ 0.35 respectively (Table 2).

There were different comorbidities identified with COVID-19. Of the COVID-19 patients, 20.59% had hypertension, 12.50% diabetes mellitus, 2.94% HIV, 1.47% malignancy, 8.09% asthma, 11.03% chronic obstructive pulmonary disease (COPD), 2.94% chronic kidney disease CKD, 5.88% chronic liver disease (CLD), 30.88% congestive heart failure (CHF) and 55.88% other comorbidities (Table 3).

The outcome status of COVID-19 positive patients during discharge revealed that 39 patients (28.68%) had died and 97 (71.32%) recovered from the disease. From the total deaths, 69.23% were males. More males (59.79%) had recovered from the disease than females (40.21%) (Table 4).

There were 58 mild, 47 severe and 31 critical cases of COVID-19 during their admission. From the mild case only one patient died, but 12 severe cases and 26 critical cases passed away. The case fatality rate (83.87%) was higher in critical patients (Figure 1).

Hematological Profile of COVID-19 Patients

The total white blood cells and differential counts of 136 COVID-19 patients were performed, and majority of them (72%) had normal white blood cell counts and 28% had abnormal leukocyte counts (5.88% low WBC count versus 22.06% high WBC counts). Among COVID-19 patients of low WBC counts, two died and six (75%) recovered. Similarly, 27.55% patients who had normal WBC count died and 72.45% recovered. However, one-third (33.33%) of the patients who had high WBC count died and 66.67% recovered.

The lymphocyte counts of the study participants indicated that 16.91% low, 77.21% normal and 5.88% high counts, respectively. The prevalence of abnormal lymphocyte counts (low and high cell counts) was about 23%. Patients with 30.43% of low and 12.5% of high lymphocyte counts died. However, patients with 69.57% of low, 70.48% of normal and 87.5% high lymphocyte counts had recovered from the disease.

Table 2 Clinical Profile of COVID-19 Patients Admitted at DMU COVID-19 Treatment Center, 2020

Variables	Category	N (%)
Asymptomatic presentation	Yes	3 (2.21)
	No	133 (97.79)
Fever	Yes	93 (68.38)
	No	43 (31.62)
Cough	Yes	128 (94.12)
	No	8 (5.88)
Respiratory distress	Yes	100 (73.53)
	No	36 (26.47)
Headache and vomiting	Yes	127 (93.38)
	No	9 (6.62)
Joint pain	Yes	130 (95.59)
	No	6 (4.41)
Loss of appetite	Yes	127 (93.38)
	No	9 (6.62)
Other symptoms	Yes	113 (83.09)
	No	23 (16.91)
	Mean	SD
Mean respiratory rate	28.87	0.56
Mean temperature	37.33	0.1
Mean pulse rate	104.80	1.86
Mean SBP	109.98	1.73
Mean DBP	68.04	0.74
Mean oxygen saturation	89.75	0.35

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; SD, standard deviation; N, number; %, percent.

The granulocyte counts of COVID-19 patients were 17.65% low, 55.15% normal and 27.21% high. From patients who had abnormal granulocyte counts (low and high counts), 16.67% of low and 35.14% of high counts died. Most of the patients who had low (83.33%), normal (70.67%) and high (64.86%) granulocyte counts were recovered.

The monocyte count of the patients indicated that only three patients had low cell counts. One of the three died and two of them recovered. About 55% of the patients had normal monocyte count; 28.30% of them had died and 71.7% had recovered. Of the total monocyte counts performed, 27.21% of the patients had high cell counts. From patients who had high counts, 29.63% died and 70.37% recovered.

The result of red blood cell (RBC) count of the study participants revealed that 11.76% had low and 18.38% high cell counts. And, 43.75% of the patients of low RBC counts and 16% of the high RBC counts died, whereas 56.25% of low and 84% of the high RBC counts recovered.

The platelet count abnormality was seen in nearly 87% (95% CI; 81–92.53) of the patients. From this abnormality, 64.71% had low and 22.06% high platelet counts. Among patients who had low platelet count, 31.82% died and 68.18% recovered. In contrast, patients with high platelet count, 20% died and most of them (80%) had recovered (Table 5).

The overall prevalence of anemia was 13.24%. The prevalence was the same in both sexes that is 6.62% (Figure 2).

The multivariable logistic regression result of hematological profile between death and recovery indicated that only red cell count (RBC) and hematocrit (Hct) were statistically significant predictors of death due to SARS-COV-2 ($P < 0.05$) (Table 6).

Discussion

The pandemic coronavirus has tremendous social, economic, environmental and health related negative impacts on different countries.^{18–20} Studies have shown that the disease severity is associated with hematological abnormalities like

Table 3 Comorbidities of COVID-19 Patients Admitted at DMU COVID-19 Treatment Center, 2020

Comorbidities	Category	N (%)
Hypertension	Yes	28 (20.59)
	No	108 (79.41)
Diabetes mellitus	Yes	17 (12.50)
	No	119 (87.50)
HIV	Yes	4 (2.94)
	No	132 (97.06)
Malignancy	Yes	2 (1.47)
	No	134 (98.53)
Asthma	Yes	11 (8.09)
	No	125 (91.91)
COPD	Yes	15 (11.03)
	No	121 (88.97)
CKD	Yes	4 (2.94)
	No	132 (97.06)
CLD	Yes	8 (5.88)
	No	128 (94.12)
CHF	Yes	42 (30.88)
	No	94 (69.12)
Other comorbidity	Yes	76 (55.88)
	No	60 (44.12)

Abbreviations: HIV, human immunodeficiency virus; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CLD, chronic liver disease; CHF, congestive heart failure; N, number; %, percent.

Table 4 Clinical Outcome Status of COVID-19 Positive Patients During Discharge

Sex	Status Outcome During Discharge		
	Death, N (%)	Recovery, N (%)	Total, N (%)
Male	27 (69.23)	58 (59.79)	85 (62.5)
Female	12 (30.77)	39 (40.21)	51 (37.5)
Total	39 (100.00)	97 (100.00)	136 (100.00)

Abbreviations: N, number; %, percent.

anemia, leukocytosis, neutrophilia, and combined neutrophilia-lymphopenia.^{21–24} However, few studies show the exact hematological abnormality predictor related to disease severity or clinical outcome.

The mean age (47.21±2.9) of the patients was in line with the study done in India (49±17.4).²⁵

The current study had shown that most of the patients (97.79%) had symptomatic clinical manifestation. This finding is higher than a study done in Wuhan, China, which was 60%.²⁶ The higher finding might be related to increased severity of SARS-COV-2 or reduced immunity of the population.

Of the manifestations: joint pain, cough, headache, vomiting, loss of appetite, respiratory distress syndrome, and fever were the common symptoms. The mean oxygen saturation level (89.75%) of the patients was lower than the National Institute of Health recommended reference range (92–96%) for both inpatient and outpatient COVID-19-positive patients.²⁷ The clinical outcome of the patients revealed that 28.68% of the patients died and majority of the deaths were males. On the contrary, the number of male patients recovered from the disease were higher than female patients. There were different comorbidities identified with COVID-19. The common comorbidities were hypertension,

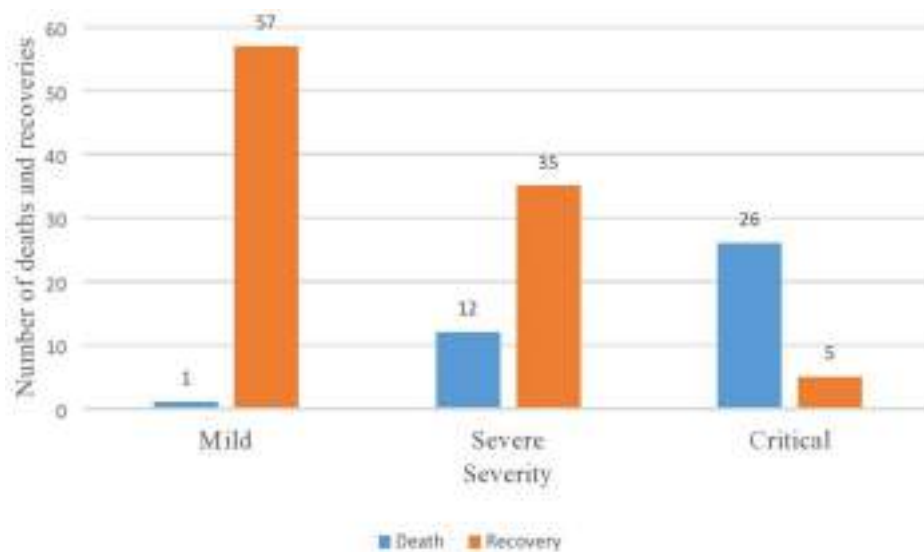


Figure 1 Severity of COVID-19 among patients admitted at DMU COVID-19 treatment center, 2020.

diabetes mellitus, HIV, malignancy, asthma, chronic obstructive pulmonary disease (COPD), chronic kidney disease CKD, chronic liver disease (CLD), and congestive heart failure (CHF). These comorbidities increased the death rate of patients in the study area.

The hematological profile of patients indicated that 28% of COVID-19 patients had abnormal WBC count; 5.88% leukopenia and 22.06% leukocytosis which is in line with a study done in Wuhan, China, indicated that 9% leukopenia

Table 5 Cell Count Abnormality of COVID-19 Patients Admitted at DMU COVID Treatment Center 2020

Cell Count Parameters	Category	Death (39)	Recovered (97)	Total
		Frequency (%)	Frequency (%)	Frequency (%)
WBC	Low	2 (25.00)	6 (75.00)	8 (5.88)
	Normal	27 (27.55)	71 (72.45)	98 (72.06)
	High	10 (33.33)	20 (66.67)	30 (22.06)
Lymphocyte	Low	7 (30.43)	16 (69.57)	23 (16.91)
	Normal	31 (29.52)	74 (70.48)	105 (77.21)
	High	1 (12.50)	7 (87.50)	8 (5.88)
Granulocyte	Low	4 (16.67)	20 (83.33)	24 (17.65)
	Normal	22 (29.33)	53 (70.67)	75 (55.15)
	High	13 (35.14)	24 (64.86)	37 (27.21)
MID	Low	1 (33.33)	2 (66.67)	3 (2.21)
	Normal	30 (28.30)	76 (71.70)	106 (77.94)
	High	8 (29.63)	19 (70.37)	27 (19.85)
RBC	Low	7 (43.75)	9 (56.25)	16 (11.76)
	Normal	28 (29.47)	67 (70.53)	95 (69.85)
	High	4 (16.00)	21 (84.00)	25 (18.38)
Platelet	Low	28 (31.82)	60 (68.18)	88 (64.71)
	Normal	5 (27.78)	13 (72.22)	18 (13.24)
	High	6 (20.00)	24 (80.00)	30 (22.06)
Total				100%

Note: *The categorization of CBC results as low, normal and high was done based on the standard operating procedure of DMCSH Laboratory.

Abbreviations: WBC, white blood cell; RBC, red blood cell; MID, middle sized white cell.

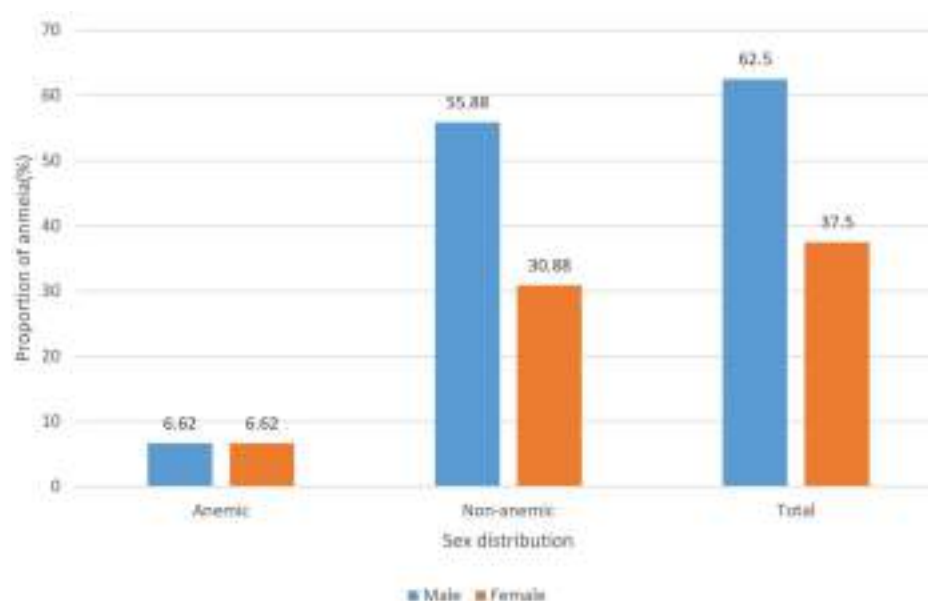


Figure 2 Prevalence of anemia among COVID-19 patients admitted at DMU COVID treatment center 2020.

and 24% leukocytosis.²⁶ However, leukocytosis in the current study is higher than 17.32%, but leukopenia is lower than 14.71% in Wuhan.²⁵ The lymphocyte count of the patients revealed that 16.9% of the patients had lymphopenia which is lower than 25.2% in China,²⁸ but lymphocytosis was seen in only 5.88% of the patients. The current finding of lymphopenia was lower than 35% in Wuhan, China.²⁶ Lymphopenia is a common feature in viral disease, which has been related to the cytopathic effect due to the affinity of the virus for angiotensin-converting enzyme inhibitors of lymphocytes.²⁹ The other possible explanation associated with lymphopenia is increased inflammatory response which leads to increased granulocyte production but lymphocyte apoptosis.³⁰ Nearly, one-third of lymphopenia patients died but only 12.5% of the patients with lymphocytosis died. This finding is related with the review done by Huang et al 2020 which stated that patients who had lymphopenia had poor outcome.³¹

Abnormal platelet count was detected nearly among 87% of the study participants in the present study which is in line with a systematic review study done by Bhattacharjee et al (84.5%).³² Nearly two-third of the platelet count

Table 6 Multivariable Logistic Regression of Hematological Profile of COVID-19 Patients, 2020

Discharge Status	CBC	Beta (Coefficient)	SE	P (Z)-value	95% CI
Death	WBC	-0.62	0.70	0.38	(-1.99, 0.75)
	Lymphocyte	0.57	0.52	0.27	(-1.58, 0.45)
	Granulocyte	0.91	0.58	0.11	(-0.22, 2.04)
	Monocyte	0.26	0.56	0.64	(-0.83, 1.35)
	RBC	-1.4	0.51	0.006*	(-2.41, -0.40)
	Platelet	-0.08	0.31	0.8	(-0.68, 0.53)
	MCV	-0.45	0.36	0.21	(-1.16, 0.26)
	MCH	0.30	0.26	0.26	(-0.22, 0.81)
	MCHC	0.12	0.27	0.67	(-0.41, 0.64)
	Hct	-1.93	0.86	0.02*	(-3.61, -0.26)
	Hgb	0.5	0.56	0.36	(-0.59, 1.59)
	RDW CV	0.31	0.3	0.3	(-0.28, 0.90)
Recovered (base outcome)	I				

Abbreviations: MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; Hct, hematocrit; Hgb, hemoglobin.

abnormalities (64.71%) were thrombocytopenia which is higher than studies done in Wuhan, China (20.7%) and by Lippi et al (55%).³³ The possible mechanism of thrombocytopenia in COVID-19 infected patients might be related to direct effect of the virus on the bone marrow cells or platelets by reducing platelet synthesis, creating dysfunctional marrow microenvironment; liver damage by the virus leading to decreased thrombopoietin production; pulmonary endothelial damage followed by platelet aggregation in the lungs, subsequent formation of microthrombi, and platelet consumption; and finally, the destruction of platelets by cross reacting antibodies.³² A study had shown that thrombocytopenia increased the risk of COVID-19 severity over five folds.^{33,34} The prevalence of anemia in the current study (13.24%±2.92) was lower than 24.7% in Austria and 25.6% and 61% in Italy, respectively.^{35–37} According to the WHO classification, the type of anemia detected among patients was mild anemia.³⁸ The difference might be related to the difference in the study population, the immunity of patients and severity of the disease or other underlying clinical conditions. The increased prevalence of anemia among COVID-19 patients are associated with SARS-CoV-2 infection which may lead to hemolytic anemia directly through cytopathic injury of red cells or indirectly through formation of auto-antibodies.³⁹

Conclusion

A concrete study has shown that COVID-19 has three critical clinical phases; phase I (initial phase), phase II (propagating phase) and phase III (complicating phase).⁴⁰ The study indicated that the hematological changes are categorized under propagating phase (Phase II)s.

The majority of the cases were recovered from COVID-19. The hematological finding of COVID-19 patients indicated that nearly one-third of the patients had abnormal WBCs, and RBC counts. Moreover, most of the patients had abnormally low platelet cell counts. Of the white cell count abnormalities, leukocytosis, lymphocytopenia, granulocytosis, and monocytosis were the common findings. Mild anemia was observed among patients. RBC count and hematocrit level were the only significant predictors of death ($P<0.05$). The case fatality rate was higher in critical cases than in any other stages of severity of the disease.

The clinician could manage cases according to the hematological findings of the patients. Further large-scale experimental studies should be conducted to determine hematological parameter changes and associated factors. The main limitation of this study is that we could not perform coagulation tests like PT, aPTT, INR and D-dimer as well as inflammatory markers due to lack of reagents and equipment during the study period in the study setting.

Abbreviations

aPTT, activated partial thromboplastin time; CBC, complete blood count; COVID-19, coronavirus disease 19; INR, international normalized ratio; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; MCH, mean cell hemoglobin; PT, prothrombin time, RBC, red blood cell; RDW, red cell distribution width; SARS-COV-2, severe acute respiratory syndrome; WBC, white blood cell.

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 complies with the Declaration of Helsinki. Ethical approval was obtained from Debre Markos University Health Science College Institutional Research Ethics Review Committee (IRERC) with reference number HSC/R/C/Ser/Co/202/11/13. Then, an informed written consent was obtained from each participant after explaining aim of the study. The study participants were briefed on the study's purpose, procedures, potential risks, and benefits. In addition, they were told that they had the right to withdraw from the study which would not endanger their access to treatment.

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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.

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Disclosure

The authors declared that there are no competing interests.

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Background and Objectives: The ABO blood group system is used to denote the presence of one, both, or neither of the A and B antigens on erythrocytes. In humans, this is the most important of the 43 different blood type (or group) classification systems currently recognized. Distribution of ABO and Rh among the Saudi population could affect many factors such as blood transfusion, prognosis, resistance and susceptibility to diseases. The impetus of this study was to develop an understanding of the distribution of the ABO and Rh types of healthy volunteer blood donors in the northern Asir region of Saudi Arabia, comparing the findings to similar studies. Understanding the frequency of different blood types in different populations is imperative.

Subjects and Methods: This study was performed during the period of January 1, 2020, to December 31, 2020, using samples from 4167 voluntary subjects obtained from the blood bank of the King Abdullah Hospital, Bisha. Commercially provided anti-A, anti-B and anti-D antibodies were used for the standard blood grouping procedure.

Results: The findings in this study showed that O was the most prominent ABO blood group, whereas AB was the least so. The frequency of the blood type A was the second highest, followed by the blood group B, whereas Rh positivity was more common than Rh negativity.

Conclusion: $O^+ > A^+ > B^+ > O^- > AB^+ > A^- > B^- > AB^-$ was found to be the order of blood group frequency in the current study.

Keywords: ABO, rhesus, donors, immunoglobulins

Introduction

Landsteiner described the ABO blood group system in the 20th century. Blood cell antigens are inherited from the parents through a single gene and have clinical significance and RBC antigens are a group of structural proteins and carbohydrates that are present on the extracellular surface of the RBC membrane.¹⁻³ To date, there are 43 blood group systems with 360 known antigens according to the ISBT, which are able to stimulate an immune response. These systems include ABO and Rh, which in the case of mismatching could result in severe forms of posttransfusion reactions.² Antibodies in the ABO system are typically found in the serum of healthy people.⁴ For example, immunoglobulins such as IgM and IgG against blood groups antigens may very well be present in individuals that express A and B antigens. However, IgM and IgG against ABO antigens are mostly present in individuals that lack the A and B antigens. Individuals are either RhD positive or RhD negative depending on the presence or absence of the RhD phenotype on the surface of the red cells. In blood transfusion therapy, antigens A, B and D are the most important red cell antigens, as donor-patient ABO mismatching could result in severe posttransfusion reactions.² Except for auto anti-D cases, Individuals whose red cells lack the D antigen cannot have anti-D in their serum unless they have been exposed to a sensitizing event that initiated the production of anti-D antibodies, in contrast to the case of A and B antigens. Overall, blood group compatibility has clinical significance that is essential for blood management in a wide variety of clinical cases. The ABO and Rh phenotype frequencies in given populations in different parts of the world have been studied. Studies that have been conducted in different parts of Saudi Arabia show similar frequencies of the ABO Rh group. For instance, a study that was conducted in the Eastern region found that the O blood group is the most prevalent, followed by A, B and AB respectively. Moreover, Rh-positive blood donors were more prevalent than Rh-negative

blood.⁵ Furthermore, several studies conducted in the Al-Jouf, Hail, Najran and Jazan provinces, showed similar patterns of ABO Rh distribution [Eweidah et al 2011⁶ and Abdullah et al 2010⁷]. Such studies may provide an idea about different hematological conditions, such as hemolytic disease of the fetus and newborn (HDFN). Blood groups are known to have some association with several clinical conditions, such as urinary tract infection and diabetes [Babiker Mohammed 2013⁸, Sinha et al 2015⁹]. The familiarity of the frequencies of ABO and Rh blood groups in the residents of a given regional area is essential for proper operative management at local hospitals and blood banks. Such a study may contribute to the effective management of a blood bank's inventory and provide safe and reliable blood transfusion services. Thus, this study purposely focused on the population of the Bisha Province in the Asir region.

Objective

This study aimed to screen and determine the frequencies of the occurrence of ABO and Rh blood groups among residents of the northern Asir region in Saudi Arabia. This information will provide an idea about the distribution of particular blood groups in the area. This study included many donors from both the urban and rural populations of the province.

Methodology

A cross-sectional study was conducted using blood samples collected from blood donors who belong to different ethnic groups.

The Saudi Ministry of Health's (MOH) criteria for blood donation was followed in this study.¹⁰ As per the MOH criteria, donors were between 18 and 65 years old (male and female) and weighed above 50 kg. The donors met the minimum hemoglobin concentration required for both genders (between 14 and 17 g/dl for males and 12–14 g/dl for females) with normal vital signs, and they were at the blood bank for various reasons. Blood collection from the donors followed a clinical examination that determined their physical wellbeing. Samples were collected over a period of 1 year, from January 1, 2020, to December 31, 2020.

All volunteers donated blood of their own free will and after fulfilling the ethical criteria of the study. All donors provided informed written consent to participate in this study. The study was conducted under the tenets of the Declaration of Helsinki. The Institution Review Board of the University of Bisha approved the study protocols. Blood typing and collection from fit, voluntary donors was performed at the blood bank of the King Abdullah Hospital. The hospital is located in Bisha city, which is an important educational and urban center for the population of the northern Asir region in Saudi Arabia. The city is located in the southern part of Saudi Arabia at altitude of 610 meters above sea level.¹¹ The ABO and Rh statuses of 4167 samples were analyzed by forward blood grouping using commercially acquired anti-A, anti-B and anti-D antisera (Beckman Coulter Inc. Clones; Anti-A 9113D10, Anti-B 9621A8, Anti-D P3X61 + P3X21223B10 + P3X290 + P3X35). Agglutination reactions were observed after mixing the blood and antibodies on the slides, and the results were recorded.

The final ABO and Rh blood groups for each sample were determined based on the presence or absence of agglutination. Using the Microsoft Excel 2019 (Office 17) software, the data on the frequency of ABO and Rh-D blood groups were reported in simple percentages.

Results

In this study, which includes 4167 Saudi and non-Saudi voluntary blood donors, the majority of blood donors within the age range of 18–65 years were male (4058), while (109) were female. The most prominent blood group was O (45.90%), followed by A (27.40%), B (21.71%) and AB (5%) (Table 1).

The most prominent Rh factor among the donors in this study was Rh positive (89.12%) followed by Rh negative (10.87%) (Table 2).

Upon complete ABO and Rh factor analysis for all donors, it was noted that the blood group O+ was the most prevalent blood type (38.68%), followed by types A+ (24.81%), B+ (20.90%), O- (7.22%), AB+ (4.72%), A- (2.59%), B- (0.81%) and AB- (0.23%) (Table 3).

Table 1 Frequency and Distribution of the ABO System

	N	O	A	B	AB
Total	4167	1913 (45.90%)	1142 (27.40%)	905 (21.71%)	207 (5%)

Table 2 Frequency and Distribution of Rh Factor

	Rh+	Rh-
Total	3714 (89.12%)	453 (10.87)

Table 3 Distribution of ABO System and Rh Factor

Blood Type and Rh	N	Percentage
A+	1034	24.81%
A -	108	2.59%
B+	871	20.90%
B-	34	0.81%
AB+	197	4.72%
AB-	10	0.23%
O+	1612	38.68%
O-	301	7.22%
Total	4167	99.96%

Discussion

The current study determined the distribution of ABO and Rh types in the northern Asir region. The vast majority of the participants were male, which is consistent with similar studies in other parts of the Arabian Peninsula.¹² The reason behind the low number of female participants according to,¹³ was an inability to reach the blood donation centers and a fear of anemia, according to Al-Johar et al.¹⁴ The most prominent hurdle preventing them from donating was the difficulty of reaching the blood bank, as they could not drive cars or use public transport alone.^{15,16} Due to the infrequent donations from females, the ABO/Rh distribution might not be an accurate assessment of the true frequency.

Knowing the ABO-Rh D frequency in a given population helps to predict the prevalence of ABO and Rh HDFN.^{4,17,18} Moreover, the essential interaction among blood groups, organ transplantation and transfusion therapy make identifying the patient's blood type one of the vital sources of information in any clinical patient's report.¹⁹

The heterozygosity of blood types is attributed to the pattern of the ABO group and Rh mode of inheritance. A and B alleles are inherited in an autosomal codominant fashion, whereas the O allele is autosomal recessive. On the other hand, the Rh factor is inherited through autosomal dominant pattern.^{3,20}

There is a well-known correlation between ABO and some diseases such as cancer, hematologic disorders, cardiovascular diseases and malaria, but there are also many factors that affect the outcome of the disease.^{21–24} Furthermore, several studies have linked blood groups to certain clinical conditions, such as stomach cancer,²⁵ and infectious diseases, such as COVID-19, HBSAg, HCV, HIV and syphilis. For example, in patients who are suffering from stomach cancer, the prevalence of blood group A was greater than that of blood group O.²⁶ Most recently, a relationship between COVID-19 and blood groups has been established. The number of COVID-19 patients with blood group A was significantly higher than patients with other blood groups. On the other hand, the number of COVID-19 patients with blood group O was much lower than that of other

individuals. This finding suggests that individuals with the blood group A are at higher risk of COVID-19 infection and more severe clinical symptoms than individuals with the blood group O.²⁷ Among blood donors, a study that analyzed the association of blood groups to HBsAg has described the blood group O as the most prevalent blood group associated with HBsAg, followed by the blood groups B, AB and A.²⁸ Another study that was conducted in Northern India has found that HBsAg is highly associated with individuals with the B blood group but is less prevalent in AB blood group donors. However, it has been concluded that ABO blood groups are not statistically associated with HBsAg positivity among their cohort.²⁹ A recent study in Pakistan found that Individuals with the blood group O and Rh-negative factor had a strong association with HCV viremia compared to other blood types.³⁰ Interestingly, patients with β -Thalassemia demonstrated a higher association between A and B blood types while those with O blood groups appeared to have less vulnerability to the virus. Moreover, HCV was more prevalent in patients with the Rh D positive factor. Furthermore, a higher correlation between the blood group O and HIV infection was described by Sayal et al (1996).³¹ However, participants with the blood group B demonstrated a lower prevalence of HIV in the same study. A more recent study³² also described a higher incidence of HIV infection among O blood group donors compared to other blood types. On the other hand,³³ Mohammadali et al (2014) failed to establish a significant association between syphilis infection and blood groups, however, a high prevalence of syphilis was noted among Rh D positive participants. Another study failed to link blood groups to obesity.³⁴ An earlier study that was conducted in the eastern region of Saudi Arabia estimated the frequency of ABO blood groups and its findings relate to the results of the present study.⁵ The O blood group was determined to be the most prominent blood group in the current study (45%) and in the eastern region at large (51%). In both studies, the blood group A was the second most common blood type (27% and 26.5% in this study and the eastern region, respectively). Similarly, the B blood group was the third most common in both regions (21% and 18%, respectively). AB was the least dominant blood group in the eastern region (4%) and the northern Asir region (5%). Individuals who were rhesus negative in the eastern region accounted for only 8%, whereas Rh positive blood donors accounted for 92%. This finding is consistent with the findings in this study.

A study that described the allele frequency at the ABO locus in Middle Eastern countries has shown a high frequency of the blood group A in the northern region of the Arabian Peninsula, whereas the frequency of the same blood group was lower in the southern part and at a minimum in the central region of Saudi Arabia. In the northeastern part of the Arabian Peninsula, the blood group B was the most prevalent blood type. A high frequency of the blood group O was noted in the southwestern Arabian Peninsula, which is in general agreement with the findings in the current study. Nevertheless, the O blood group is considerably less frequent in the northeastern part of the region of interest.¹²

Several studies have found that the B blood group is the most prominent in central and western Africa, whereas O is the most common blood group in southern and eastern Africa.²⁷ According to one study, the blood group B is the most common in Nepal. The most common blood group in the United Kingdom is O, and the least common is AB.²⁷ Another study in Morocco showed a different ABO Rh frequency from this study.³⁵ Benahadi et al (2013) have found the A blood group to be the most prominent, followed by B, AB and O at 32.86%, 15.80%, 4.53% and 46.80%, respectively. Similar to the findings in the current study, the Rh-negative factor was less prevalent (0.09%) than the Rh-positive factor (91.00%) among the participants. In disagreement with the findings in the current study, in Greece,³⁶ found that the blood group A was the most prevalent (36.11%), followed by O (35.13%), B (15.53%) and AB (4.64%). However, most of the donors were Rh-positive (91.41%), while the Rh-negative factor was less common (8.59%) among the participants, which is in agreement with the current study. These geographical differences can be interpreted using factors such as genetic mapping, ethnic background and migration frequency of a given population.

The findings of this study define the frequency of blood groups in the kingdoms north of the southwest regions. This area of the kingdom is not known to have a prevalence of diseases that are related to the blood, such as thalassemia and sickle cell anemia.^{25,37}

Furthermore, this study had grouped the donors based on their gender because some studies have indicated that the variation in blood group inheritance between men and women could be attributed to some sex determining genes alongside the mode of inheritance of ABO-Rh genes.^{38–40}

Conclusion

The current study provides a better understanding of the ABO and Rh distribution among the residents of the northern Asir region, Saudi Arabia. This study identified the O+ blood group to be the most prevalent and AB- to be the least so in the studied area. The findings of this study contribute to the improvement of resource management and efficient health planning. Given the recognized connection between certain clinical conditions and blood groups, such studies could be helpful in the prediction of disease prognosis in certain geographical regions.

Limitations of the Study

Overall, since this study was conducted during the COVID-19 pandemic, the participants experienced some difficulties in reaching the collection site to donate blood. The other limitation of this study is the limited number of female participants in comparison to male donors.

Ethical Statement

This study was approved by the ethical committee at the University of Bisha.

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Disclosure

The author declares no conflicts of interest in relation to this work.

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Structure and Function of Recombinant versus Plasma-Derived von Willebrand Factor and Impact on Multimer Pharmacokinetics in von Willebrand Disease

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Structure and Function of Recombinant versus Plasma-Derived von Willebrand Factor and Impact on Multimer Pharmacokinetics in von Willebrand Disease

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Background: Recombinant von Willebrand factor (rVWF, vonicog alfa) is a purified VWF concentrate produced from Chinese hamster ovary cells. rVWF is not exposed to the VWF-cleaving protease ADAMTS13 and so is not subject to proteolytic degradation of large (L) and ultra-large (UL) VWF multimers by that enzyme.

Purpose: To compare the structure and function of rVWF with the human plasma-derived VWF [pdVWF] concentrates Haemate P[®]/Humate-P[®], Voncento[®], Wilate[®]/Eqwilate[®], and Wilfactin[®]/Willfact[®], to investigate the relationship between VWF multimeric pattern and VWF:ristocetin cofactor (VWF:RCo) activity through population pharmacokinetic (PK) modeling in patients with severe von Willebrand disease (VWD) treated with rVWF.

Methods: Analyses included VWF:RCo activity, VWF:collagen-binding activity, VWF:platelet glycoprotein Ib receptor binding, factor VIII (FVIII) binding capacity, and VWF-mediated platelet adhesion under flow conditions. VWF multimeric structure was determined by agarose gel electrophoresis. Population PK models describing the activity-time profile of small, medium, and L/UL multimers following intravenous administration of rVWF in patients with severe VWD were developed.

Results: Findings demonstrate that rVWF contains a non-degraded VWF multimer pattern including the UL multimers not present in pdVWF concentrates. rVWF displayed higher specific platelet-binding activity, and faster mediation of platelet adhesion to collagen under shear stress versus pdVWF concentrates. rVWF also demonstrated higher FVIII binding capacity than Haemate P[®], Voncento[®] and Wilate[®]. Modeling provided evidence that VWF:RCo activity in patients with severe VWD treated with rVWF is associated with L/UL VWF multimers in the circulation.

Conclusions: Findings suggest that the L and UL multimers preserved in rVWF contribute to high biological activity and might be important for providing hemostatic efficacy.

Keywords: agar, electrophoresis, factor VIII, plasma, von Willebrand diseases, von Willebrand factor

Introduction

von Willebrand factor (VWF) is a large adhesive glycoprotein synthesized in endothelial cells (ECs) and megakaryocytes.¹ Following post-translational modifications in the Golgi apparatus, up to 95% of VWF is constitutively secreted into the plasma and the remainder is stored in EC Weibel-Palade bodies or in the α -granules of platelets.¹ VWF is essential for hemostasis: under high shear flow it mediates initial platelet adhesion by acting as a bridge between subendothelial collagen and platelet surface glycoproteins. VWF also functions as a carrier for factor VIII (FVIII), protecting it from rapid clearance and increasing its plasma half-life.^{2,3}

Plasma VWF circulates as a series of multimers of different sizes that are comprised of identical subunits linked by disulfide bonds. The linking of two subunits produces a dimer of approximately 500 kDa; these are further combined with disulfide bonds to produce multimers ranging in size from 500 to over 10,000 kDa.^{4–6} In healthy individuals, ultra-large (UL) multimers (>10,000 kDa, >20 mers) are secreted into the plasma and are degraded by the naturally occurring VWF protease ADAMTS13.⁷ Large (L) multimers (5500–10,000 kDa, 11–20 mers) and UL multimers are the most hemostatically active, as measured by binding capacity for collagen and platelet receptors, and platelet aggregation under conditions of high fluid shear.^{8–12}

von Willebrand disease (VWD) is an inherited bleeding disorder caused by a deficiency of VWF or functional or structural defects in the protein.^{13,14} Management of bleeding in VWD involves the mainstay treatments of desmopressin (1-deamino-8-D-arginine vasopressin) and replacement therapy with VWF-containing concentrates from human plasma (plasma-derived VWF [pdVWF]). However, desmopressin is not effective in severe VWD, as well as in certain subtypes of VWD, and is associated with tachyphylaxis.^{15,16} VWF-containing concentrates from human plasma vary in the content and composition of VWF, FVIII, and other constituents.^{3,17,18} Recombinant VWF (rVWF, vonicog alfa, Vonvendi® [US]/Veyvondi® [Europe]; Baxalta US Inc., a Takeda company, Lexington, MA, USA) is a replacement therapy approved for on-demand treatment and control of bleeding episodes, as well as perioperative management of bleeding in adults diagnosed with VWD.^{19,20} In the US, rVWF has also been approved by the FDA for routine prophylaxis to reduce the frequency of bleeding episodes in adult patients with severe type 3 VWD receiving on-demand therapy.¹⁹ It is a purified VWF protein produced in Chinese hamster ovary cells without the addition of any exogenous human- or animal-derived protein.²¹ At administration, rVWF has an intact multimer pattern, including UL multimers, because it is not exposed to proteolytic degradation during manufacture, particularly by ADAMTS13.²²

Although comparisons of pdVWF and rVWF concentrates have been published,^{3,23–26} a comprehensive analysis of multimers and functional characteristics of VWF products is lacking. The objective of this study was to conduct a structural and functional comparison of rVWF versus the clinically used pdVWF (Wilfactin®/Willfact®) and pdFVIII/VWF (Haemate P®/Humate-P®, Voncento®, Wilate®/Eqwilate®) products, hereafter collectively referred to as pdVWF concentrates. In addition, population pharmacokinetic (PopPK) modeling was used to investigate the relationship between VWF multimeric pattern and VWF:ristocetin cofactor (VWF:RCo) activity in patients with severe VWD treated with VWF.

Methods

Preparation of VWF Products

The VWF products (Table 1) were reconstituted according to the manufacturer's instructions, using diluents provided by the manufacturer. Briefly, exact volumes of each diluent were added to sample vials. Vials were held at room temperature until complete dissolution of the product. Aliquots of the reconstituted materials were stored at –60°C or below, and quick-thawed for analysis.

Desmopressin Challenge Study

The effect of desmopressin on the multimeric structure of VWF was investigated using samples from a previously published study.²⁷ In that study, changes in ADAMTS13 activity were measured after infusion of desmopressin in 10 healthy volunteers and three patients with VWD.²⁷ The present analysis used plasma samples from the healthy volunteers, which comprised seven men and three women (median age, 29.5 years) who received an infusion of desmopressin (Octostim®; Aventis AG, Vienna, Austria) at a dose of 0.3 µg per kilogram of body weight in 50 mL saline over 30 minutes. Blood was collected at baseline and 1, 2, 6, and 24 hours after infusion was initiated. Platelet-poor citrated plasma was prepared according to standard methods and was analyzed for VWF multimers.

VWF Assays

The VWF:RCo activity, VWF:collagen-binding activity (VWF:CB), and VWF:platelet glycoprotein Ib receptor binding (VWF:GPIbM) assays were performed as published previously²⁸ and are described below in brief. One vial per product/strength was reconstituted and liquid aliquots were frozen. For analysis, two independent measurements of VWF:RCo

Table I VWF Products Analyzed in This Study

Product	Units per Vial (Diluent Volume)	Concentration After Reconstitution	Manufacturer
pdFVIII/VWF concentrates ^a Haemate P [®] /Humate-P [®] antihemophilic factor/von Willebrand factor complex (human) Voncento [®] human coagulation factor VIII/human von Willebrand factor	500 IU FVIII / 1200 IU VWF (10 mL) 1000 IU FVIII / 2400 IU VWF (15 mL) 500 IU FVIII / 1200 IU VWF (5 mL) 1000 IU FVIII / 2400 IU VWF (10 mL)	50 IU/mL FVIII / 120 IU/mL VWF 66.6 IU/mL FVIII / 160 IU/mL VWF 100 IU/mL FVIII / 240 IU/mL VWF 100 IU/mL FVIII / 240 IU/ml VWF	CSL Behring, King of Prussia, PA, USA
Wilate [®] /Eqwilate [®] von Willebrand factor/coagulation factor VIII complex (human)	500 IU VWF:RCO / 500 IU FVIII (5 mL) 1000 IU VWF:RCO / 1000 IU FVIII (10 mL)	100 IU/mL VWF:RCO / 100 IU/mL IU FVIII 100 IU/mL VWF:RCO / 100 IU/mL IU FVIII	Octapharma, Lachen, Switzerland
pdVWF concentrate ^a Wilfactin [®] /Willfact [®] human von Willebrand factor	1000 IU VWF:RCO (10 mL)	100 IU/ml VWF:RCO	LFB Biomedicaments, Courtaboeuf, France
Vonicog alfa (rVWF) Vonvendi [®] /Veyvondi [®] (von Willebrand factor [recombinant])	650 IU VWF:RCO (5 mL) 1300 IU VWF:RCO (10 mL)	~130 IU/mL VWF:RCO ~130 IU/mL VWF:RCO	Baxalta US Inc., a Takeda company, Lexington, MA, USA

Notes: ^apdFVIII/VWF and pdVWF concentrates are collectively referred to as pdVWF concentrates in this manuscript.

Abbreviations: FVIII, factor VIII; IU, international units; pdFVIII/VWF, plasma-derived factor VIII/von Willebrand factor; pdVWF, plasma-derived von Willebrand factor; rVWF, recombinant von Willebrand factor; VWF:RCO, von Willebrand factor:ristocetin cofactor activity; VWF, von Willebrand factor.

and VWF:GPIbM were taken from a fresh aliquot, and the mean of the two assay runs was reported. Single assay runs with multiple dilutions tested were performed for VWF:Ag, VWF:CB and VWF:FVIII-binding and the mean of the different dilutions tested was reported. To allow comparison between products, the specific VWF activity was calculated as the ratio relative to VWF antigen (VWF:Ag).

VWF:Ag

VWF:Ag was determined by enzyme-linked immunosorbent assay (ELISA) using an in-house assay and had a lower limit of quantification (LLOQ) of 4 IU/dL. VWF was captured using polyclonal rabbit antihuman VWF (DAKO, Glostrup, Denmark) coated on microplate wells. Polyclonal rabbit antihuman VWF/horseradish peroxidase (HRP) conjugate (DAKO) was added and incubated. After a washing step, bound VWF/HRP conjugate complex was detected using tetramethyl-benzidine reagent. The absorbance was read at 450 nm using a microplate reader. VWF:Ag was calculated relative to a human plasma reference (citratid; Siemens AG, Munich, Germany) calibrated versus the World Health Organization plasma standard.²⁹

VWF:RCO

VWF:RCO was measured using a von Willebrand reagent containing lyophilized platelets and ristocetin (BC reagent, Siemens AG, Munich, Germany) on an automated analyzer (BCS[®] XP, Siemens AG, Munich, Germany). The VWF:RCO activity was calculated from a reference curve constructed by different dilutions of the in-house rVWF reference standard, calibrated versus the World Health Organization 2nd International Standard for VWF Concentrate (National Institute for Biological Standards and Control [NIBSC] code 09/182).²⁹ The LLOQ of the assay was 20 IU/dL.

VWF:GPIbM

VWF activity via GPIb binding was measured on an automated instrument (BCS[®] XP, Siemens AG, Munich, Germany) using a commercial assay kit (Innovance[®] VWF Ac, Siemens AG, Munich, Germany) with minor modifications. The

product-specific in-house reference standard was calibrated versus NIBSC code 09/182.²⁹ The LLOQ of the assay was 19 IU/dL.

VWF:CB

VWF:CB was measured using the ZYMUTEST™ VWF:CBA ELISA (Hyphen BioMed, Neuville-sur-Oise, France). VWF was captured by fibrillar collagen (type 1 and 3) coated on microplate wells. Following a washing step, collagen-bound VWF was detected by adding polyclonal antihuman VWF antibodies coupled with peroxidase. Peroxidase activity was detected by a substrate (tetramethylbenzidine) and absorbance read at 450 nm. The VWF:CB of the samples was calculated relative to the assay reference standard (normal human plasma [citrate]; Siemens AG, Munich, Germany) calibrated against World Health Organization Plasma Standard²⁹) and expressed as VWF:CB IU/mL. The LLOQ of the assay was 12 IU/dL.

VWF Binding to FVIII

FVIII binding capacity of VWF products (VWF:FVIII) was measured using the ELISA Asserachrom® VWF:FVIII test kit (Stago, Asnières-sur-Seine, France). VWF was captured by antihuman VWF antibodies coated on microplate wells. Endogenous FVIII of the sample was eliminated by a high-salt washing step, and the remaining immobilized VWF was incubated with an excess amount of recombinant FVIII. After washing, FVIII bound to VWF was detected by antihuman FVIII antibodies labeled with peroxidase. The VWF:FVIII of the samples was calculated relative to the assay reference standard (normal human plasma [Siemens AG, Munich, Germany] calibrated against the World Health Organization Plasma Standard²⁹) and expressed as a percentage of VWF:FVIII.

VWF-Mediated Platelet Adhesion (Under Flow Conditions)

Platelet adhesion to collagen under shear stress (flow conditions) was measured in a perfusion chamber (BioFlux 1000z System, Fluxion BioSciences, San Francisco, CA, USA) using a 48-well plate. Citrated blood from healthy volunteers obtained from donations made to the Austrian Red Cross (consent was provided by donors as part of the routine blood donation process) was spiked with VWF product samples at a concentration of 1 IU/mL VWF:Ag, or with buffer only, and perfused over wells coated with fibrillar collagen type 1 (Horm collagen reagent, Takeda, Linz, Austria). All perfusion experiments were carried out with fluorescent-labeled platelets (calcein) at a wall shear rate of 1500 s^{-1} (60 dyn/cm^2). The degree of platelet adhesion was expressed as a percentage of coverage of the total surface.

Semi-Quantitative Structural Analysis of VWF Multimer Composition

Sodium dodecyl sulfate (SDS)-agarose gel electrophoresis was performed with home cast low-resolution 1% and high-resolution 2.5% SDS-agarose gels (SeaKem, Lonza, Basel, Switzerland) to visualize the multimerization grade of VWF concentrates and assess the presence of satellite bands resulting from ADAMTS13 cleavage. In a separate set of experiments, a 3% SDS-agarose gel was used for higher resolution of the multimer-band structure in human plasma samples. Multimer bands were detected by in-gel two-step immunostaining with an anti-VWF antibody (DAKO) and a goat antirabbit alkaline phosphatase conjugate (Jackson, West Grove, PA, USA). Multimer bands were visualized using an alkaline phosphatase conjugate substrate kit (Bio-Rad, CA, USA). Normal human plasma pool (citrate; Siemens AG, Munich, Germany) was analyzed for reference. Agarose electrophoresis and immunodetection were run twice per sample; displayed gels are representative for the analysis.

Low-resolution multimer patterns were evaluated visually and by densitometry (GS-900, Bio-Rad). Patterns were divided into two categories: (i) all multimers up to the highest multimer of normal human plasma and (ii) exceeding normal plasma up to the top end of the separation gel and thus representing UL multimers. UL multimers were defined as multimers larger than those found in normal human plasma. Relative volumes (area under the curve) were calculated for small (S) (1–5 mers), medium (M) (6–10 mers), and L/UL (>10 mers) multimers. High-resolution agarose gels were evaluated visually for appearance and structure of ADAMTS13-mediated satellite bands.

VWF concentrates were also analyzed with a Hydrasys 2 system using the Hydragel von Willebrand Multimers kit (Sebia, Lisses, France) to validate findings from the home cast gel electrophoresis. This semi-automated instrument

allowed assessment of the overall size distribution of VWF multimers using 2% agarose gel electrophoresis, direct immunofixation, and visualization with peroxidase-labeled antibodies. Analysis was performed according to the manufacturer's instructions and evaluated visually. This method separates VWF multimers in the high to UL range less well than the home cast 1% agarose gels but is less time-consuming and is based on ready-to-use gels and reagents.

rVWF Multimer PopPK Model

PopPK models describing the activity-time profile of small (S) (1–5 mers), medium (M) (6–10 mers), and L/UL (>10 mers) multimers following intravenous administration of rVWF were developed for a prototypic patient with a body weight of 75 kg, based on pharmacokinetic (PK) data from patients with type 1/2 or type 3 VWD. L/UL multimers were analyzed together because of the semi-quantitative nature of the densitometric method. Guidelines on PopPK modeling from the United States Food and Drug Administration and the European Medicines Agency were followed.^{30,31} The multimer PK profiles were derived through scaling the proportion of S, M, and L/UL multimers by the total antigen levels; they were calculated as $\text{VWF:Ag} \times \text{VWF:S}$, $\text{VWF:Ag} \times \text{VWF:M}$, and $\text{VWF:Ag} \times \text{VWF:L/UL}$, respectively, where the input dose for the multimer PK models was the rVWF dose as measured by VWF:RCo activity in international units. The proportion of S, M, and L/UL multimers was determined by densitometric scanning of agarose gels as described previously.³² These models were used to compare multimer kinetics with that of VWF:RCo. The data for the development of multimer PopPK models originated from multimer distribution data collected in the phase 3 study of patients with severe VWD receiving rVWF for on-demand treatment of bleeding (NCT01410227),³³ whereas the information on rVWF PopPK was obtained from a previously presented PopPK/pharmacodynamic model.³⁴

Ethics Approval and Informed Consent

Patient samples for the Desmopressin Challenge Study and Multimer PopPK Model were derived from two previously published clinical studies,^{27,33} and subjected to secondary analyses at the time of those studies. As described in the original articles, all participants provided written informed consent prior to any screening procedures. Approvals to conduct the studies were granted by the institutional review boards or independent ethics committees of all participating sites. The trials were conducted in accordance with the Declaration of Helsinki.

Results

Functional Activity of VWF Concentrates

For all pdVWF concentrates, the ratios of VWF:RCo to VWF:Ag were below 1, whereas rVWF had a ratio above 1, indicating a higher functional activity than the pdVWF concentrates (Figure 1A). Similar results were obtained for the ratios of VWF:CB to VWF:Ag. For VWF:GPIbM measurements, ratios obtained for rVWF were also higher than that for pdVWF concentrates. The coefficient of variation of the ELISA VWF:FVIIIb method was 8.9% (intermediate precision), FVIII binding capacity of rVWF was expressed as the percentage of the FVIII binding of normal human plasma. Although the FVIII binding capacity of rVWF (113% and 106%) was similar to that of Willfact (100%), it was higher than that of the other concentrates tested (71% to 92%) (Figure 1B).

VWF-Mediated Platelet Adhesion to Collagen Under Shear Stress (Flow Conditions)

When human whole blood was spiked with pdVWF or rVWF samples to a final VWF:Ag concentration of 1 IU/mL, the increase in platelet binding above baseline indicated a time-dependent increase in platelet adhesion to immobilized collagen. This increase varied between VWF products, and rVWF was highly effective at mediating platelet adhesion to collagen, displaying a higher mean efficacy compared with the pdVWF concentrates (Figure 2).

VWF Multimers in Human Plasma Samples

Multimer analysis of human plasma from healthy donors with a 3% SDS-agarose gel showed a characteristic satellite band pattern comprising an intense main band with one faster- and one slower-migrating satellite band. A schematic representing cleavage of UL multimers by ADAMTS13 into smaller molecules is included alongside the image of the gel

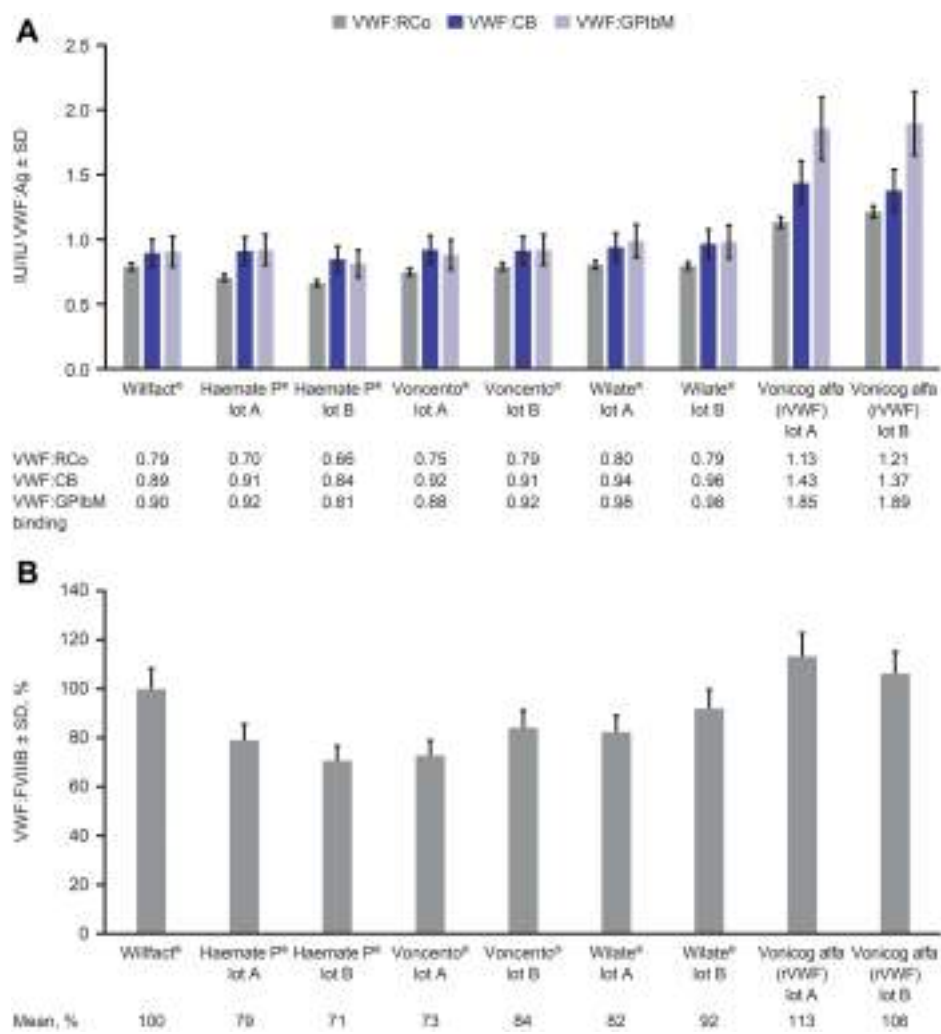


Figure 1 Static binding activity of VWF concentrates. **(A)** Binding to platelet receptor and collagen. **(B)** FVIII binding capacity. Data in both graphs represent mean $\pm$ SD. Error bars were calculated from the known imprecision of the assays with relative SDs between 3.7% and 13.0% depending on the assay. Numbers below graph **(A)** are mean ratios (IU/IU VWF:Ag). Numbers below graph **(B)** are mean VWF:FVIII binding percentages.

Abbreviations: FVIII, factor VIII; rVWF, recombinant von Willebrand factor; SD, standard deviation; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen-binding activity; VWF:FVIII, von Willebrand factor-factor VIII binding capacity; VWF:GP1bM, von Willebrand factor platelet glycoprotein 1b mutant receptor binding; VWF:RCo, von Willebrand factor:ristocetin cofactor activity.

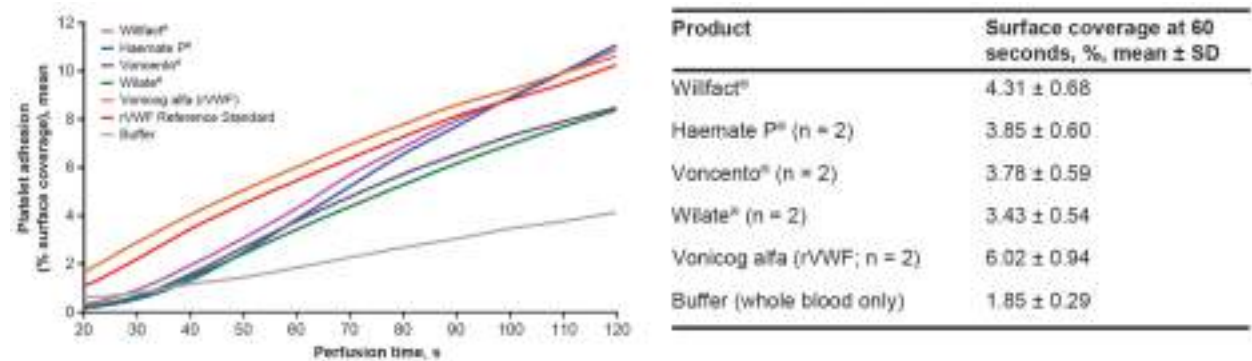


Figure 2 VWF activity: platelet adhesion to collagen under shear stress (flow conditions). VWF-mediated platelet adhesion to immobilized collagen type I under shear stress (1500 s^{-1}). Human whole blood was spiked with VWF samples at a final concentration of 1 IU/mL VWF antigen (or buffer) to determine time-dependent platelet binding. Perfusion time was 0–120 seconds. Comparison was based on the same amount of VWF antigen. The data are means of three independent measurements, with average values calculated for two product lots of each VWF sample, except for Willfact®, for which one lot was tested.

Abbreviations: rVWF, recombinant von Willebrand factor; SD, standard deviation; VWF, von Willebrand factor.

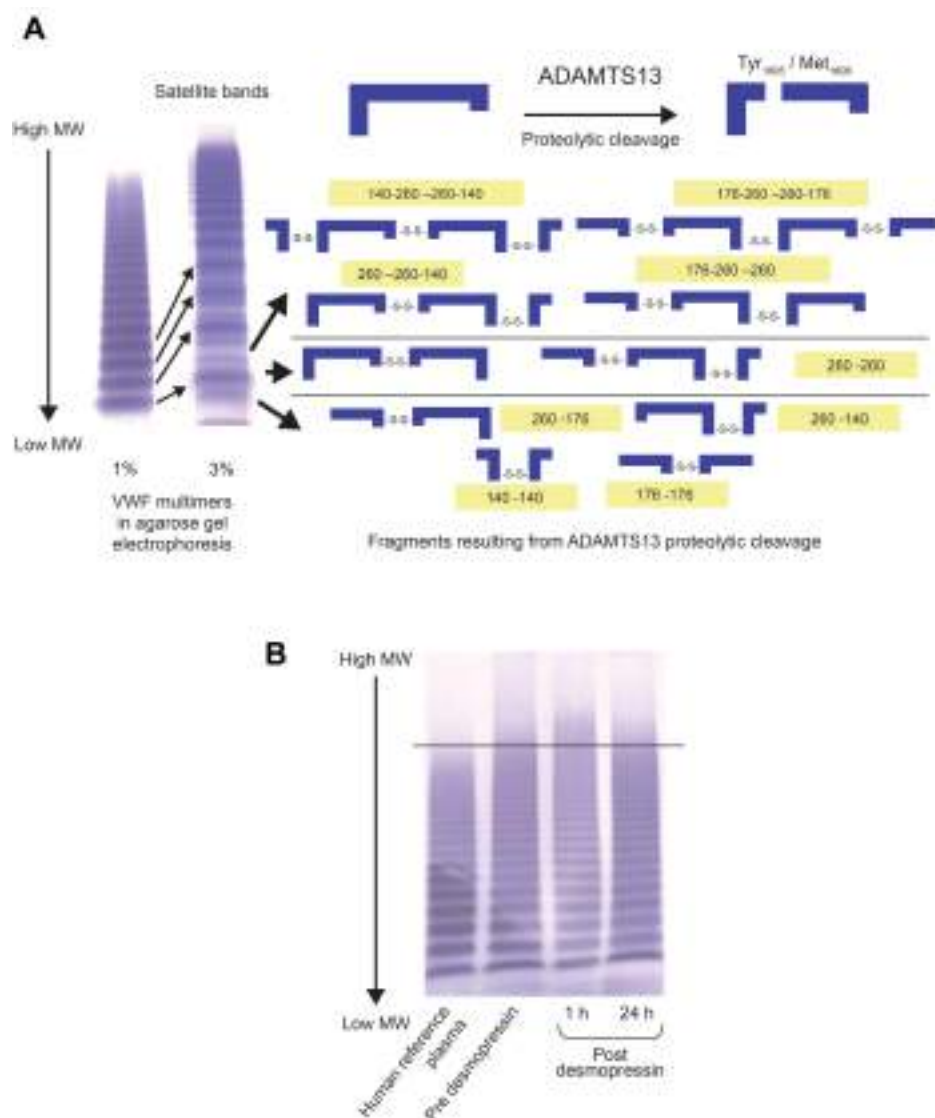


Figure 3 Multimeric analysis of plasma samples from healthy volunteers. **(A)** In the plasma of healthy human donors, normal VWF fragments are produced by ADAMTS13 proteolysis (representative sodium dodecyl sulfate–agarose gel shown). The schematic shows that cleavage at Tyr₁₆₀₅/Met₁₆₀₆ generates fragments linked via disulfide bonds with different sizes/MWs relative to the central band of each multimer and the corresponding satellite bands. **(B)** Representative gel showing VWF multimers from the plasma of a desmopressin-treated healthy volunteer versus a pretreatment plasma sample and a human reference plasma pool.

Abbreviations: MW, molecular weight; VWF, von Willebrand factor.

(Figure 3A). Desmopressin infusion stimulated the secretion of UL multimers, which was observed at 1 hour post-baseline but had declined by 24 hours. Similar images of VWF multimers were seen in all healthy volunteers. Figure 3B shows a representative SDS-agarose gel from a single healthy volunteer.

VWF Multimers in VWF Concentrates

Low-Resolution Agarose Gel Electrophoresis

Visual inspection of the immune-stained low-resolution agarose gels showed that rVWF contains UL multimers not present in human plasma or in pdVWF concentrates. In rVWF, the lowest-molecular-weight multimers (up to hexamer) were present in relatively low amounts, whereas pdVWF concentrates showed intense low-molecular-weight multimers (Figure 4). Semi-quantitative densitometric evaluation showed that the relative percentage area of UL multimers after separation on agarose gel was higher for rVWF samples than for the pdVWF concentrates (Figure 4). This result was verified by the semi-automated

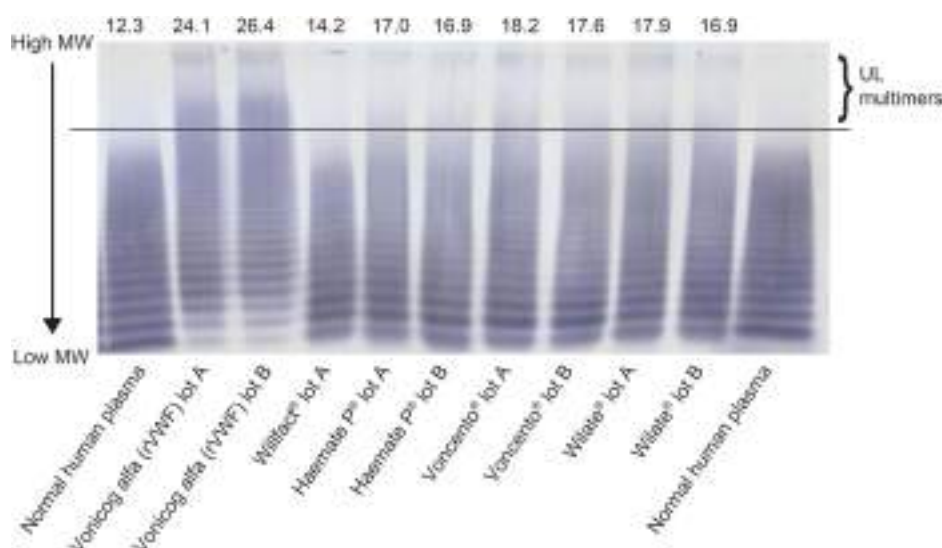


Figure 4 Multimer composition of VWF concentrates: low-resolution (1%) agarose gel electrophoresis. The relative percentage area of UL multimers (percentage area above highest plasma multimers) after separation on agarose gel are shown above each lane. Although normal human plasma and the pdVWF concentrates do not contain UL multimers, densitometry of the separation lanes gives an inherent background of 12.3% UL area for normal human plasma and a range of 14.2–18.2% UL area for the pdVWF concentrates. Vonicog alfa (rVWF) shows UL multimers clearly above background.

Abbreviations: MW, molecular weight; rVWF, recombinant von Willebrand factor; UL, ultra-large; VWF, von Willebrand factor.

electrophoresis system, which showed that rVWF contains UL multimers that are not observed in human plasma in circulation or in pdVWF concentrates, as well as an absence of the lowest-molecular-weight multimers (data not shown).

High-Resolution Agarose Electrophoresis

Electrophoresis of human plasma demonstrated a typical satellite band pattern as expected on a high-resolution agarose gel. Voncento® and Haemate P® had a different pattern from human plasma: the faster- and central-migrating bands were more intense, whereas the slower-migrating satellite band was very faint (Figure 5). This pattern could be indicative of degradation occurring during purification from plasma. No satellite bands were observed with rVWF indicating the intact multimer profile for this concentrate.

Comparison Between VWF:RCo Activity and S, M, and L/UL rVWF Multimer PK

A PopPK analysis was undertaken to characterize the contribution of various-sized multimer populations to the overall hemostatic potential of rVWF. The PopPK models for VWF:Ag $\times$ VWF:S, VWF:Ag $\times$ VWF:M, and VWF:Ag $\times$ VWF:L/UL were two-compartment models with first-order elimination from the central compartment. Endogenous levels were described as a constant background with identified covariates of the effect of VWD type on endogenous levels (type 3 versus type 1 and 2) and allometric body-weight scaling of clearances and volumes of distribution. Of note, the structural models for VWF:Ag $\times$ VWF:S, VWF:Ag $\times$ VWF:M, and VWF:Ag $\times$ VWF:L/UL were identical to the model for VWF:RCo.³⁴

Visual comparison between the population predictions of VWF multimers and VWF:RCo obtained from the respective final models (Figure 6) suggested a good agreement, as indicated by parallel PK curves, between VWF:RCo and VWF:Ag $\times$ VWF:L/UL, up to quantifiable VWF:Ag/VWF:RCo activity levels in both VWD subgroups, whereas VWF:Ag $\times$ VWF:M and VWF:Ag $\times$ VWF:S were generally characterized by a slower PK. The difference between absolute VWF:RCo and VWF:Ag $\times$ VWF:L/UL activity levels could partly be attributed to the assumption regarding the use of VWF:RCo dose as input for the multimer models, although the effect of this is unknown. Another explanation is that the smaller multimer fractions also contribute, to a lesser degree, to the total VWF:RCo activity-time profile. However, the model-based comparison between VWF:RCo and VWF:Ag $\times$ VWF:S, VWF:Ag $\times$ VWF:M, and VWF:Ag $\times$ VWF:L/UL PK revealed that L/UL VWF multimers displayed the highest degree of similarity with VWF:

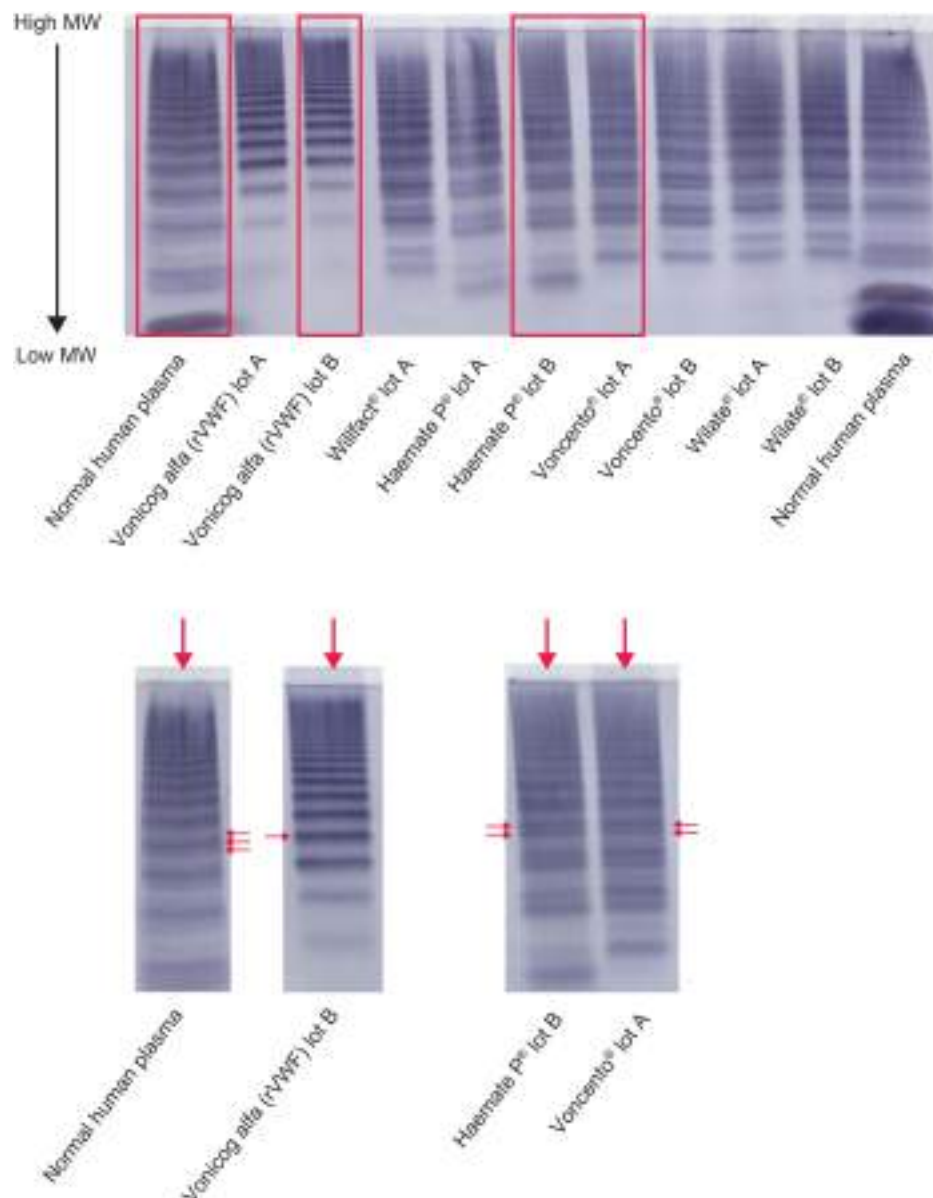


Figure 5 Multimer composition of VWF concentrates: high-resolution agarose electrophoresis. Enlarged images of selected lanes are shown in the lower panel; Voncento[®] and Haemate P[®] exhibit a different satellite band pattern from normal human plasma, as indicated by the arrows. The faster- and central-migrating bands appear more intense, whereas the slower-migrating satellite band is very faint. Vonicog alfa (rVWF) is composed of intact multimers without satellite bands.

Abbreviations: MW, molecular weight; rVWF, recombinant von Willebrand factor; VWF, von Willebrand factor.

RCo across the three multimers sizes, as indicated by parallel PK curves. This provides evidence that VWF:RCo activity is largely associated with the persistence of L/UL multimers in the systemic circulation.

Discussion

Patients with VWD who have an inadequate response or intolerance to desmopressin are potential candidates for replacement therapy with VWF concentrates. The integrity (lack of degradation) and content of VWF in these products is important for their effectiveness. With respect to composition, it is established that the L and UL multimers have higher platelet- and collagen-binding affinity than smaller multimers.^{8–12} rVWF is the only purified recombinant human VWF approved for adults with VWD for the treatment of bleeding episodes in the on-demand and perioperative settings,

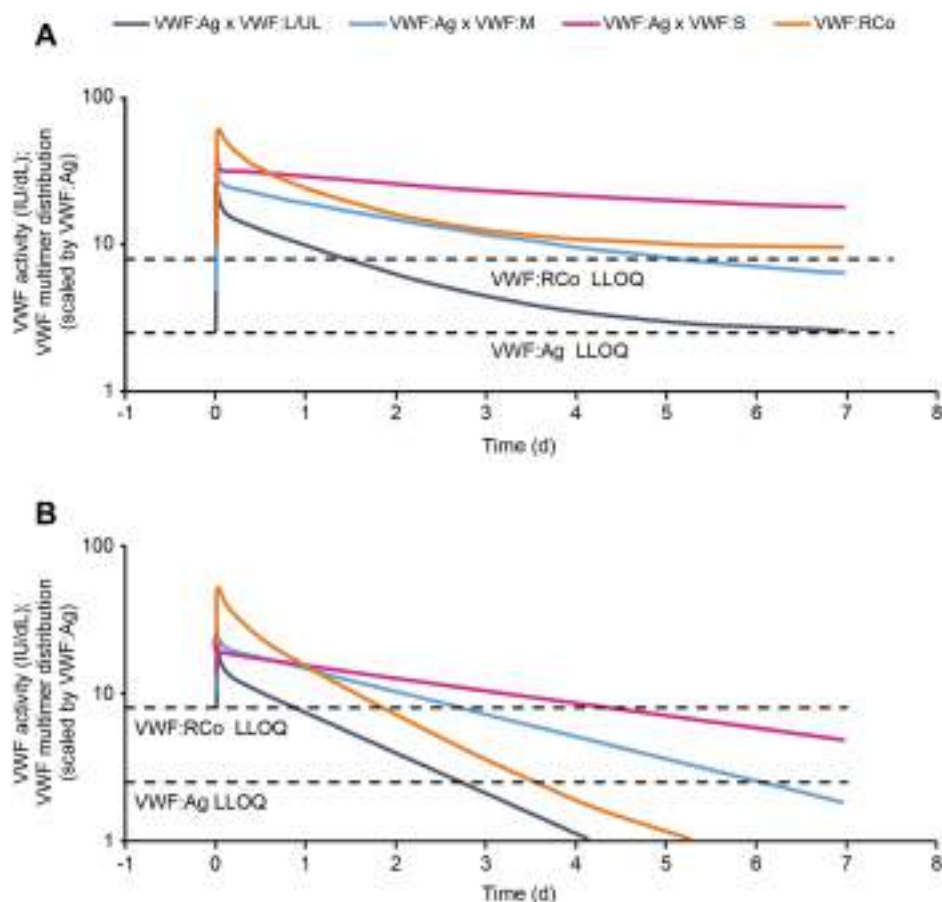


Figure 6 Population PK predictions of VWF:RCo and VWF multimer activity (VWF:Ag $\times$ VWF:S, VWF:Ag $\times$ VWF:M, and VWF:Ag $\times$ VWF:L/UL activity) over time after a 30 IU/kg (VWF:RCo) vonicog alfa (rVWF) dose for a patient with a body weight of 75 kg with VWD subtypes 1/2 or 3. **(A)** Type 1/2 VWD. **(B)** Type 3 VWD. Note that for type 1/2 VWD **(A)**, visual assessment may be distorted after day 3 as curves approach endogenous levels of VWF:Ag and VWF:RCo. This not an issue in type 3 VWD **(B)** as VWF:Ag and VWF:RCo are undetectable in type 3 VWD. Dotted lines represent LLOQs for VWF:RCo and VWF:Ag (IU/dL) for analysis of human plasma. The graph shows modeled Pop PK data and therefore curves partially cover a range below the LLOQ of the respective methods.

Abbreviations: LLOQ, lower limit of quantification; L/UL, large/ultra-large; M, medium; S, small; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor:ristocetin cofactor activity.

and for routine prophylaxis to reduce the frequency of bleeding episodes in adult patients with severe type 3 VWD receiving on-demand therapy.^{19,20} In this study, the structure and function of rVWF and clinically used pdVWF concentrates were compared under static and dynamic flow conditions.

rVWF is manufactured in the absence of human plasma proteins, thus avoiding the risk of transmission of human blood-borne pathogens and eliminating the effects of co-purifying plasma proteins. As rVWF is not exposed to proteolytic enzymes from human blood during the manufacturing process, it preserves the L and UL multimers.²¹ Thromboembolic reactions can occur with rVWF, especially in patients with thrombotic risk factors.^{19,20} However, clinical trial results indicate that the risk is low. During the clinical development of rVWF, two patients treated with rVWF experienced thromboembolic events. Of these, one patient experienced thrombosed hemorrhoid not considered related to rVWF in a Phase 3 study (NCT01410227). A second patient experienced 2 adverse events of deep vein thrombosis in the perioperative setting, of which one was considered possibly related to rVWF.³⁵ The risk of thrombosis with pdVWF products is potentially greater because the exogenous FVIII in the product adds to the endogenous FVIII stabilized by VWF replacement and can lead to FVIII accumulation in the plasma.³⁶

The current study of the multimer composition and functional activity of VWF concentrates demonstrated that rVWF contained UL multimers not present in pdVWF concentrates and displayed single-band multimers because of no previous exposure to ADAMTS13. In a phase 1 clinical trial that evaluated the pharmacokinetics of rVWF:rFVIII in patients with

severe VWD, VWF cleavage products were present in patient samples as early as 15 mins post-infusion indicating rapid proteolysis of VWF multimers.²⁴ However, UL multimers were present in samples from rVWF-treated patients for up to 3 hours after infusion but not in samples from pdVWF-treated patients at any post-infusion timepoint.²⁴ This suggests that the most active multimeric fraction for promoting primary hemostasis is present in the plasma for a short period after infusion of rVWF.

The functional activity of the VWF concentrates for static binding of platelets was measured using the VWF:RCo, VWF:GPIbM, and VWF:CB assays. The VWF:RCo assay measures the ability of a sample to bind to GPIb on fixed normal platelets after ristocetin is added *in vitro*.³⁷ The GPIbM assay provided relatively higher values for rVWF compared with the VWF:RCo assay, regardless of whether the calibrated in-house rVWF reference or the World Health Organization reference standard²⁹ were used (latter not shown). This suggests greater sensitivity of the GPIbM assay, which is based on the binding of VWF to recombinant gain-of-function mutated GPIb that does not require ristocetin.³⁷ Regardless of assay used, the results were consistent, demonstrating that the ratio of measurements of VWF activity to VWF protein content (VWF:Ag) were close to or above 1 for rVWF, whereas pdVWF concentrates had ratios below 1. In normal human plasma, the ratio of VWF:RCo/VWF:Ag is close to 1.³ Therefore, ratios below 1 in the pdVWF concentrates indicate some degree of VWF multimer degradation. VWF binding to collagen was also higher for rVWF, indicating a high functionality of L and UL VWF multimers present in the recombinant product. With respect to FVIII binding capacity, the results of the VWF:FVIII ELISA demonstrated higher binding capacity for rVWF than the majority of pdVWF concentrates tested. Overall, the results from these binding experiments are in line with those from other studies,^{3,25} indicating that the specific activity for binding of collagen and platelets is higher for rVWF than for pdVWF products.

VWF circulates in an inactive globular form, and requires sufficient shear force to change its conformation to an extended form that exposes domains critical for collagen and platelet receptor binding.³⁸ In this study, primary hemostatic activity of rVWF was demonstrated by VWF-induced platelet adhesion to collagen under shear stress (flow conditions), which was faster than for the pdVWF concentrates. This suggests that the presence of UL multimers in rVWF results in improved platelet binding under dynamic flow conditions, presumably because larger-sized multimers have more available binding sites for collagen and GPIb binding³⁹ and/or these sites are more available to their respective ligands in the context of larger molecular structures. This is also consistent with previous reports of a linear correlation between the content of L multimers in VWF concentrates and the specific activity of VWF:RCo.^{3,26}

Visual inspection and semi-quantitative densitometric evaluation of low-resolution agarose gels of VWF concentrates indicated that rVWF contained UL multimers not present in pdVWF concentrates. High-resolution multimer analysis of rVWF displayed single-band multimers, thus demonstrating an integral multimer pattern of VWF that is not degraded by proteases. The amount of lowest-sized multimers was reduced in rVWF as a result of the manufacturing process, which includes chromatography steps aimed at purifying rVWF to homogeneity and preserving the intact multimer pattern of rVWF secreted into the cell culture medium. However, these multimers support platelet aggregation less effectively than larger ones and, therefore, do not affect overall functionality.²⁵

High-resolution multimer analysis of the normal human plasma samples and pdVWF concentrates demonstrated a characteristic triplet structure consisting of a central band and two flanking bands. In fact, it is known that each pdVWF multimer is accompanied by two slower- and two faster-migrating bands, but further resolution is required to observe the quintuplet structure.⁴⁰ The satellite bands are degradation products of VWF resulting from physiologic proteolysis by ADAMTS-13 in plasma.^{4,40,41} For some of the pdVWF concentrates, the satellite structure was altered compared with normal human plasma, ie, the quantity of the slower-migrating band was lower, whereas the faster-migrating satellite band was more intense. These results are in line with those from previous studies that reported variable VWF multimeric patterns compared with normal human plasma in six²⁶ and twelve³ different pdVWF concentrates analyzed using SDS-agarose gel electrophoresis, and suggest that some degree of degradation occurs during purification from plasma.

Multimer analysis of plasma samples from desmopressin-treated healthy volunteers showed that desmopressin induced secretion of VWF multimers, including a fraction containing UL multimers observable at 1-hour post-stimulation. The human reference sample had a lower content of UL multimers than a fresh pre-sample collected before

administration of desmopressin. The reference plasma was a pool from healthy volunteers, whereas the pre- and post-desmopressin samples were from single individuals. Therefore, plasma pretreatment could vary, explaining differences seen in the multimer pattern because VWF is extremely sensitive to minor differences in manipulation, resulting in variable degradation by ADAMTS13 in the human plasma samples. Preparation of a plasma pool requires at least one additional manipulation step, which is unnecessary when a single plasma sample is freshly frozen following centrifugation to remove cells.

A PopPK model described activity-time profiles of the different-sized multimers using data from patients with severe VWD treated with rVWF in a phase 3 clinical trial (NCT01410227).³³ In the model-based comparison between VWF biological activity (VWF:RCo) and VWF:Ag $\times$ VWF:S, VWF:Ag $\times$ VWF:M, and VWF:Ag $\times$ VWF:L/UL PK, L/UL multimers displayed the highest degree of similarity with VWF:RCo PK compared with S and M multimers. These results indicate that VWF:RCo activity in patients with severe VWD treated with rVWF is associated with the persistence of L/UL VWF multimers in the systemic circulation, which are known to be the most effective multimers with respect to interactions with collagen and platelet receptors, and also display increased FVIII binding capacity.^{8–12}

In healthy individuals, VWF is derived from multiple sources: plasma, platelet α -granules, and EC Weibel-Palade bodies.⁴² Although the relative contribution of each of these sources to hemostasis remains the subject of investigation, the lower-molecular-weight multimers that predominate in the plasma pool are not nearly as efficient at mediating platelet adhesion as the UL forms in the EC storage granules.^{8–12} In type 3 VWD and most forms of type 2, the EC storage pools of UL forms are absent, and the management of bleeding in affected patients depends principally on the repletion of VWF in the plasma compartment.^{7,43} The results of the present studies provide a clearer basis for the hemostatic efficacy demonstrated by rVWF at a molecular level in recent clinical studies.^{33,35}

Conclusions

This analysis of the multimer composition and functional activity of VWF concentrates demonstrated that rVWF contained UL multimers not present in pdVWF concentrates and displayed single-band multimers because of no previous exposure to ADAMTS13. rVWF had higher specific platelet-binding activity and faster mediation of platelet adhesion to collagen under shear stress relative to pdVWF concentrates. rVWF also demonstrated higher FVIII binding capacity than Haemate P[®], Voncento[®] and Wilate[®]. The results of the model-based analysis provide supporting evidence that the VWF:RCo activity in patients with severe VWF deficiency treated with rVWF is associated with the persistence of L and UL VWF multimers in the systemic circulation. Taken together, these findings suggest that the L and UL multimers preserved in rVWF contribute to high biological activity and might be important for providing hemostatic efficacy.

Abbreviations

EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; FVIII, factor VIII; HRP, horseradish peroxidase; L, large; M, medium; NIBSC, National Institute for Biological Standards and Control; pdVWF, plasma-derived VWF; PK, pharmacokinetic; PopPK, population pharmacokinetic; rVWF, recombinant VWF; S, small; SDS, Sodium dodecyl sulfate; UL, ultra-large; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:CB, VWF collagen binding; VWF:FVIIIb, FVIII binding capacity of VWF products; VWF:GPIbM, VWF platelet glycoprotein Ib mutant receptor binding; VWF:RCo, VWF ristocetin cofactor.

Data Sharing Statement

The datasets analyzed in this study are available upon reasonable request from the corresponding author.

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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.

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Disclosure

HG, GS, and NW are employees of Baxalta Innovations GmbH, a Takeda company, and hold Takeda stock or stock options. BM is an employee of Takeda Development Center Americas, Inc., and holds Takeda stock or stock options. BE was an employee of Takeda Development Center Americas, Inc., during the analysis and development of the manuscript, and holds Takeda stock. PLT is an employee of Baxalta Innovations GmbH, a Takeda company, and holds relevant Takeda patents and Takeda stock or stock options. The authors report no other conflicts of interest in this work.

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Primary Bone Marrow Lymphoma: De Novo and Transformed Subtypes

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Abstract: Diffuse large B-cell lymphoma (DLBCL) is well known for selectively involving certain extranodal locations such as the central nervous system (CNS), testes, and skin. DLBCL or high-grade B-cell lymphoma selectively involving the bone marrow is rare and has been sparsely reported in the medical literature. We report two cases of lymphoma presenting with primary bone marrow involvement without evidence of involvement of any other sites. The first case represents de novo DLBCL. The patient achieved complete remission with initial treatment, had a bone marrow-only relapse three years later, and achieved a second complete remission following non-transplant salvage therapy. The second case had findings consistent with “double hit” Richter’s transformation of chronic lymphocytic leukemia with translocation of *c-MYC* and *BCL-2*. This patient had an aggressive clinical course characterized by rapid progression with CNS involvement within three months resulting in the demise of the patient. These two cases represent two distinct subtypes of primary bone marrow lymphoma: de novo and transformed. Further research is necessary to gain a better understanding of this rare lymphoma entity and develop novel therapies.

Keywords: diffuse large B-cell lymphoma, high-grade B-cell lymphoma with *MYC* and *BCL-2* rearrangements, bone marrow involvement, cytopenias

Plain Language Summary

- Primary bone marrow lymphoma (PBML) is confined to the bone marrow with or without a leukemic component and represents a distinct, aggressive type of extranodal lymphoma.
- PBML is very rare and has been sparsely reported in the medical literature.
- PBML appears to consist of two subtypes: de novo PBML and PBML derived from transformation of bone marrow-based low-grade lymphomas.
- PBML should be distinguished from other aggressive lymphomas which can primarily involve the bone marrow such as Burkitt lymphoma, lymphoblastic lymphoma, blastoid mantle cell lymphoma, and intravascular large B-cell lymphoma.
- PET imaging is useful for monitoring lymphoma activity in the bone marrow.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is well known for selectively involving a number of extranodal anatomic sites including the central nervous system (primary CNS lymphoma), testicles (primary testicular lymphoma), and skin (primary cutaneous DLBCL, leg type). Primary bone marrow lymphoma (PBML) is an exceedingly rare lymphoma and can also be called lymphoma with primary bone marrow involvement.¹ It is characterized by lymphomatous bone marrow involvement without focal bone lesions or disease elsewhere except for a leukemic component in the peripheral blood.^{1–5}

The true incidence of PBML is uncertain as definitions have varied, but it appears to represent a distinct, aggressive type of extranodal lymphoma.^{1–3,6,7} The majority of cases are DLBCLs.^{1,6,8} Patients with PBML often present with

B-symptoms (fevers, night sweats, and weight loss) and cytopenias.¹ Diagnosis requires a combination of imaging and bone marrow biopsy which helps distinguish it from primary bone lymphoma (PBL) and leukemias/lymphomas with known primary or secondary bone marrow involvement.^{1–5,9–11}

PBL is defined as either a single bony lesion with or without regional node involvement or multifocal bony disease without nodal or visceral involvement.⁹ PBL is associated with bone destruction, while PBML does not result in bony destruction but demonstrates diffuse enhancement within the marrow on imaging.^{1,9} PBML and PBL are also distinct from secondary bone lymphoma and leukemias and lymphomas with known primary bone marrow involvement.^{1,3,4,9–11}

PBML should be differentiated from other lymphomas which can also involve the bone marrow and mimic high-grade B-cell lymphoma (HGBCL) such as Burkitt lymphoma, lymphoblastic lymphoma, and blastoid mantle cell lymphoma.^{1,4,11} PBML diagnosis also requires exclusion of secondary bone lymphoma which arises from infiltration of the marrow in the setting of disseminated disease in 16–20% of lymphomas.^{3,9,10}

We report two cases of PBML from our institution.

Case Reports

Case #1

A 71-year-old female was referred for evaluation of leukopenia. She endorsed weight loss related to poor appetite but was otherwise asymptomatic. Her medical history was significant for immune-mediated thrombocytopenia treated with intravenous immunoglobulin, steroids, and platelet transfusions with normalization of her counts 5 years prior to presentation. Laboratory evaluation was significant for mild anemia with a hemoglobin of 10.6 g/dL, leukopenia with a white blood cell (WBC) count of 2.8×10^9 /L, and mild neutropenia with an absolute neutrophil count of 1.11×10^9 /L. The lactate dehydrogenase (LDH) was elevated at 321 U/L (normal 122–222 U/L). Given the leukopenia, unremarkable peripheral smear, and low suspicion for a leukemic component, ancillary studies including peripheral blood flow cytometry (PBFCM), cytogenetics, and molecular studies were deferred. Serum protein electrophoresis demonstrated a monoclonal IgM kappa with an M-spike of 0.2 g/dL. Bone marrow biopsy revealed a hypercellular marrow, 70% cellularity with grade 3+ myelofibrosis secondary to the lymphoma.¹² Erythropoiesis and granulopoiesis were decreased, and the lymphocytes were increased. There was diffuse involvement (80%) of the bone marrow with DLBCL. The malignant cells were positive for CD20 (Figure 1A and B) and negative for CD5, CD25, CD30, CD34, BCL-1, and MUM-1. Epstein–Barr virus-encoded RNA (EBER) in situ hybridization (ISH) was also negative. ¹⁸F Fluorodeoxyglucose (FDG) positron emission tomography-computed tomography (PET-CT) demonstrated bone marrow involvement without evidence of other disease (Figure 2A and B). She was diagnosed with stage IV primary bone marrow (PBM)-DLBCL with a revised-international prognostic index (R-IPI) of 3 and central nervous system (CNS)-IPI of 3. She received chemoimmunotherapy with six cycles of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) and high-dose (HD) methotrexate (MTX) for CNS prophylaxis in alternate cycles for a total of three cycles. She achieved complete remission (CR) after four cycles (Figure 2C and D). The IgM monoclonal protein resolved with treatment.

Approximately 3 years after completion of therapy, she developed fatigue, anorexia, dyspnea on exertion, and presyncope. Laboratory evaluation revealed anemia, leukopenia, and a recurrent, small monoclonal IgM kappa protein on immunofixation. A bone marrow biopsy confirmed relapsed DLBCL with 60% involvement by a nodular and paratrabecular infiltrate of recurrent B-cell lymphoma. The Ki-67 was 60%. Immunohistochemistry (IHC) studies showed that the neoplastic B lymphocytes were positive for BCL-2 and negative for CD10 and MYC (Figure 1C–F). They were also negative for BCL-6 and MUM1. The immunophenotype was most consistent with a non-germinal center phenotype. Ancillary studies were not performed due to a dry tap with bone marrow biopsy and the absence of circulating neoplastic lymphocytes. FDG PET-CT did not show evidence of extraosseous disease. Given her advanced age and poor performance status, she was deemed ineligible for autologous hematopoietic stem cell transplantation. She was treated with a combination of an experimental Bruton tyrosine kinase (BTK) inhibitor, pomalidomide, and everolimus on a clinical trial and achieved transfusion independence with improvement in her cytopenias. This therapy was discontinued after 2 months due to severe mucositis. She was transitioned to polatuzumab, bendamustine, and rituximab

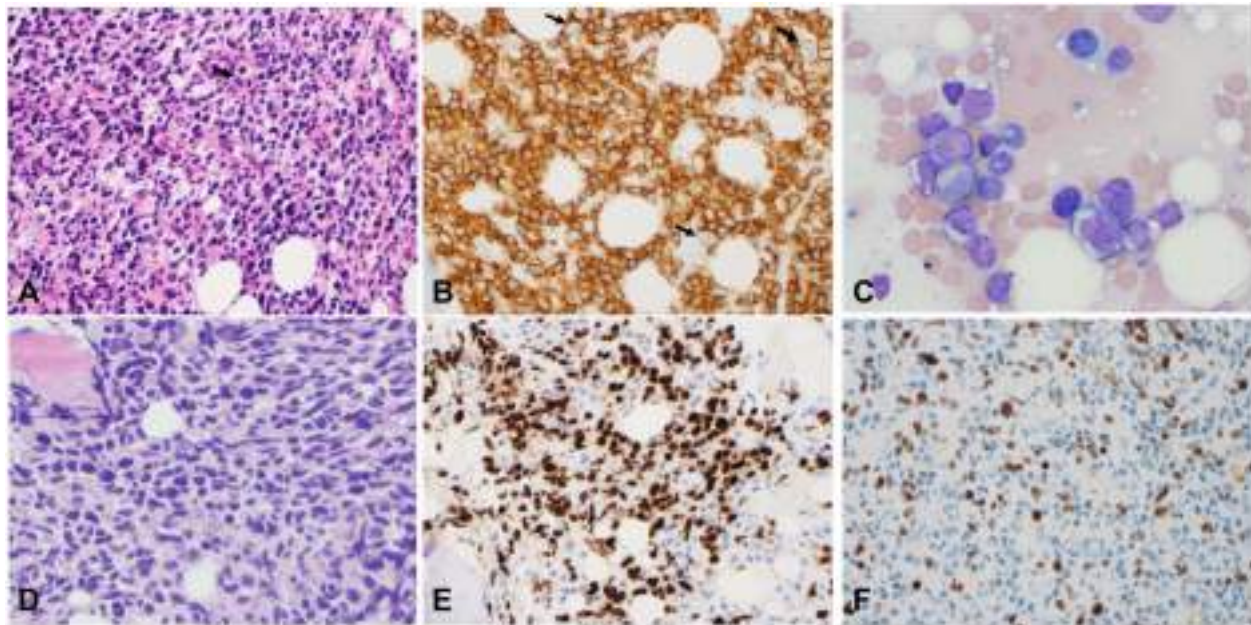


Figure 1 Bone Marrow Biopsy Case #1. The initial bone marrow biopsy of case #1 revealed infiltrating large, atypical lymphocytes with open chromatin and prominent nucleoli (A, H&E stain $\times 40$) which were positive for CD20 (B, IHC $\times 40$). The black arrows in A and B indicate frequent mitosis. Three years later, repeat bone marrow biopsy with roll prep-smear demonstrated recurrent lymphoma (C, WG stain $\times 50$; D, H&E $\times 40$). The neoplastic lymphocytes were positive for PAX5 (E, IHC $\times 20$) and showed a high proliferative rate with a Ki-67 of 60% (E and F, IHC $\times 40$).

Abbreviations: H&E, hematoxylin and eosin; IHC, immunohistochemistry; WG, Wright-Giemsa.

(PBR) and completed 6 cycles of therapy. Bendamustine was not given with the first four cycles. She achieved transfusion independence with excellent blood counts halfway through the treatment. The repeat bone marrow biopsy after PBR treatment showed CR. To date, she has remained in CR and is 4 months post completion of therapy.

Case #2

A 75-year-old female presented with a 1-month history of intractable back pain that failed to respond to oral steroids and a corticosteroid injection and more recently fatigue, night sweats, and a 4 kg weight loss. Computed tomography of the chest, abdomen and pelvis was unrevealing. Magnetic resonance imaging (MRI) of the lumbar spine demonstrated multifocal marrow signal abnormalities concerning for malignancy. Laboratory evaluation was significant for thrombocytopenia with a platelet count of $69 \times 10^9/L$ and leukocytosis with a WBC count of $10.1 \times 10^9/L$. There was an absolute lymphocytosis with 8% blastoid cells on peripheral smear (Figure 3A and Inset). LDH was elevated at 4890 U/L (normal 122–222 U/L), and ferritin was 9979 mcg/L (normal 11–307 mcg/L). PBFCM revealed two distinct B-cell populations (Figure 4A–E). There was a $1.40 \times 10^9/L$ monoclonal kappa restricted B-cell population that was positive for CD10 but negative for CD5, CD23, CD11c, and CD103. This population showed high forward scatter (large size) compatible with a high-grade B-cell neoplasm. The second population consisted of $0.97 \times 10^9/L$ monoclonal dim kappa restricted B-cells with co-expression of CD5 and CD23 and low forward scatter (small size), representing a chronic lymphocytic leukemia (CLL) phenotype. Bone marrow biopsy revealed a hypercellular marrow with extensive, near 100%, involvement by HGBCL with a Ki-67 of 80%. IHC studies showed the neoplastic lymphocytes were positive for CD10, CD20, CD79a, PAX-5, BCL-2 (90%), and MYC (90%) (Figure 3B–F). They were negative for CD23, BCL-6, cyclin D1, TdT, CD30, and EBER ISH. Chromosome analysis on peripheral blood revealed a complex karyotype including a balanced t(14;18) consistent with BCL2/IGH rearrangement and an abnormal chromosome 8 consistent with MYC rearrangement. Lymphoma fluorescence in situ hybridization (FISH) testing was consistent with HGBCL with BCL-2 and C-MYC rearrangements, the so-called “double hit” lymphoma (Figure 4F–G).¹³ FISH for CLL was positive for 13q deletion in 22% of cells (Figure 4H). Cerebrospinal fluid (CSF) cytology was negative for malignancy on 2 occasions. Staging FDG PET-CT demonstrated increased uptake throughout the axial and proximal appendicular skeleton

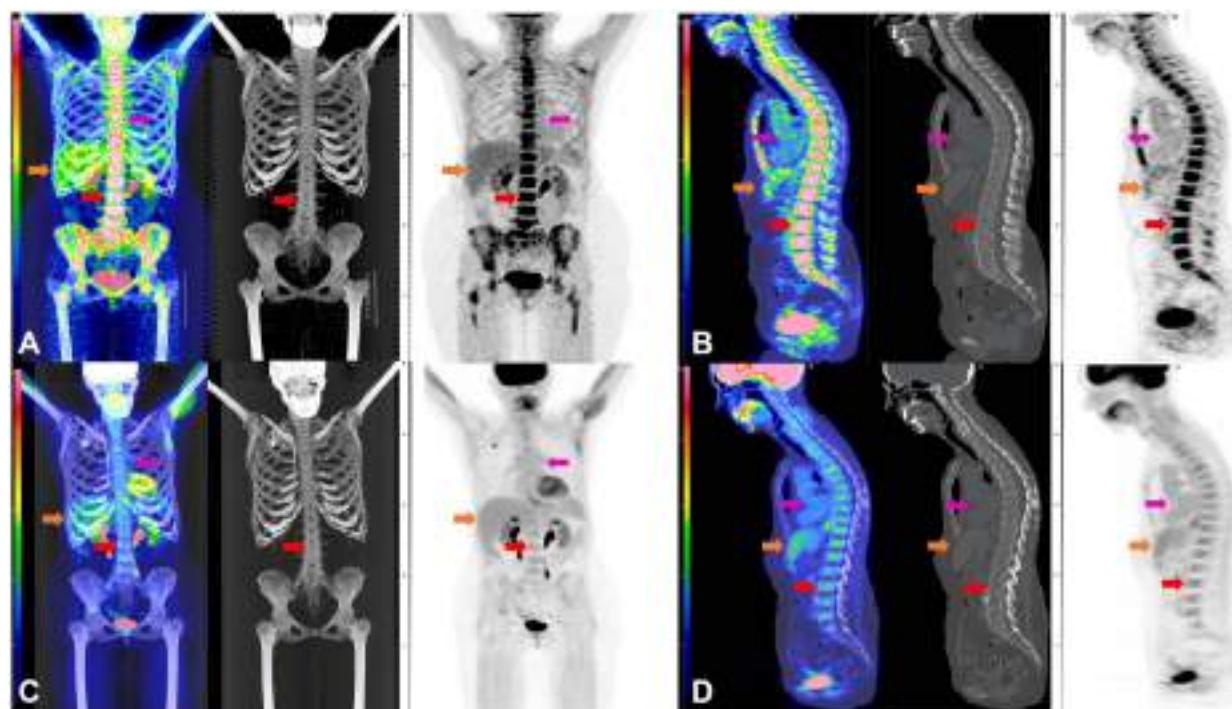


Figure 2 Initial FDG PET-CT and Post-Treatment FDG PET-CT for Case #1. Composite FDG PET-CT images of case #1 at initial diagnosis (**A** and **B**) and after R-CHOP and high-dose MTX (**C** and **D**). Coronal view of fused FDG PET-CT MIP, CT, and PET (**A**) and sagittal fused FDG PET-CT, CT, and PET (**B**) demonstrates intense FDG accumulation in the bone marrow of the axial and appendicular skeleton at initial diagnosis without a specific focus of FDG accumulation, nor lytic or sclerotic lesions on CT. Reference L3 vertebral body SUVmax of 9.9 (red arrow). Physiologic FDG accumulation is seen in the liver (Orange arrow) and blood pool (magenta arrow) and spleen. No hypermetabolic lymph nodes or other soft tissue to suggest lymphomatous involvement. Post treatment fused FDG PET-CT MIP, CT, and PET (**C**) and sagittal fused FDG PET-CT, CT, and PET (**D**) demonstrate normalization of FDG accumulation in the bone marrow of the axial and appendicular skeleton (red arrow) with the L3 vertebrae (SUVmax of 2.3), and unchanged FDG accumulation in the liver (Orange arrow) and blood pool (magenta arrow).

Abbreviations: CT, computed tomography; FDG, fluorodeoxyglucose; MIP, maximum intensity projection; MTX, methotrexate; PET, positron emission tomography; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone.

consistent with involvement of the bone marrow (Figure 5A and B). Based on these findings, she was diagnosed with stage IV PBM-HGBCL (“double hit” Richter transformation of CLL with primary involvement of the bone marrow with leukemic component). Her R-IPI score was 3 and CNS-IPI was 3. She initiated treatment with R-CHOP while awaiting the results of FISH testing. The treatment was later changed to dose-adjusted etoposide, doxorubicin, vincristine, cyclophosphamide, prednisone, and rituximab (DA-EPOCH-R) with intrathecal (IT) MTX. Interim bone marrow biopsy demonstrated low-level residual CD5-, CD10+, kappa-light chain restricted B cell population consistent with HGBCL (<5%). The CD5+, CD10- B-cell population was absent. The patient had a rapid deterioration 3 months after her initial diagnosis, and she developed progressive right-sided weakness, gait instability, and numbness in her hands and feet. MRI spine showed diffuse leptomeningeal enhancement in the cervical and thoracic regions. CSF cytology was positive for HGBCL. She was treated with rituximab, HD MTX, and dexamethasone for one cycle. She had a progressive functional decline and was enrolled in hospice, expiring shortly thereafter.

Discussion

We report two interesting cases of PBML. It is an exceedingly rare lymphoma entity; however, the true incidence is unknown.^{1,2,5} PBML has not been recognized in the International Consensus Classification (ICC) or World Health Organization (WHO) classification.^{5,14,15} Currently, most cases of PBML would be classified as DLBCL not otherwise specified.¹⁴ One review suggested that 1.16% of lymphomas are PBM-DLBCLs; however, it included patients with hepatosplenomegaly which would be excluded with the current definition.⁶

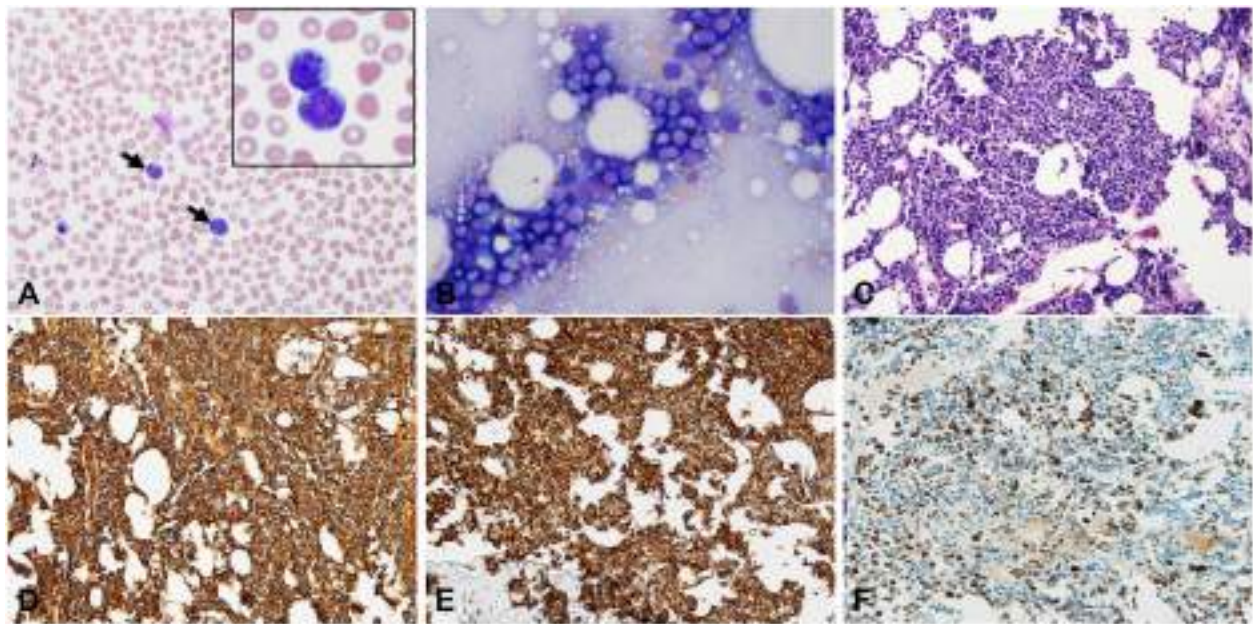


Figure 3 Peripheral Smear and Bone Marrow Biopsy Case #2. For case #2, circulating atypical large lymphocytes (arrows) were present in the peripheral blood smear (A, WG stain, x 40; inset x 60). The roll prep smear and bone marrow biopsy revealed diffusely infiltrating lymphoma cells (B, WG stain x 40; C, H&E stain x 20). IHC showed the lymphoma cells were positive for CD79a and CD10 with a high proliferative rate by Ki-67 (80%) (D, E, F, x 20).

Abbreviations: H&E, hematoxylin and eosin; IHC, immunohistochemistry; WG, Wright-Giemsa.

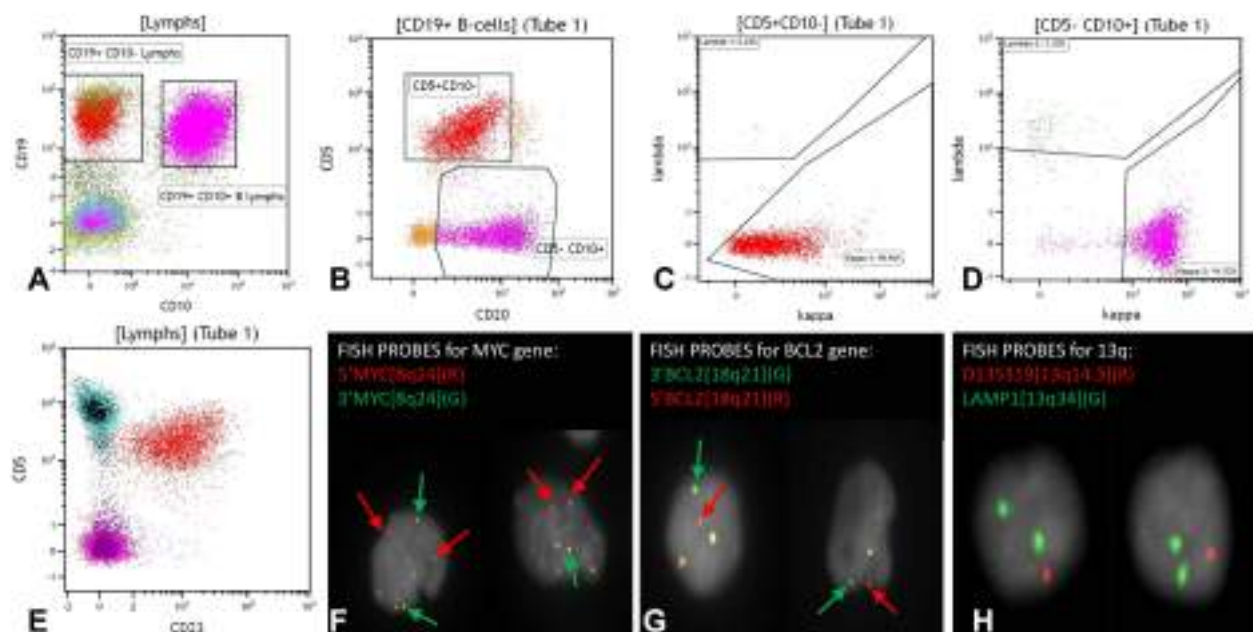


Figure 4 Peripheral Blood Flow Cytometry and FISH Testing for Case #2. For case #2, ancillary studies were performed on the peripheral blood as the bone marrow was a dry tap. Flow cytometry analysis identified two distinct B-cell populations (A–E). One B-cell population (red color) expressed CD19, CD5, and CD23 but was negative for CD10. The second B-cell population (pink color) was positive for CD10 but negative for CD5 and CD23. Both populations showed kappa light chain restriction (C and D). FISH break-apart probes showed MYC gene rearrangement and BCL2 gene rearrangement (F and G, green and red arrows indicate break apart signals) and identified deletion of 13q14.3 (H). **Abbreviation:** FISH, fluorescence in situ hybridization.

The pathologic and biological understanding of PBML is evolving. There have been no definitive diagnostic criteria established as it is very rare and has been reported sparsely in the medical literature. It is generally defined as primary lymphomatous bone marrow involvement with or without a leukemic component by HGBCL without focal bony lesion

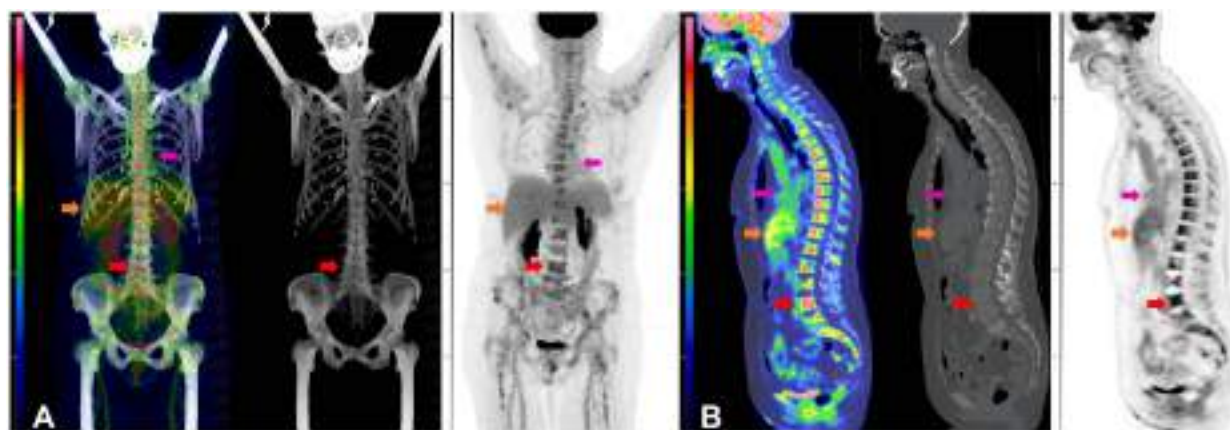


Figure 5 FDG PET-CT of Case #2 After Cycle 1 of R-CHOP. Composite FDG PET-CT images of case #2 after cycle 1 of R-CHOP chemotherapy. Coronal view of fused FDG PET-CT MIP, CT, and PET (**A**) and sagittal fused FDG PET-CT, CT, and PET (**B**) demonstrates moderate intense FDG accumulation in the bone marrow of the axial and appendicular skeleton without a specific focus of FDG accumulation nor lytic or sclerotic lesions on CT. Reference L4 vertebral body SUVmax of 5.9 (red arrow). Physiologic FDG accumulation is seen in the liver (Orange arrow) and blood pool (magenta arrow) and spleen. No hypermetabolic lymph nodes or other soft tissue to suggest lymphomatous involvement.

Abbreviations: CT, computed tomography; FDG, fluorodeoxyglucose; MIP, maximum intensity projection; MTX, methotrexate; PET, positron emission tomography; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone.

or disease elsewhere.^{1,3–5} A diagnosis of PBML also requires exclusion of intravascular lymphoma, leukemias and other aggressive B-cell lymphomas known for primary bone marrow involvement and secondary bone lymphoma which arises from infiltration of the marrow in the setting of disseminated disease.^{1,4,5,9–11,16} The majority of reported PBML cases were DLBCLs; however, they were diagnosed before the widespread use of HGBCL as a diagnostic entity.^{1,6,8} We have used HGBCL confined to the bone marrow with or without leukemic component as the operational diagnostic criteria irrespective of the underlying lymphoma subtype.

Our first case represents de novo PBML without an apparent leukemic component and no evidence of an underlying low-grade lymphoma. Pathologically, it is consistent with DLBCL, non-germinal center phenotype. Additional molecular and FISH testing could not be performed given the dry tap. The interesting finding is a small IgM kappa monoclonal protein in the blood. The relationship between the lymphoma and IgM monoclonal gammopathy is not definitively known. No evidence of lymphoplasmacytic lymphoma was found in the bone marrow. As such, she probably had IgM monoclonal gammopathy of unknown significance (MGUS) related to her age. It has been reported, however, that HGBCL with IgM monoclonal gammopathy is associated with high CNS risk.¹⁷ She has not had any CNS involvement. Bone marrow involvement by DLBCL has not been definitively proven to be associated with high CNS risk; however, leukemic manifestation with bone marrow involvement is associated with high CNS risk.^{18–20} Unlike the second case, this patient did quite well, achieving CR for three years after R-CHOP and getting back into CR following a late bone marrow-only recurrence with non-transplant treatment.

Our second case most likely represents a transformed subtype of PBML (“double hit” Richter’s transformation of CLL confined to the bone marrow) with a leukemic component on initial diagnosis. Richter’s transformation presenting as leukemic HGBCL with extensive bone marrow involvement has been reported in the setting of widely disseminated disease.¹⁸ There were two clonal B cell populations in the blood: a CD5+ and CD10- clone likely related to CLL and a CD5- and CD10+ clone related to HGBCL with *c-MYC* and *BCL-2* translocations. These two clonal B-cell populations shared the same kappa light chain restriction. Although the peripheral blood leukemic B-cell count did not meet the criteria for CLL, the immunophenotypic findings and the presence of 13q deletion were suggestive of a CLL clone in the peripheral blood. The CD5+ clonal B-cell population in the blood can be labeled as monoclonal B-cell lymphocytosis with CLL immunophenotype. Interestingly, we did not find any evidence of CLL on the careful examination of the bone marrow. The clinical course was very aggressive with the rapid development of CNS (spinal leptomeningeal) involvement three months following the initial diagnosis. Double-hit lymphoma has been associated with a high CNS risk.²¹ It is likely that the bone marrow involvement, leukemic manifestation, and Richter’s transformation resulted in higher CNS

risk. Leukemic HGBCL carries a high CNS risk.¹⁸ Abnormal laboratory findings including very high LDH and ferritin supported the aggressive clinical manifestation.

Based on our two cases, it appears that PBML has two subtypes: de novo (d-PBML) and transformed (t-PBML).^{6,22} The de novo subtype may have tissue tropism for the bone marrow. Our first case remained confined to the bone marrow on relapse without extramedullary manifestations. The transformed subtype can potentially arise from primary bone marrow low-grade lymphomas such as CLL, follicular lymphoma, lymphoplasmacytic lymphoma, and marginal zone lymphoma.^{1,4,11} Our two cases also demonstrate that PET imaging is an excellent tool for monitoring lymphoma in the bone marrow.

In the pre-PET/CT era, bone marrow biopsy was performed regularly in the staging of lymphoma. Currently, a routine bone marrow biopsy is no longer performed in the absence of abnormal bone marrow metabolic activity on PET/CT. According to the National Comprehensive Cancer Center guidelines, PET/CT may also be used to monitor response to treatment in FDG-avid lymphomas like DLBCL and Hodgkin Lymphoma.²³

As a rare lymphoma entity, the best therapeutic approach to PBML is not known. There is no clear standard regimen for the management of PBML. Treatment should be individualized according to Eastern Cooperative Oncology Group performance status, age, histology, and other risk factors. Given the aggressive nature of this lymphoma and its tendency for relapse, several studies have recommended R-CHOP therapy with CNS prophylaxis followed by consolidation with autologous stem cell transplant.^{7,22,24} R-CHOP chemotherapy has been used with variable success.^{7,25,26} In a study by the International Extranodal Lymphoma Study Group (IELSG), the majority of PBM-DLBCL patients received CHOP-regimens with or without rituximab with a CR of 33%. Two of the 5 CRs relapsed.³ It has been suggested that poor response to R-CHOP or early relapse following R-CHOP therapy may be associated with elevated LDH, high Ki-67, TP53 mutations, and rearrangements of *MYC* and *BCL-2*.^{26,27} In patients with these high-risk features, more intensive therapy with front-line DA-EPOCH-R or chimeric antigen receptor T-cells could be considered.^{26,27} Reed et al demonstrated good response to rituximab, cyclophosphamide, vincristine, doxorubicin, and dexamethasone (R-Hyper-CVAD) in a young woman with PBM-DLBCL and high-risk of CNS disease.²⁸ PBML has been reported to have a median overall survival (OS) ranging from 8 months to 1.8 years.^{3,6,7,11,29} According to the IELSG, prognosis differs by histologic subtype with a median OS <1.5 years for those with PBM-DLBCL.³

While PBML is not included in the ICC or WHO classification of lymphomas, separation of DLBCL or HGBCL confined to extranodal anatomic sites should be considered. This subclassification could enhance research on the biology of these cancers and optimize their management.

Conclusions

PBML is an extranodal lymphoma confined to the bone marrow with or without a leukemic component. It has not been recognized in the most recent ICC or WHO classification. Biologically, it appears to have two subtypes: de novo and transformed. The transformed subtype appears to have a high CNS risk. Further research is necessary to gain a better understanding of this rare lymphoma entity and identify novel therapeutic approaches.

Abbreviations

BTK, Bruton tyrosine kinase; CLL, chronic lymphocytic leukemia; CNS, central nervous system; CNS-IPI, central nervous system-international prognostic index; CR, complete remission; CSF, cerebrospinal fluid; DA-EPOCH-R, dose-adjusted etoposide, doxorubicin, vincristine, cyclophosphamide, prednisone, rituximab; DLBCL, diffuse large B-cell lymphoma; d-PBML, de novo primary bone marrow lymphoma; EBER, Epstein-Barr virus-encoded RNA; FDG, fluorodeoxyglucose; FISH, fluorescence in situ hybridization; HD, high-dose; HGBCL, high-grade B-cell lymphoma; ICC, International Consensus Classification; IELSG, International Extranodal Lymphoma Study Group; IHC, immunohistochemistry; ISH, in situ hybridization; IT, intrathecal; LDH, lactate dehydrogenase; MGUS, monoclonal gammopathy of undetermined significance; MRI, magnetic resonance imaging; MTX, methotrexate; OS, overall survival; PBFCM, peripheral blood flow cytometry; PBL, primary bone lymphoma; PBM-HGBCL, primary bone marrow high-grade B-cell lymphoma; PBR, polatuzumab, bendamustine, rituximab; PET-CT, positron emission tomography-computed tomography; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; R-Hyper-CVAD, rituximab,

cyclophosphamide, vincristine, doxorubicin, dexamethasone; R-IPI, revised-international prognostic index; t-PBML, transformed primary bone marrow lymphoma; WBC, white blood cell; WHO, World Health Organization.

Consent for Publication

The study participants or their next of kin have given written informed consent to participate as well as written informed consent to publish the case details and accompanying images. Institutional approval was not required to publish the case details.

Disclosure

The authors report no conflicts of interest in this work.

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Impact of Abnormal Leukocyte Count in the Pathophysiology of Sickle Cell Anemia

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Impact of Abnormal Leukocyte Count in the Pathophysiology of Sickle Cell Anemia

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Background: Sickle cell disease (SCD) is a hereditary disease that causes deoxygenated erythrocytes to stiffen, resulting in vaso-occlusive crises, endothelial damage, organ failure and systemic consequences. An abnormal leukocyte count is associated with a worse clinical picture in SCD.

Methods: I studied the link between an abnormal leukocyte count and sickle cell disease. I used the PRISMA guidelines to conduct a systematic review of 5 different clinical literatures that discussed the link. Use of databases including Web of Science, Scopus, Cochrane Library, MEDLINE, ScienceDirect, EMBASE, and JSTOR to make our findings.

Findings: SCD is a red cell disorder, but an elevated leukocyte count plays a significant role in its pathophysiology. Sickled red cells adhere better to leukocytes when compared to normal red cells, which explains the worse clinical picture seen in sickle cell patients. Hydroxyurea therapy inhibits the recruitment and invasion of neutrophils, which lowers vaso-occlusion.

Interpretations: An elevated leukocyte count is responsible for worse clinical manifestations in SCD, eg, clinical stroke, acute chest syndrome, end organ damage, and infarction pain. Leukocytes attach to erythrocytes and vascular endothelium to cause vaso-occlusion, responsible for these effects. They also elaborate inflammatory mediators to aid the process. The higher the leukocyte count, the worse the effects. Hydroxyurea is very helpful in disrupting several pathways of leukocyte contribution to SCD pathophysiology. Therefore, more research on other pharmacologic agents that interfere with this disease process is necessary. Lastly, there is a need for further research into the effects of a reduced leukocyte count in SCD pathophysiology.

Keywords: SCD pathophysiology, leukocyte count, infarction, effects, sickle cell

Introduction

In Sickle Cell Anemia (SCA), valine is abnormally switched out for glutamine at position 6 of the hemoglobin chain (SCA). Any person who expresses this mutation homozygously has the genotype associated with Sickle Cell Disease (SS).¹ The aberrant sickle shape that the erythrocytes adopt is due to a genetic abnormality, which also causes the endothelial damage, vas blockage, hemolysis, ischemia, and inflammation that appear around 5 to 6 months of age after the loss of fetal hemoglobin (HbF).² The disease's clinical manifestation differs from person to person and is more prevalent in the black race while having a clearly characterized monogenic inheritance pattern.³

Although there is no doubt that SCD is caused by a red cell hemoglobin mutation, SCD patients frequently have an abnormal white blood cell count.

When the WBC count is inadequate, infections are more likely to spread and are more difficult to treat. Her white blood cell count will often be normal or higher if the child has sickle cell disease. The WBC count, however, might briefly increase or decrease as a result of disease and some medications. White cell control-focused treatments may be advantageous for patients. Approximately half of the 290 SCD patients in a 2016–2017 study by Ahmed et al⁵ in Saudi Arabia exhibited abnormally increased or decreased white cell counts of 116 or 15, respectively. Other studies have revealed that an elevated white cell count is the most common observation in SCD patients with abnormal white cell counts.^{6–8} By adhering to vascular endothelium and obstructing the lumen, leukocytes participate in the SCD process.

They achieve this by stimulating the production of vascular endothelial ligands for the blood cell adhesion molecules in order to be successful. That results in tissue damage, inflammation, and ultimately vaso-occlusion.⁹

This study will review the pertinent literature in order to comprehend how an unusually high or low white cell count contributes to sickle cell anemia at the tissue level.

Method

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses standards were followed for this study's systematic literature review.¹⁰

Information Sources

I searched these databases for relevant literature: Google Scholar, PubMed, Web of Science, Scopus, Cochrane Library, MEDLINE, ScienceDirect, EMBASE, and JSTOR.

Search Strategy

The terms "sickle cell anemia", "sickle cell illness", "white blood cell", and "sickle cell disease" were used in the search.

Eligibility Criteria

The inclusion criteria for selection include studies that attempt to establish a link between abnormal leukocyte count and sickle cell anemia between January, 2002 and May, 2022; literature published in English; and those with an observational, qualitative, or systematic review design.

Selection Process

Studies unrelated to the link between abnormal leukocyte count and sickle cell anemia, duplicated ones, and abstract-only papers were excluded.

Results

A total of 20 studies that satisfied my inclusion criteria were identified, of which I selected 4. The study selection process goes as follows: After identifying 20 studies, 15 passed the screening stage, while the other 5 were duplicates. Of these 15 studies, only six were eligible, as the remaining nine were abstract-only studies. I arrived at the inclusion of 2 studies and 3 articles^{6,9,11} following a careful review of all options. The characteristics of the samples, types of intervention, and outcomes of the 2 studies are shown in Table 1.

Okpala I,⁹ in his article titled "The Intriguing Contribution of White Blood Cells to Sickle Cell Disease – a Red Cell Disorder", reviews relevant literature, totaling 67, in an attempt to establish a direct link between leukocytes and the disease process in SCDA. Wun T.¹¹ takes it a step further and also explores the role of inflammatory cytokines alongside leukocytes in "The Role of Inflammation and Leukocytes in the Pathogenesis of Sickle Cell Disease." Zhang D.⁶ decides to focus on a particular class of leukocytes in his article "Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology."

Results of Individual Studies

After a thorough search of the listed databases, three experienced reviewers selected the relevant studies for this review based on the title and abstract. They passed on the selected studies to two other reviewers, who read the full text of the selected studies and approved the most suitable options.

As Okpala I⁹ demonstrates, there's ample evidence to suggest that white blood cells contribute to the clinical manifestations seen in SCD. Despite the diverse phenotypes seen in a monogenic disorder such as SCD, the most consistent finding directly related to clinical severity remains an elevated leukocyte count, particularly neutrophils.¹⁴⁻¹⁷ Furthermore, a drop in leukocyte count reduces the severity of the hemoglobinopathy, consistent with reports indicating SCD is exacerbated by increased leukocyte levels. The therapeutic impact of hydroxyurea in SCA is associated with a decrease in the polymorphonuclear (neutrophil) count even in people with stable hemoglobin F levels.¹⁸ SCD vaso-occlusion is thought to be caused by

Table 1 Characteristics of the Samples, Types of Intervention, and Outcome of the 2 Studies

Authors/Year	Objectives of Study	Type of Study	Sample	Protocol	Result
Okpala I, Odoemene D, Goldman J, Daniel Y, Haynes R ¹² 2002	To explain the link between the expression of Adhesion Molecules (AM) by leukocytes and SCD crises	Randomized Clinical Trial	N=100 patients	The connection between leukocyte Adhesion Molecule (AM) expression and clinical symptoms of SCD were assessed in 100 SS patients and 34 AA controls. The impact of hydroxyurea on AM expression was also investigated.	(1) Leukocytes that highly express aMb2 integrin and I-selectin predispose SCD patients to worse symptoms. (2) Increased Adhesion Molecule expression in leukocytes above normal levels contribute to crisis development. (3) The early clinical relief associated with hydroxyurea therapy goes beyond increasing HbF levels. There's a high index of suspicion for decreased expression of Adhesion Molecules in leukocytes.
Aslihan Turhan, Narla Mohandas, Paul S. Frenette, Linnea A. Weiss, and Barry S. Collier ¹³ 2002	To determine the role of leukocyte adherence in sickle cell vaso-occlusion.	Animal Trial	Berkeley mice	Intravital microscopy	Vasooocclusion of cremasteric venules occurs when sickled erythrocytes attach to leukocytes in inflamed venules. The SS mice without E- and P-selectins and this is probably because of deficient recruitment of white blood cell to the vessel wall.

a complex interaction involving white blood cells, red blood cells, platelets, plasma proteins, and blood vessel walls, which explains why a high leukocyte count has such a negative influence on clinical severity. The attachment of leukocytes to the endothelium of postcapillary venules is thought to diminish the diameter of the vessels, which is worsened by the attachment of erythrocytes, both sickled and un-sickled, as well as platelets.¹⁹

The neutrophils are the most notorious leukocytes, well known for attaching to the vessels' walls. It is the body's most common leukocyte but it has a short circulation time, totaling about 6 hours. The huge number of white blood cells in the diapedesis process reveals one key fact: the lumen of postcapillary venules is constantly shrinking. The more adherent cells inside the vessel, the higher the leukocyte count. As a result, HbSS patients with greater leukocyte counts have a worse prognosis. White blood cells do not only attach to the walls of blood vessels. They also attach to one another and to other blood cells like red blood cells and platelets. In this way, they form cell aggregates that reduce the diameter of small arteries more effectively. Moreover, there are higher chances for entanglement of sickled red blood cells in this matrix, leading to vascular occlusion. The stability of these blood cell aggregates rests on the CD36–TSP, CD31–CD31, and CD62L–CD162 connections.^{20–23}

Leukocytes may indirectly precipitate vascular occlusion in SCD by activating the endothelial layer of blood vessels. The inflammatory mediators TNF- α and interleukin-1 β , for example, are secreted by monocytes and activate the vascular endothelium.²⁴ The inflammatory response is heightened, and the danger of tissue damage is increased. The endothelium of blood vessels is stimulated during inflammation, and leukocytes and other blood cells express more adhesion molecule ligands. In turn, that increases the risk of lumen blockage, ischemia, organ damage, dysfunction, and infarction pain associated with SCD.

Wun T.¹¹ remarks that raised white cell counts in SCD patients with no signs of infection, pain, or inflammation are documented.²⁵ He goes further to list reasons for this, such as functional hyposplenism, asplenia, and bone marrow stimulation for red cell production due to the hemolysis in SCD. Furthermore, an elevated leukocyte count in SCD indicates a worse clinical picture, with an increased risk of stroke²⁶ and acute chest syndrome.²⁷ According to the article, what role do leukocytes play in SCD pathogenesis? Activated and stiff PMN can initiate vascular occlusion by impeding blood flow in the small caliber arterioles and increasing the time needed for the sickled erythrocytes to transit. All these happen in a bid to increase the chances of HbS polymerization. Furthermore, SS-RBC adhered to neutrophil monolayers better than control red cells (AA-RBC). In the case of SSRBC, autologous plasma-enhanced adhesion, but not in the case of AA-RBC. This revealed that SSRBC attached more easily to PMN, and that the plasma contained chemicals that aided this attachment. Surprisingly, the dense fraction had the most adhesive SSRBC, but the thin fraction had the most adherent SSRBC to active endothelium. Activated PMNs release free oxygen radicals, which increase the expression of AMs on the vascular endothelium, causing vascular injury.²⁸

Monocytes are not left out. In sickle cell disease, monocytosis is prevalent. They attach to active platelets and SS-RBC. This most likely activates them and has been linked to the etiology of vascular disease.^{29–31} In vitro investigations show that monocytes from SCD patients produce greater superoxide anion in response to agonists than controls, leading to vascular injury. Finally, in sickle cell disease patients, the action of procoagulants is enhanced, with practically all of the procoagulant activity found in the mononuclear component of the blood.

According to Zhang D,⁶ the identification of absolute contraindications for myeloid growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in SCD patients suggests a role for neutrophils in SCD pathogenesis. Early reports indicate that SCD patients given either growth factors to cure leg ulcers, mobilize hematopoietic stem cells, or correct diminished neutrophil counts experienced severe or fatal crises.^{32–34} A patient with SCD who had severe congenital neutropenia was recently found to have significantly improved clinical symptoms when compared to his brothers and sister. However, things changed when he was given G-CSF for neutropenia, as his condition quickly worsened.³⁵ The significance of hydroxyurea in SCD therapy is also explained in further detail in the article. Sickled RBC adherence to the endothelium is reduced by hydroxyurea treatment, as are the levels of soluble vascular cell adhesion molecule (VCAM)-1 in patient plasma.³⁶ Furthermore, current research reveals that hydroxyurea treatment increases NO species, which is probably related to HbF induction.^{37,38} Furthermore, things remain the same in patients who do not have a detectable increase in HbF as hydroxyurea medication is still helpful. On the other hand, patients who have a positive clinical response to hydroxyurea therapy have lower neutrophil counts.³⁹ Furthermore, the correction of neutrophil activation indicators reveals that hydroxyurea treatment inhibits neutrophil activation.⁴⁰ Additional data suggests that hydroxyurea treatment lowers vascular occlusion in sickle cells by limiting the recruitment and activation of PMNs via the NO-cGMP pathway.⁴¹ The discovery that sickled erythrocytes bind to PMN in-vitro adds to the growing body of evidence indicating neutrophils may have a direct impact on SCD pathogenesis. In vivo studies in SCD mice have discovered that intravital imaging can be used to study the pattern of blood cell flow in the cremasteric microvasculature. Sickled erythrocytes primarily interact with attached leukocytes in postcapillary venules in this model. Surgical trauma induces these interactions, which are increased and extended by the administration of tumor necrosis factor (TNF), resulting in a fatal vaso-occlusive crisis (VOC). In the test subjects lacking E- and P-selectins, which usually block leukocyte migration to the vascular endothelial layer, VOC is not seen.³⁹ First, inflammatory stimuli from within and outside the body activate endothelial cells, allowing circulating neutrophils to be attracted to postcapillary venules. The attached PMNs then connect with circulating sickled erythrocytes via activated Mac-1, restricting venular blood flow for a short or long time. Increased RBC transit time, ischemia, erythrocyte sickling, the recruitment of neutrophils, and interaction within this blood cell recruitment all result in vascular occlusions, which can lead to life-threatening crises.

The role of monocytes is not ignored by Zhang D. Monocytes with SCD have a functional characteristic that encourages inflammation—they can activate the nuclear factor B (NF- κ B) pathway in the cells of the endothelium. By enhancing the production of leukocyte AMs like E-selectin, ICAM-1 (intercellular adhesion molecule), and VCAM-1, the NF- κ B pathway increases mononuclear leukocyte attachment to endothelial cells. Endothelial cells are stimulated by monocytes via proinflammatory cytokines produced by monocytes rather than cell-cell contact. When tumor necrosis

factor- α and IL-1 from monocytes are blocked by using neutralizing antibodies, there is a decrease in the expression of E-selectin by the endothelial cells.^{42,43}

The in-vivo study of Turhan et al¹³ to demonstrate that sickled erythrocytes attach to adhering leukocytes in inflamed venules has already been covered briefly in previous study reviews. They used intravital microscopy to view the microvasculature of genetically engineered mice that exclusively express human hemoglobin S (HbS). These mice have irreversibly sickled circulating cells and exhibit several symptoms of SCD, such as anemia, increased reticulocyte counts, and end-organ damage. Using two separate animal models of SCD, the researchers discovered that sickled erythrocytes interact with white blood cells attached to the cremasteric postcapillary and collect venules. These interactions were sustained or increased when TNF- α was given shortly after the surgical alteration (30 minutes). Although most interactions between the sickled erythrocytes and white blood cells were short-lived, some lasted several seconds, withstood blood shear, and resulted in vascular occlusion. These findings show that the attachment of leukocytes to the walls of the blood vessels has a direct impact on the arterial occlusions seen in SCD.

Okpala et al¹² looked at 100 HbSS patients, 43 males and 57 females between the ages of 18 and 65. There were 34 HbAA controls in all, 11 men and 23 females between 19 and 60. The connection between leukocyte adhesion molecules and SCD clinical symptoms was investigated using flow cytometry. HbSS patients with any condition other than SCD, HbF levels greater than 10%, and controls with any known medical diagnosis were all excluded from the study. SCD and control participants were both excluded if they had been pregnant or had given birth in the previous three months. Significant levels of aMb integrin expression in neutrophils and l-selectin expression in lymphocytes and neutrophils were seen in patients with SCD ($P < 0.03$).

The neutrophils of sickle nephropathy patients had a high level of CD18 expression ($P = 0.018$), whereas the lymphocytes of stroke patients had a high level of l-selectin ($P = 0.03$). Monocyte l-selectin increased ($P = 0.04$) in the sickle cell crisis compared to steady-state. Within one month of starting hydroxyurea therapy, there was a decrease in aLb2 integrin expression by leukocytes-neutrophils, monocytes, and lymphocytes ($P = 0.05$), with clinical improvement in the patients with a 3.3% increase in HbF level. They also discovered that the steady-state expression of neutrophil aMb2 integrin and l-selectin was much higher in patients with complications than in people without problems. Because adhesion molecules mediate the attachment of white blood cells to the vascular endothelial layer, this discovery is very likely. As the number of neutrophils in the blood increases, SCD becomes more severe, and leukocytosis increases the chances of the worst complication-death. The first contact of PMNs and leukocytes with the blood vessel wall is controlled by selectin (CD62L).⁴⁴ CD11b and CD18 together make up the aMb2 integrin molecule, mediating leukocyte adhesion to vascular endothelium.⁴⁵ It should come as no surprise that HbSS patients with elevated levels of leukocyte selectin and aMb2 integrin are more likely to develop SCD complications.⁴⁶

Based on their data, they came to the following conclusions: (1): Leukocytes with high aMb2 integrin and l-selectin steady-state expression predispose patients to severe symptoms. (2) AM expression in leukocytes increased above steady-state levels and may have contributed to the beginning of the crisis. (3). Other processes, such as decreased AM expression in leukocytes, may be involved in the early clinical improvement associated with hydroxyurea therapy. (4) Other therapies that reduce leukocyte AM expression may benefit patients.

Discussion

An abnormal leukocyte count and the SCD disease process have only sometimes been directly linked in research. Due to our review of the articles, we have presented quite a few. First, there is a ton of evidence that leukocytes play a part in the development of SCD. Adults with SCD and elevated white cell counts were more likely to visit the Emergency Department (ED) as a result of sickle cell crises, according to Curtis et al.⁴ Leukocytes, like erythrocytes, may aid in SCD vaso-occlusion by sticking to the endothelium. The severity of SCD is highly correlated with the number of neutrophils in the blood. Unsurprisingly, among all leukocytes, neutrophils have the greatest effect on the SCD disease process. This location is closely followed by monocytes. Other leukocytes, such as basophils and eosinophils, are also present. Blood cell-vessel wall contacts and intercellular interactions are potentiators of the process.

Leukocytosis has been associated with an elevated mortality rate from illness. In some SCD patients, the advantages of hydroxyurea (HU) therapy are accompanied with a decreased neutrophil count without an increase in hemoglobin F levels.

Leukocytes undoubtedly contribute to SCD vaso-occlusion, which results in serious clinical conditions, as shown by the aforementioned study as well as several other ones that have already been mentioned.

Limitations

This study could not link the effect of reduced leukocyte count in the disease process of SCD. Furthermore, the impossibility of in-vivo human studies limits us to the use of studies in mice, which may not necessarily be an exact representation of the pathophysiology in humans.

Conclusion

The involvement of abnormal leukocyte counts in the etiology of SCD is not only supported by evidence. There is also a lot of proof that therapies that focus on these pathways enhance the clinical results of SCD patients. But there is little proof to prove that a low leukocyte count contributes to the pathophysiology of SCD.

Disclosure

The author reports no conflicts of interest in this work.

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Evaluation of Minimal Optimal Dose of Intravenous Ferric Carboxymaltose for Treatment of Iron Deficiency Anemia and Risk of Transient Hyperferritinemia

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Evaluation of Minimal Optimal Dose of Intravenous Ferric Carboxymaltose for Treatment of Iron Deficiency Anemia and Risk of Transient Hyperferritinemia

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Background: Iron supplementation is administered orally or intravenously to treat iron-deficiency anemia (IDA). Ferric Carboxymaltose (FCM) “Ferinject®” is an intravenous (IV) iron preparation that has emerged as a safe therapeutic option for treating IDA in the past decade.

Aim: This study aimed to evaluate safety and efficacy of carboxymaltose in a cohort of patients with IDA not responding to oral therapy.

Methods: This 12-month retrospective study included 106 patients with IDA, with-or without bariatric surgery, who received (single or multiple doses) of Carboxymaltose 500mg/10mL. Data points included patients’ demographics, baseline data for Hb, platelet, ferritin, and MCV pre-and at 1, 2, and 3 months following different doses of IV-Carboxymaltose. Changes in Hb, MCV, platelets, and ferritin levels were recorded in response to Carboxymaltose to assess the optimal dose, risk of hyperferritinemia, and hypophosphatemia.

Results: At three months (95 days) follow-up, the median change pre-and post-therapy in hemoglobin was from 9.5 to 11.9g/dL ($p < 0.01$), MCV 73.6–80.5fL ($p < 0.01$), and ferritin 5.3–93.8ng/mL. A significant difference was observed between platelet count of patients who underwent bariatric surgery and those who did not. An optimal ferritin response ($>30\text{ng/mL}$) was observed in 87.8% of patients who received first dose, and none of the full three doses showed no response. 37% of patients who received two doses developed hyperferritinemia. Serum phosphate levels were assessed in 28 cases, and hypophosphatemia was observed in 25% of these patients.

Conclusion: Carboxymaltose is a reliable option for IDA. IV-FCM therapy helps achieve significant improvement in hemoglobin concentration and MCV from the first dose carrying a low reversible risk of hyperferritinemia following multiple doses. An interesting finding of this study is the discovery of a population of IDA patients requiring periodic assessment for iron reinfusion to sustain normal levels, mostly post-bariatric surgery. Changes in serum phosphate levels reported to occur consecutively with FCM treatment should be further studied.

Keywords: iron deficiency, anemia, ferrous carboxymaltose, hypophosphatemia

Impact of Findings on Practice Statements

- We identified the minimal optimal dose of Ferric Carboxymaltose for correcting anemia, which may be helpful in clinical practice in managing noncompliant patients.
- The hyperferritinemia which developed after multiple dose infusion proved to be transient, and resolved at follow-up. This should minimize the concerns among physicians and patients.
- Hypophosphatemia developing in a small portion of the cohort after infusion also proved to be transient on follow-up.

Introduction

Optimal iron balance plays a critical role in disease management, as iron is an integral component of hemoglobin-mediated oxygen transport essential for survival and health. Iron deficiency is a major cause of anemia, affecting one-third of the world population,¹ while 50% of anemia cases worldwide are due to iron deficiency anemia (IDA).² Iron deficiency can be absolute (lack of total iron stores in the body) or functional (impaired iron bioavailability).³ Usually, iron deficiency develops gradually and does not have clinically apparent symptoms until anemia becomes severe. IDA is the fifth leading cause of disability, affecting 1.24 billion people.¹ Iron deficiency results from prolonged iron imbalance due to nutritional deficiency, impaired iron absorption, worm infestations, chronic blood loss, or multiple pregnancies.⁴

Oral or intravenous (IV) iron supplementation or rarely blood transfusion is prescribed to combat iron deficiency. The oral route of iron administration is historically favorable; however, in addition to stomach upset including (pain, bloating and sometimes vomiting) and constipation side effects, poor absorption and compliance can compromise the effectiveness of the interventions. Second, there are multiple risks associated with blood transfusion, including transmission of infections such as human immunodeficiency virus, mismatched transfusions, immune modulation, and other risks.⁵ IV iron therapy is safe and more cost-effective than blood transfusion and overcomes the many hurdles faced by oral supplementation, such as poor absorption and gastrointestinal side effects with few side effects mainly allergic reactions.

The prevalence of anemia in Europe is around 20%.⁶ In contrast, regional studies on anemia present varied results, with the prevalence of anemia in adolescents in the Kingdom of Saudi Arabia (KSA) as 16–34%.⁷ IDA is the most common cause of anemia, especially among females, in KSA. However, the prevalence of IDA is not well established by epidemiological surveys in KSA, with reports limited to single institutes with specific age groups or gender indicating a wide 6–56% prevalence.^{8–12} In a recent multicenter epidemiological study, the prevalence of IDA was found to be 10.7% among Saudi university students.¹³

Aim

Although iron supplementation is often prescribed, the most effective route for this patient population is unclear to attain stable hemoglobin (Hb) levels. Therefore, the current study aimed to describe the response to IV iron in different approved doses. It also estimates the minimal optimal dose and the risk of hyperferritinemia through evaluating the efficacy and safety of IV iron supplementation (carboxymaltose) with its effect on Hb, Mean Corpuscular Volume (MCV), ferritin, and platelet levels in IDA patients in both bariatric and non-bariatric subgroups and evaluate the related health conditions such as menorrhagia, ulcerative colitis, anticoagulation or thalassemia.

Methodology

Study Design and Data Collection

This retrospective study was approved by our institutional review board. The inclusion criteria were age >18 years with a confirmed IDA diagnosis based on Hb <12 g/dL for women and <13 g/dL for men with ferritin levels <30 ng/mL, who could not tolerate oral iron or had poor response defined with no improvement after 2-month therapy.

This study was conducted in a hematology outpatient clinic for 12 months at a major secondary care center, where data was collected from the medical records system for 106 patients with IDA who received (single or multiple doses) of Carboxymaltose “Ferinject®” 500 mg/10 mL at a dose of 500mg in 200 mL normal saline over 30 minutes. A total of 94 (88.7%) patients were included in the statistical analysis, while 12 (11.3%) patients were excluded from the analysis because they had no follow-up data for the primary efficacy endpoint assessment. Data points included patients’ demographics and baseline data for Hb, platelet, ferritin, and MCV pre- and at 1, 2, and 3 months following different doses of IV Carboxymaltose.

Statistical Methods

Categorical variables are presented as counts and percentages (eg, sex, bariatric surgery, medical history, and total administered dose of IV iron). Numerical variables are presented as means and standard deviations if normally distributed

(eg, age). Laboratory results are presented as the median, interquartile range (IQR), and range. The Wilcoxon signed-rank test was used to compare the laboratory values pre- and post-therapy with Carboxymaltose “Ferinject”. The magnitude of change was recorded for laboratory results such as Hb, MCV, platelets, and ferritin in response to different doses of Carboxymaltose to assess the optimal dose, risk of hyperferritinemia, and hypophosphatemia as an outcome. The magnitude of change was compared between the subgroups (age groups, bariatric surgery, and total administered dose) using the Kruskal–Wallis test. Statistical significance was set at $p < 0.05$. IBM SPSS (Statistical Package for the Social Sciences; IBM Corp, Armonk, NY, USA) was used to perform all statistical calculations, version 21 for Microsoft Windows. No data imputation was carried out for missing data.

Results

The mean age of the cohort was 37 ± 12.3 years. There was a female preponderance, with 95.7% of subjects being female. The median pre-therapy Hb concentration and MCV in our subjects were 9.5 g/dL and 73.6 fL, respectively, which increased to 11.9 g/dL and 80.5 fL post-therapy. Of our cohort, 3.2% had menorrhagia, 4.3% were on anticoagulants with bleeding, and 2.1% had a concomitant vitamin B12 deficiency. 39.4% of subjects received one dose, 53.2% received two doses, and 7.4% received three doses of Carboxymaltose “Ferinject” 500 mg/10 mL. Only 32 (34%) patients underwent bariatric surgery (Table 1).

A median (IQR) increase of 1.8 (2.5) g/dL was observed in hemoglobin levels. The median change observed after three months of follow-up among patients who received three doses of IV iron was significantly higher ($p = 0.003$) than those who received one or two doses of IV iron (Table 2). However, no significant difference was found between patients who underwent bariatric surgery and those who did not ($p = 0.655$). Similar to bariatric surgery, no significant difference

Table 1 Socio-Demographic Variables

Parameter	Categories	Count	Percent
Gender	Male	4	4.3%
	Female	90	95.7%
Age (years)		Mean $\pm$ SD	37.0 $\pm$ 12.3
Bariatric surgery	Yes	32	34.0%
	Sleeve	22	23.4%
	Bypass	9	9.6%
	Gastric band	1	1.1%
	No	62	66.0%
Medical history	Yes*	17	18.1%
	Multivitamin Deficiency	2	2.1%
	Menorrhagia	3	3.2%
	Ulcerative colitis	1	1.1%
	Anticoagulation	4	4.3%
	Thrombocytopenia	2	2.1%
	MVR	1	1.1%
	TTP	1	1.1%
	Vitamin B12 deficiency	2	2.1%
	vWD	1	1.1%
	Thalassemia	1	1.1%
	No	77	81.9
Total received dose of Carboxymaltose “Ferinject”	1 dose	37	39.4%
	2 doses	50	53.2%
	3 doses	7	7.4%

Note: *One patient has more than two diseases as medical history.

Table 2 Hemoglobin Level (g/dl) Before and After Treatment in the Total Population and in the Different Subgroups

Patients' Subgroup		Count	Before Treatment		3 Months Post Treatment		Change		P-value*	P-value**
			Median	IQR	Median	IQR	Median	IQR		
Total population		94	9.5	2.63	11.9	1.9	1.8	2.5	<0.001	
Bariatric surgery	Yes	32	9.7	1.7	12.0	1.5	2.0	2.4	<0.001	0.655
	No	62	9.5	1.9	11.8	1.5	1.8	2.6	<0.001	
Number of administered doses	1 dose	37	10.6	1.9	12.0	1.5	1.3	2.6	<0.001	0.003
	2 doses	50	9.4	1.6	11.9	1.6	1.9	2.1	<0.001	
	3 doses	7	7.4	0.6	11.4	0.8	3.5	1.1	0.018	
Age group	<40 years	57	9.5	2.0	12.0	1.5	1.9	2.7	<0.001	0.481
	≥40 years	37	9.5	1.6	11.4	1.6	1.8	2.1	<0.001	

Notes: *Wilcoxon signed-rank test was used to compare hemoglobin levels before and after treatment in the total population and all subgroups at 0.05 level of significance.
 **Kruskal–Wallis test was used to compare the magnitude of change observed in hemoglobin levels between the different subgroups at 0.05 level of significance.

was found between the different age groups regarding the increase in Hb levels ($p = 0.481$). Among the total population, the median hemoglobin concentration pre and post treatment was significantly increased from 9.5 g/dL to 11.9 g/dL ($p < 0.01$) after almost three months (95 days) of follow-up (Table 2).

Among the total population, the median MCV was significantly increased from 73.6 fL to 80.5 fL ($p < 0.01$) after almost three months (95 days). A statistically significant increase was observed among all subgroups. Further assessment after 133 days (~ 4.5 months) post-baseline showed a median (IQR) MCV of 82.3 (12.3) fL with a significant increase from the first post-baseline assessment ($p < 0.001$) (S1).

A significant drop in the median platelet count from $301.5\text{--}272 \times 10^9/\text{L}$ was observed among the total population after almost three months (95 days) of the therapy, regardless of the age group or bariatric surgery ($p < 0.05$). The total administered doses of IV iron were found to significantly affect the decrease in platelet count in patients who received two doses of IV iron, showing a significant drop in their median platelet count ($p < 0.001$), while those receiving one did not show a significant drop ($p = 0.502$). The sample size of those receiving three doses was much smaller than that of their single- or double-dose counterparts; hence, the statistical comparison was not significant ($p = 0.236$). Further assessment of platelet count after 133 days (~ 4.5 months) post-baseline revealed a median (IQR) value of 242 (65.0) $\times 10^9/\text{L}$ with a statistically non-significant decrease from the first post-baseline assessment ($p = 0.935$) (Tables 3, S2).

Among the total population, the median ferritin level was significantly increased from 5.3 ng/mL to 93.8 ng/mL ($p < 0.01$) at three months (95 days). A statistically significant increase was observed among all subgroups (Figure 1). In a subgroup analysis of patients who received a single dose of Ferric Carboxymaltose (FCM), the assessment of ferritin level 133 days (~ 4.5 months) post-baseline showed a median (IQR) of 17.5 (30.1) ng/mL with a significant decrease from the first post-baseline assessment ($p < 0.047$) (Tables 4, S3).

In a subgroup analysis, we found that 37% of the patients who received two doses developed hyperferritinemia. Of these, 19.6% had ferritin levels <300 ng/mL, whereas 17.4% had ferritin levels >300 ng/mL. Approximately 9.1% of patients receiving one dose developed hyperferritinemia, but the level in all patients was <300 ng/mL. Among those who received the three doses, 57.1% developed hyperferritinemia, with 28.6% having >300 ng/mL. An optimal ferritin response (>30 ng/mL) was observed in 87.8% of the patients receiving one dose. This can be explained by the fact that the first dose was successfully administered to most of the patients, while the second and third dose was given to a portion of the cohort. One dose was insufficient to replenish ferritin stores in 3% of the patients, while none of the other full three doses resulted in nonresponse (Tables 5, S5). Serum phosphate levels were assessed in 28 patients 3-months post-infusion, of which seven (25%) had hypophosphatemia. Of the patients in whom the assessment was performed, four patients (14.3%) received one dose, two patients (7.1%) received two doses and only one patient (3.6%) received three doses.

Table 3 Platelets Counts Before and After 3 Months Treatment in the Total Population and the Different Subgroups

Population		Timing	Count	Median	IQR	Min.	Max.	P-value*
Total population		Before treatment	94	301.5	122.0	132.0	828.0	<0.001
		After treatment	94	272.0	94.4	86.0	552.0	
Bariatric surgery	Yes	Before treatment	32	303.5	113.7	132.0	605.0	0.002
		After treatment	32	269.0	108.9	86.0	552.0	
	No	Before treatment	62	300.5	126.9	152.0	828.0	0.002
		After treatment	62	281.5	86.9	117.0	513.0	
Total administered dose	1 dose	Before treatment	37	262.0	97.8	132.0	531.0	0.502
		After treatment	37	278.0	100.1	86.0	513.0	
	2 doses	Before treatment	50	312.5	135.6	152.0	828.0	<0.001
		After treatment	50	269.0	83.5	117.0	552.0	
	3 doses	Before treatment	7	428.0	70.5	258.0	463.0	0.236
		After treatment	7	323.0	121.0	220.0	497.0	
Age group	<40 years	Before treatment	57	296.0	130.3	132.0	828.0	0.004
		After treatment	57	280.0	104.3	86.0	552.0	
	≥40 years	Before treatment	37	311.0	109.7	152.0	735.0	0.001
		After treatment	37	264.0	75.6	141.0	496.0	

Note: *Wilcoxon signed-rank test at 0.05 level of significance.

A median (IQR) increase of 5.8 (10.9) fL was observed in the MCV level. The median change observed among patients who received three doses of IV iron was significantly higher ($p = 0.036$) than that observed among patients who received one or two doses of IV iron. On the other hand, no significant difference was found between patients who underwent bariatric

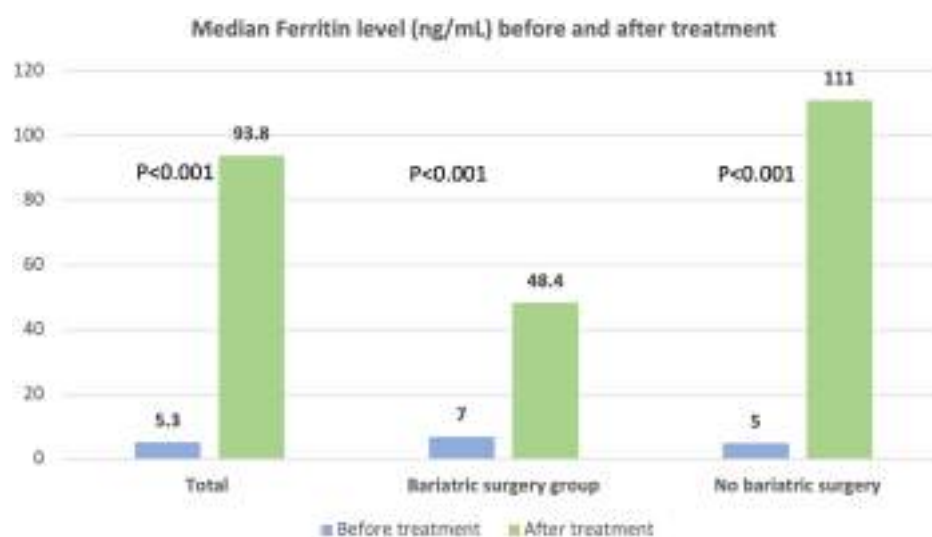
**Figure 1** Median Ferritin level (ng/mL) before and after treatment.

Table 4 Ferritin Level (ng/mL) Before and After 3 Months Treatment in the Total Population and the Different Subgroups Descriptive

Population		Timing	Count	Median	IQR	Min.	Max.	P-value*	P-value**
Total population		Before treatment	90	5.3	32.1	1.00	199.3	<0.001	
		After treatment	86	93.8	173.8	1.48	875.30		
Bariatric surgery	Yes	Before treatment	31	7.0	25.5	1.00	112.6	<0.001	0.212
		After treatment	31	48.4	133.2	3.01	581.90		
	No	Before treatment	59	5.0	35.3	1.0	199.3	<0.001	
		After treatment	55	111.0	190.6	1.48	875.30		
Total administered dose	1 dose	Before treatment	35	5.8	48.3	1.00	199.29	<0.001	<0.001
		After treatment	33	31.8	82.7	3.9	291.1		
	2 doses	Before treatment	48	5.6	12.1	1.00	63.9	<0.001	
		After treatment	46	116.9	198.3	1.48	875.30		
	3 doses	Before treatment	7	1.8	1.6	1.0	4.5	0.018	
		After treatment	7	238.3	182.8	53.4	521.6		
Age group	<40 years	Before treatment	53	5.3	38.0	1.0	199.3	<0.001	0.929
		After treatment	51	105.8	134.6	3.01	521.64		
	≥40 years	Before treatment	37	5.3	21.7	1.00	112.75	<0.001	
		After treatment	35	42.9	220.3	1.5	875.3		

Notes: *Wilcoxon signed-rank test was used to compare ferritin levels before and after treatment in the total population and all subgroups at 0.05 level of significance. **Kruskal–Wallis test was used to compare the magnitude of change observed in ferritin levels between the different subgroups at 0.05 level of significance.

Table 5 Comparing the Prevalence of Hyperferritinemia Between the Different Doses

Hyperferritinemia	Yes		No		P-value
One dose (37)	3	9.1	30	90.9	0.005
Two doses (50)	17	37.0	29	63.0	
Three doses (7)	4	57.1	3	42.9	
Total (68)*	24	27.9	62	72.1	

Note: *Eight patients with missing ferritin data at first assessment post-treatment.

surgery and those who did not undergo bariatric surgery ($p = 0.191$). Like bariatric surgeries, no significant difference was found between the different age groups with respect to the increase in the MCV level ($p = 0.889$) (S4).

A median (IQR) decrease of $29.5 (72.3) \times 10^9/L$ was observed in platelet count. The median decrease observed among patients who received two doses of IV iron was significantly higher ($p = 0.024$) than that observed among patients who received one dose of IV iron. The sample size of those receiving three doses was much smaller than that of their single- or double-dose counterparts; hence, the statistical comparison was not significant. However, no significant difference was found between patients who underwent bariatric surgery and those who did not undergo bariatric surgery ($p = 0.503$).

Similar to bariatric surgeries, no significant difference was found between the different age groups regarding the decrease in platelet count ($p = 0.254$) (S2).

Changes were observed in ferritin level after the first dose with a median (IQR) increase of 59.6 (193.2) ng/mL. The median change observed among patients who received three doses of IV iron was significantly higher ($p < 0.001$) than that observed among patients who received one or two doses of IV iron. In contrast, no significant difference was found between patients who underwent bariatric surgery and those who did not ($p = 0.212$). Like bariatric surgery, no significant difference was found between the different age groups regarding the increase in ferritin levels ($p = 0.929$).

Discussion

In the current study, we aimed to investigate the efficacy and safety profiles of various doses of IV iron to identify the minimal optimal dose without the risk of hyperferritinemia in IDA patients, both post-bariatric and non-bariatric subgroups of patients suffering from related health conditions such as menorrhagia, ulcerative colitis, anticoagulation, thalassemia, or bariatric surgery.

FCM is a novel, next-generation complex for IV iron treatment with several advantages over previously used IV preparations including reduced administration time, optimal pH range and lack of reactivity with dextran antibodies.¹⁴ We have found that hemoglobin levels increased significantly in the study group. Compared to orally administered ferrous sulfate, FCM is better tolerated, prompts a more rapid hemoglobin response, especially at low or very low Hb levels, and is more reliable.¹⁵ The formulation releases a minimal amount of iron into the circulation taken up by macrophages in a controlled manner and delivered via physiological iron metabolism pathways to transferrin and ferritin.¹⁴ Other IV iron preparations are also marketed and prescribed in various cases, but they usually cause increased adverse events, including hypersensitivity reactions.¹⁶ IV iron sucrose, for instance, carries side effects affecting Hb level enhancement.¹⁷ Iron dextran, another IV iron preparation, carries the risk of anaphylactic reactions.¹⁸

Although oral iron supplementation is the first-line treatment for IDA, some patients cannot tolerate oral iron owing to gastrointestinal side effects. For some patients with IDA, the rate of chronic loss is greater than the replacement rate of oral iron supplementation.¹⁹ A recent observational study published by Das et al compared the safety and efficacy profiles of oral iron with those of IV iron. The increase in Hb and serum ferritin levels was higher for IV iron than for oral iron.^{20,21} FCM is typically recommended in cases in which the oral preparations are ineffective or cannot be used. FCM can be administered as a single dose providing up to 1000 mg of iron as a rapid infusion or in multiple doses (100–200 mg) without the need for a test dose.²² FCM is a suitable method of iron supplementation, especially in very low Hb levels or cases where multiple doses of IV iron are not convenient, or there is a patient compliance challenge.^{22,23}

In addition to Hb levels, MCV evaluation is also recommended to assess the efficacy of iron supplementation by comparing pre- and post-treatment values.²⁴ Reduced MCV is a marker of anemia. Similar to the increase in Hb levels, we found a significant increase in the levels of MCV in our study group. However, the increase in MCV levels was significantly higher for the individuals receiving three doses of FCM than those receiving a single dose. To our knowledge, this is a novel finding that has not been investigated to date, and the precise molecular mechanisms that lead to increased long-term efficacy of multiple FCM doses in terms of MCV require further investigation. Overall, these findings suggest that FCM can effectively treat iron deficiency in patients with IDA caused by various pathologies.

The risk of developing IDA is higher both pre- and post-operatively in individuals undergoing bariatric surgery due to increased consumption of high-calorie food with low iron content before the procedure,²⁵ and reduced oral intake and decreased iron absorption due to the decline in gastric acid production and bypass of the proximal small intestine after the surgery.^{26,27} FCM is helpful in the treatment of persistent or relapsing IDA after Roux-en-Y gastric bypass.²⁸ In a retrospective review of five Phase III studies, FCM effectively increased hemoglobin and ferritin levels. The observed adverse effects were similar to those observed for other IV iron products.²⁹ Most studies published to date have evaluated the safety and efficacy profiles of iron supplementation in individuals who underwent bariatric surgery. We evaluated the efficacy of IV FCM administration in individuals undergoing bariatric surgery and found no statistically significant difference between the two groups; however, our results suggested periodic re-evaluation with a possible need for maintenance of IV iron, especially in this group of patients. The hemoglobin concentration and MCV did not differ significantly between the two study groups.

An increase in circulating platelets, referred to as thrombocytosis, may occur secondary to iron deficiency.³⁰ Therefore, correction of IDA leads to a reduction in platelet count to the normal range. We observed a significant decrease in the median platelet count among study subjects. Similar to the Hb concentration and MCV, no significant difference was noted for individuals who underwent bariatric surgery. The decrease was higher for individuals receiving multiple doses of IV iron than for those receiving a single dose, which may indicate that even though one dose can achieve an adequate level of Hb and ferritin, it may not be sufficient to replenish the deficient stores. This finding is similar to that of MCV, indicating at long-term influence on iron homeostasis.

Hypophosphatemia has been reported as a side effect of FCM more often than of other iron formulations.³¹ FCM treatment leads to a transient increase in the plasma levels of phosphaturic hormone intact FGF23 (iFGF23) which stimulates renal phosphorus excretion.³² As mentioned in various case reports and cohort-based studies, extensive renal excretion of phosphorus leads to low serum phosphate levels.^{31,33} We also observed that some of our subjects had hypophosphatemia; however, there were fewer cases of hypophosphatemia in patients receiving increased doses. This can be attributed to the relatively small number of patients receiving increasing doses. The hypophosphatemia associated with FCM reportedly resolves spontaneously after 6–12 weeks.³⁴

IV FCM has been reported to produce a rapid and pronounced initial increase in serum ferritin levels that decline over time but remain higher than baseline.^{35–38} Our study findings are in concordance with the reported data, whereby the ferritin levels remained significantly elevated during the first three months after treatment with FCM but then dropped gradually. In contrast, other forms of IV iron therapy are associated with only a slight increase in serum ferritin level.^{35,39} To our knowledge, the effects of single and multiple doses of FCM on hyperferritinemia have not been reported in the literature. We found that multiple doses carry an increased risk of transient hyperferritinemia compared with a single dose of IV FCM. Thus, the optimal response is inversely related to the number of doses administered. Interestingly, a population of IDA patients requires periodic review for reinfusion of iron to sustain the level above the required level of >30 ng/mL. Further subgroup studies are needed to determine the optimal dose and the relationship between the number of doses and increase in ferritin levels and the various modifiers of this relationship. This study is limited by its retrospective design and smaller sample size.

Conclusion and Future Prospects

FCM is a robust and reliable option for the IDA treatment. Therapy with IV FCM helps achieve quick significant improvements in Hb concentration, MCV, and reactive thrombocytosis. The risk of transient hyperferritinemia associated with IV FCM administration was tolerable and reversible. Furthermore, hypophosphatemia has been reported in some cases following FCM treatment. We observed that the full two or three doses of IV iron resulted in decrease in platelet count although not statistically significant. We recommend conducting a prospective large-scale study to confirm our findings. An interesting finding of this study is the discovery of a population of IDA patients requiring periodic assessment for iron reinfusion to sustain normal levels, mostly post-bariatric surgery.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Dr. Sulaiman Al Habib Medical Group.

Consent to Participate

Informed consent was obtained from all individual participants included in the study.

Consent to Publish

All authors consent for publication. The authors also affirm that human research participants provided informed consent for publication related to their data.

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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. All authors read and approved the final manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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Acquired Haemophilia A: A Review of What We Know

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Abstract: Autoantibodies against plasma coagulation factors could be developed by some individuals inducing severe and sometimes fatal bleedings. This clinical entity is called acquired haemophilia. It should be suspected in subjects with acute abnormal bleedings, without personal or familiar history of congenital bleeding disorders with an unexplained prolonged aPTT. It is rare disease, although its incidence may be underestimated due to the low knowledge about it by many specialists, the frequent use of anticoagulant or antiplatelet therapies in the affected population that can mask the diagnosis and, sometimes, a so withering effect that avoid its confirmation. Mortality ranges between 9% and 33% depending on the series in the first 2 months after diagnosis. This mortality is attributed in up to 40% of the cases to infections in the context of immunosuppressive treatments used to eliminate the inhibitor. Factor VIII levels below 1% and high inhibitor titers are conditions of worse response rates. Advanced age, patient's ECOG, and underlying conditions are key prognostic factors for response to treatment and patient survival. To reduce morbidity and mortality in these patients, it is important to have clinical knowledge and access to guidelines to achieve an early diagnosis and to optimize the haemostatic and immunosuppressive treatment. This review aims to contribute to the dissemination of basic concepts on the epidemiology etiopathogenesis, diagnosis, treatment and management of these patients, as well as risk factors to get remission and the longest overall survival to allow individualized care. Especial awareness will be proposed in patients with some underlying conditions like cancer, autoimmune diseases, children, pregnancy or drugs.

Keywords: acquired haemophilia, inhibitors, coagulopathy, autoimmune, bleeding

Key Ideas

- AHA should be suspected in any patient with an unjustified prolonged aPTT and abnormal acute bleeding symptoms, with no personal or family history of coagulopathy.
- Do not exclude AHA as a potential diagnosis in patients anticoagulated or with antiplatelet treatment with abnormal bleeding or a change in their routine bleeding profile and the laboratory suggest it.
- Mortality and morbidity in AHA depend on the patient's age, the underlying pathology, hemoglobin at diagnosis, and response to eradication therapy.
- Immunosuppressive therapy should be started as soon as the diagnosis is made and should be individualized based on the characteristics of the patient.

Introduction

In a subject with abnormal bleeding in amount or location, no personal history of coagulopathy, and an unexplained prolonged activated partial thromboplastin time (aPTT), the presence of acquired haemophilia should always be ruled out.¹ This is an autoimmune organo-specific bleeding disorder secondary to the presence of autoantibodies against plasma coagulation factors. The most common antibodies are those directed against factor VIII (FVIII), that is why when we talk about acquired haemophilia in general, we do reference to acquired haemophilia A (AHA). It is included among the group of rare diseases, although its

incidence may be underestimated due to the limitations of the available registries, the lack of knowledge about it, the high prevalence of concomitant anticoagulant or antiplatelet treatment given the advanced age of the patients, and, finally, to a clinical presentation so fulminant that it prevents its confirmation in some cases.² There are basically two groups of affected subjects, women during postpartum and the largest group consisting of aging people. Mortality ranges between 7% and 38% depending on the series.^{3–8} Mortality is mainly related to bleedings during the first days after diagnosis and to infections related to immunosuppressive treatment indicated to eradicate the inhibitor or underlying conditions of patients.^{3–8,10} To reduce morbidity and mortality, it is important that the physician responsible for the patient management knows the guidelines to follow to obtain an early diagnosis.

This review focuses on acquired haemophilia secondary to autoantibodies directed against FVIII, AHA. With this work, we intend to expose, updated, the cornerstones of the diagnosis and approach to AHA, based on a comprehensive review carried out on the available bibliography: through MEDLINE/PubMed, all identifiable works have been searched in Spanish and English using the terms “acquired h(a)emophilia”, “acquired factor VIII inhibitor(s)”, “acquired inhibitors”, “autoantibodies” and “haemophilia with inhibitor” [“h(a)emophilia with inhibitor(s)”], until July 2022. The objective is to bring AHA closer to health professionals, especially to non-specialists in hemostasis, since, without clinical suspicion, the diagnosis of this entity is delayed, which poses a risk to the patient.

Epidemiology

The incidence of AHA ranges from 1 to 6 cases per million inhabitants per year.^{3,9–11} These data should be treated with caution given the paucity of records and because the diagnosis may be underestimated in the absence of a high rate of clinical suspicion. The average age of onset is 65 years old, but it has a biphasic distribution. A first peak comprises young women starting in the postpartum period or in the presence of autoimmune systemic diseases. The second peak affects patients over 60 years of age with no clear gender differences.^{3,7} Some pediatric cases have been reported with an estimated incidence of 0.045 per million per year.^{12,13}

Although more than 50% of the cases are idiopathic (Table 1), AHA has been associated with postpartum, drugs and underlying diseases like autoimmune disorders such as rheumatoid arthritis or systemic lupus erythematosus, malignancies and infectious diseases.^{14–18} Medications reported to be associated with AHAs include penicillin, sulfonamides, phenytoin, interferons and fludarabine.¹⁴

In the case of autoimmune diseases, the development of autoantibodies against FVIII occurs mainly in cases of systemic involvement (rheumatoid arthritis, SLE, Sjögren's syndrome, Goodpasture's syndrome, dermatomyositis or graft-versus-host disease in allogeneic liver or hematopoietic progenitors' transplantation).^{19–21} Inhibitors in this group of patients, and in particular those associated with rheumatoid arthritis, are high titer ones, traditionally associated with few spontaneous remissions and poor response to steroids.^{19,22}

In AHA secondary to neoplasia, there is no predominant oncological entity, although it seems more frequent among solid organ neoplasms.²³ Sometimes it precedes tumor diagnosis in months, so it can be labeled in this context as a paraneoplastic syndrome.^{23–32} Sometimes, the detection of an inhibitor takes place after the start of treatment of the specific neoplasm, and it is very difficult to exclude the influence of other factors such as immunosuppressive treatment, chemotherapy and radiotherapy used in its genesis. Another hypothesis to take into account is the non-causal association between neoplasms and AHA, since both are pathologies of advanced age that could coexist.^{23,29}

In the context of AHA and pregnancy, AHA generally occurs during the postpartum period, between 1 and 4 months, although cases have been described up to 1 year after delivery.^{21,33–35} The most common symptoms are abnormal bleeds during the postpartum period; the rest of the clinical profile is similar to that of other patients with AHA.³⁴ Exceptionally, these inhibitors have been described during pregnancy, delivery or abortions. In these rare cases, severity is extreme because high risk of severe uterine bleeding and hysterectomy in most cases.^{36–39} If we look at the EACH2 data, half of these women classified as postpartum AHA had an aPTT in the upper limit of normal already at the time of delivery, when in these circumstances it should be shortened, so probably AHA could be diagnosed in advance.³⁵ The etiopathogenesis of AHA in the context of pregnancy is not clear. Its usual presentation in the postpartum period suggests the possibility of an immune response from the mother when she is exposed to FVIII from the child during delivery.⁴⁰ Against this theory, there is low relapse rate because of anamnestic response in subsequent deliveries.⁴¹

Table I Underlying Conditions Associated with Acquired Hemophilia A

Idiopathic	Dermatologic diseases • Psoriasis • Pemphigus
Postpartum/Abort/Pregnancy	Pulmonary diseases • Asma • EPOC
Autoimmune diseases • Systemic lupus erythematosus • Multiple sclerosis • Rheumatoid arthritis • Temporal arteritis • Sjogren's syndrome • Autoimmune hemolytic anemia • Goodpasture syndrome • Myasthenia gravis • Graves disease • Autoimmune hypothyroidism • Inflammatory bowel disease • ITP	Diabetes
Drugs Penicillins and derivatives sulfonamides and quinolones, griseofulmin, Phenytoin, Chloramphenicol, methyldopa, Levodopa, alpha interferon, pegylated interferon, Fludarabine, BCG vaccine, Clopidogrel, Antidepressants (Thioxanthines, Flupenthixol, Fluphenazine), hydralazine acetaminophen	Infectious diseases HIV, HVB, HVC, SARS-CoV-2
Hematologic diseases • Chronic lymphocytic leukemia • Non-Hodgkin's lymphoma • Multiple myeloma • Waldenstrom's disease • Myelodysplastic syndrome • Myelofibrosis • Erythroleukemia	Solid tumors Prostate, Lung, Colon, Pancreas, Stomach, Bile duct, Head Neck, Cervix, Melanoma, Kidney

In a recent review that brings together pregnancy and postpartum AHA cases published up to 2021, in a total of 56 pregnancies, 69% were first pregnancies.⁴² Of these women, only 13% had second and subsequent pregnancies, developing a new episode in 22% of them. The median time to CR was 12 months in women treated with steroids and 8 months with combinations of immunosuppressants, relapse rate 29% in the first year and 22% in a subsequent pregnancy. These data together with a mortality of 1.7%, although they define a more benign profile than other AHA, should warn us about the importance of ruling out other associated autoimmune and neoplastic pathologies and being more aggressive in inhibitor elimination treatments if the evolution is not adequate.

Dewarrat et al describe three neonates with incidents:⁴² one with intracranial hemorrhage treated with intravenous immunoglobulins (iv Ig) and FVIII, a second one with post-puncture musculocutaneous hemorrhage that did not require haemostatic treatment and a third neonate with a gastrointestinal bleed treated with rFVIIa and FVIII with good evolution.

The etiopathogenesis of drug-associated AHA is not well understood.^{14,43} They mostly occur after hypersensitivity reactions and, despite generally high inhibitor titers, they remit after a short period after drug withdrawal with a remission rate close to 80%.

Physiopathology of AHA

The mechanism of loss of tolerance to autologous FVIII that causes AHA remains to be elucidated. Tolerance against self-antigens is the result of the balance of different immunological processes. These include the elimination of autoreactive T lymphocytes during the maturation of the immune system and the anergy or loss of T and B lymphocytes with antigenic specificity. Like many other proteins, FVIII has epitopes that are universally recognized by CD4⁺ T lymphocytes. This response is modified over time and seems to depend on structural variability and the amount of antigen exposed in each case.⁴⁴ Tolerance is related to anergy due to CD4⁺ apoptosis secondary to continuous stimulation of circulating FVIII¹⁴ (Figure 1). In AHA, the loss of tolerance causes the control of the response to FVIII to be compromised. There are several proposed triggering factors:^{45–47}

- Modifications of T regulatory (Treg) lymphocytes, decreasing their response in favor of T helper (Th) lymphocytes. As justification, it has been observed in high inhibitor titer, higher proportion of anti-FVIII antibodies IgG4 (Th2 predominance) versus IgG1 (Th1 predominance).
- Existence of Th lymphocytes with receptors against FVIII related to more intense immune response (receptors V β 2, V β 5, V β 9).
- Incidence of specific polymorphisms of the cytotoxic T lymphocyte antigen (CTLA-4) such as +49 A/G is higher in AHA population and could increase activity and expansion of Th lymphocytes.
- Polymorphisms of the FVIII gene and HLA could induce an abnormal recognition of FVIII. In patients with AHA associated with transfusion and postpartum, polymorphisms of the FVIII gene have been described, a substitution in domain B (D1241E) of FVIII. The combination of this polymorphism with certain profiles of the class II alleles of the HLA system could give rise to the loss of tolerance to FVIII in the subject himself.⁴⁸

In 15% of the healthy population, low titers of anti-FVIII antibodies can be found. These are asymptomatic and have no underlying disease that justifies them.⁴⁹ These antibodies are IgG1 and IgG2 and are directed against the C2 domain of FVIII in most cases. The *in vitro* study of the plasma of these subjects confirms the inhibition capacity of FVIII by these antibodies, although it does not have a haemostatic effect. This is because they produce anti-idiotypic antibodies that neutralize circulating autoantibodies against FVIII. This anti-idiotypic neutralizing capacity is greater in the plasma of multiparous women and older men. Some authors have identified them in situations of sepsis, related to good prognosis for their recovery. It is theorized about a relationship between antibodies against FVIII and defense capacity against pathogens.⁵⁰

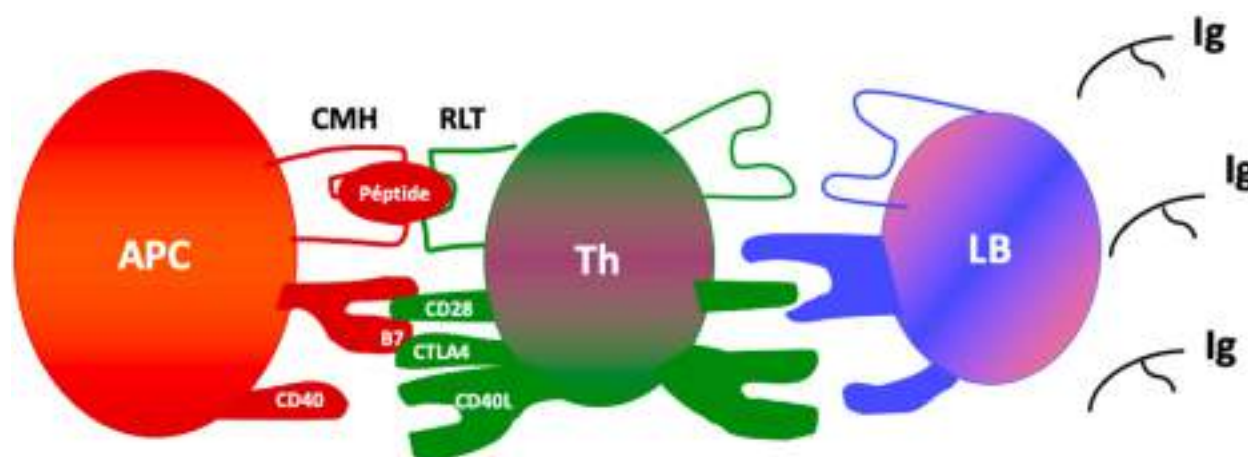


Figure 1 Mechanism of immune response to FVIII. FVIII is processed by the antigen-presenting cell (APC). Its function is to recognize and internalize it, giving rise to an endosome inside, which after merging with a lysosome leads to digestion of FVIII into peptide fragments that can bind to class II molecules of the major histocompatibility system. In case it is not assembled, the class II molecule will be degraded; otherwise, the complex will be transported to the surface of the APC, which presents it to the T cell receptor (TCR). The activation of T lymphocytes requires this union and the signals resulting from the interaction between various molecules such as CD28/B7 (pro-activation) or CTLA4/B7 (anti-activation). After this, there is an expansion of clone responsible for presenting FVIII to B lymphocytes. B lymphocytes (BL) are responsible for the production of specific antibodies (immunoglobulins [Ig]).

Autoantibodies Against FVIII Characteristics

Inhibitory autoantibodies against FVIII are usually polyclonal IgG4 molecules and they have shown specificity against a single epitope of the FVIII molecule.⁵¹ Clonality is a rare phenomenon but with a predominance of kappa light chains⁵² and in these cases there is specificity against functional epitopes of more than one domain of the FVIII⁵³ protein. The identification of autoantibodies of the IgA and IgM isotypes is an epiphenomenon typical of AHA associated with lymphoid neoplasms and paraproteinemias.⁵⁴ Approximately, 60% of the inhibitors are directed against the A2 or C2 domain of the FVIII molecule, not against both at the same time. This is a difference compared to inhibitors of congenital haemophilia, in which 85% have specificity against several domains at the same time.^{56,57} Inhibitors against A3 and B domain epitopes are sporadic.

FVIII inhibitors neutralize their procoagulant activity through different pathways: inhibiting the procoagulant function of FVIII directly by binding to functional regions of the protein or accelerating its catabolism and clearance.⁵⁸ These inhibitors are characterized by being time and temperature dependent in their action. FVIII–autoantiFVIII complexes do not fix complement, so there is no possibility of tissue damage secondary to deposits of immune complexes. Finally, unlike alloantibodies against FVIII, autoantibodies against FVIII are present in most cases with nonlinear inactivation kinetics type II or second order.^{53,59–61} In this type of kinetics, FVIII is incompletely neutralized with an initial phase of rapid inactivation followed by a slower equilibrium phase where sometimes small amounts of FVIII can be detected because there is no complete saturation of the inhibitor (Figure 2).

Signs and Symptoms, Laboratory Diagnosis

Patients with AHA present with acute or recent abnormal bleeding symptoms, without a previous bleeding diathesis or family history, and laboratory tests showing an isolated prolonged activated partial thromboplastin time (aPTT).¹⁵ Characteristic bleeding pattern is subcutaneous bleeds (Figure 3) being the most common (observed in 80% of the patients), followed by muscle, gastrointestinal, genitourinary, and retroperitoneal bleeds.^{3,5,10,43} A relevant fact, revealed by the Spanish registry,⁵ is that up to a third of the patients received anticoagulant or antiplatelet treatment at the time of diagnosis, due to the high age of onset. The use of anticoagulant or antiplatelet drugs could delay diagnosis by confusing bleeds because of AHA with bleeds because of these drugs.

A prolonged aPTT may be due not only to decreased FVIII but also to other causes,^{62,63} including deficiency of other factors of the intrinsic pathway, Willebrand disease or the effect of some anticoagulant drugs such as heparin or dabigatran. Therefore, an unexplained aPTT prolongation should be investigated if it is encountered before surgery or

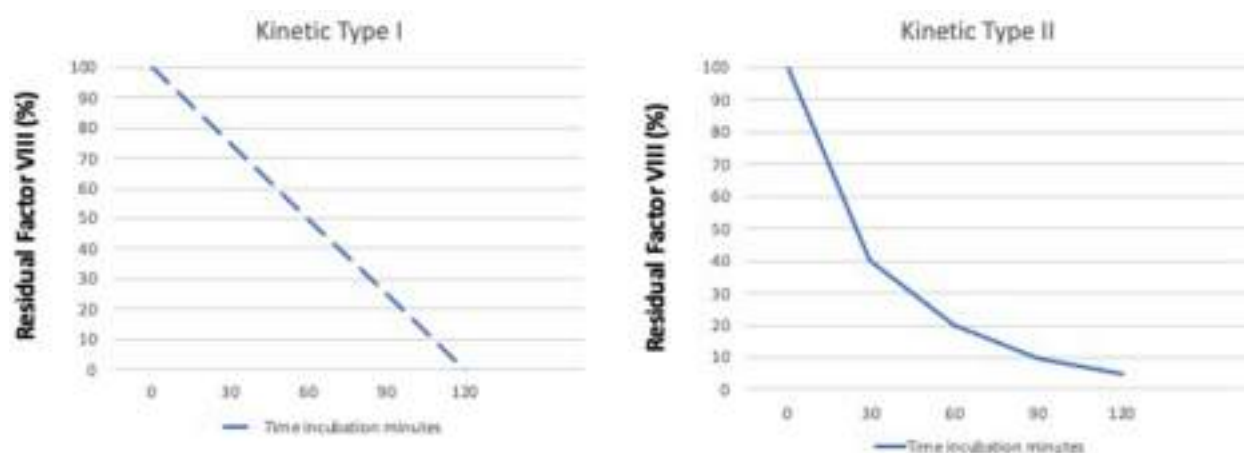


Figure 2 Kinetics of inhibition of FVIII activity by autoantibodies. The inhibition profile of FVIII activity which most often exhibit the autoantibodies characteristic in acquired haemophilia responds to a second order kinetics (triangles), in which a first phase of inhibition resulting from a linear pattern is followed by an equilibrium phase in which the inhibition rate slows markedly, a fact that allows the detection of residual FVIII activity in vitro. However, autoantibodies directed against FVIII rarely interact with this following a first-order kinetics (circles), in which the inhibition profile corresponds to a linear pattern and leads to the complete disappearance of FVIII activity, this being a more typical behavior of anti-FVIII alloantibodies that can occur in congenital haemophilia.



Figure 3 Examples of AHA bleeding profile. **(A)** Lingual and soft palate haematoma in patient with AHA. **(B)** Haemorrhagic infiltration of subcutaneous tissue due to abductor muscle bleeding in a patient with AHA.

in bleeding patients.¹⁴ Measuring FVIII, factor IX or factor XI levels and testing for conditions able to prolong aPTT such as lupus anticoagulant (LA) and factor XII deficiency may be performed.¹⁵

The aPTT coagulation waveforms generated by coagulation analyzers using optical detection methods provide freely available information that can help in the detection of acquired FVIII inhibitors.⁶⁴ The aPTT derivative maxima and minima curve differed significantly between acquired FVIII inhibitors and other diagnostic categories. The presence of an abnormal double-peaked first derivative curve had a sensitivity of 83.3% and specificity of 81.6% for identification of acquired factor VIII inhibitors in cases with aPTT >50 seconds.⁶⁴

To distinguish a factor deficiency from the presence of an inhibitory substance, mixing tests should be performed. FVIII inhibitors from AHA are time- and temperature-dependent, so aPTT results obtained immediately following the 1:1 volume

mixture of normal and patient plasma and after a 2 hours incubation at 37°C should be compared.⁶⁵ A significant prolongation of the incubated mixture identifies a circulating inhibitor in the patient plasma. Further investigation is always required, and specific factor activity assays should be performed in parallel to detect the factor neutralized by the inhibitor and allow early diagnosis. An isolated low FVIII level suggests diagnosis of AHA. However, other causes of low FVIII as von Willebrand disease, congenital haemophilia A or acquired von Willebrand syndrome, must be excluded.⁶⁶

Lupus anticoagulant (LA) behaves as a circulating anticoagulant; it can be excluded by a negative diluted Russell viper venom test (DRVVT), which is typically not affected by FVIII inhibitors.⁶⁷ On the other hand, interference of LA on FVIII activity can be excluded by using chromogenic substrate assays that are usually insensitive to LA.⁶⁸ Alternatively, a normal chromogenic assay FVIII excludes AHA in cases in which LA decreases one-stage FVIII assay results. Fluorescence immunoassay is a technique under development that could be useful to differentiate an inhibitor from a LA.⁶⁹ Viscoelastic tests could also be useful for this purpose, since the prolonged clotting time (CT) is clearly greater in the AHA than in the presence of a LA,⁷⁰ although the real value could be the verification of shortening CT (ROTEM®) or R (TEG®) with bypass agents treatment.⁷¹ However, it should be noted that AHA and LA are both autoimmune disorders that can co-exist in the same patient.⁷²

AHA must also be differentiated from disseminated intravascular coagulation (DIC). Although symptoms could be quite similar, in DIC an haemostatic exhaustion occurs due to the consumption of multiple coagulation factors, not just a specific one, and the prolonged APTT is corrected by mixing the patient's plasma with normal plasma. There is also a decrease in the platelet count and an increase in D-dimer level.⁷³

There are several tests for the detection and quantification of inhibiting autoantibodies,⁷⁴ but the most used is Bethesda assay.⁷⁵ The inhibitor titer is equal to the reciprocal of the plasma dilution that results in 50% inhibition of FVIII in normal plasma after incubation for 2 hours at 37°C.⁷⁶ Inhibitor titers are measured in Bethesda units (BU) where 1 BU is equal to the amount of antibody that neutralizes 50% of FVIII activity in the normal pooled plasma.¹⁴ It was developed to detect and quantify FVIII alloantibodies in congenital haemophilia A that display linear type 1 kinetics, but it may be also useful in detecting FVIII inhibitors in AHA, although these often display a complex non-linear type 2 kinetics (Figure 3) and so the assay may not be able to estimate the true potency of the autoantibody.^{16,61} An important consequence is that a lack of correlation between FVIII or inhibitor titer and bleeding phenotype in AHA has been described in many studies.^{3,9,10} Sensitivity and specificity of the Bethesda assay is improved by the Nijmegen modification (buffering the normal plasma) and heat inactivation of the patient's plasma at 56°C (to precipitate circulating residual FVIII) prior to assessment of the test.^{77,78} The incubation time can also have an effect on the estimation of the inhibitor by facilitating its binding to FVIII.⁷⁹ Samples exhibited inhibitory effects within 0.5 hours, with the higher titer sample being more inhibitory; then the curve in their polygram tended to be flat at >1.0 hours, and the inhibitory trends were almost the same for both levels. Altogether, it is feasible to incubate 1.0–2.0 hours for the aPTT mixed test and the Bethesda assay.⁷⁹

Finally, if recombinant porcine FVIII (rpFVIII) is a therapeutic option, then a Bethesda assay specific to rpFVIII should be considered as it may help guide treatment decisions.¹⁵

Enzyme-linked immunosorbent assays (ELISA) can be used to diagnose FVIII antibodies, but these cannot distinguish neutralizing capacity and are seen to some degree in the normal population.¹⁴

Thus, diagnosis should be made by a stepwise approach.^{14–16} A proposal for a diagnostic algorithm is presented in Figure 4.

General Treatment Recommendations

In AHA, the risk of bleeding is independent of the inhibitor titer and the residual FVIII. Until FVIII levels are at least greater than 50% and inhibitor is undetectable, the risk of bleeding remains.^{1,80} Therefore, early diagnosis and the prescription of immunosuppressive treatment to eliminate the inhibitor are key to patient safety, up to day. Delays in diagnosis are associated with a greater consumption of haemostatic agents.⁸¹

Treatment of acquired haemophilia has four pillars: Prevent bleeding episodes and basic care, treatment of the underlying disease, haemostatic treatment and eradication of the inhibitor. General recommendations could be:^{1,10,16,82–84}

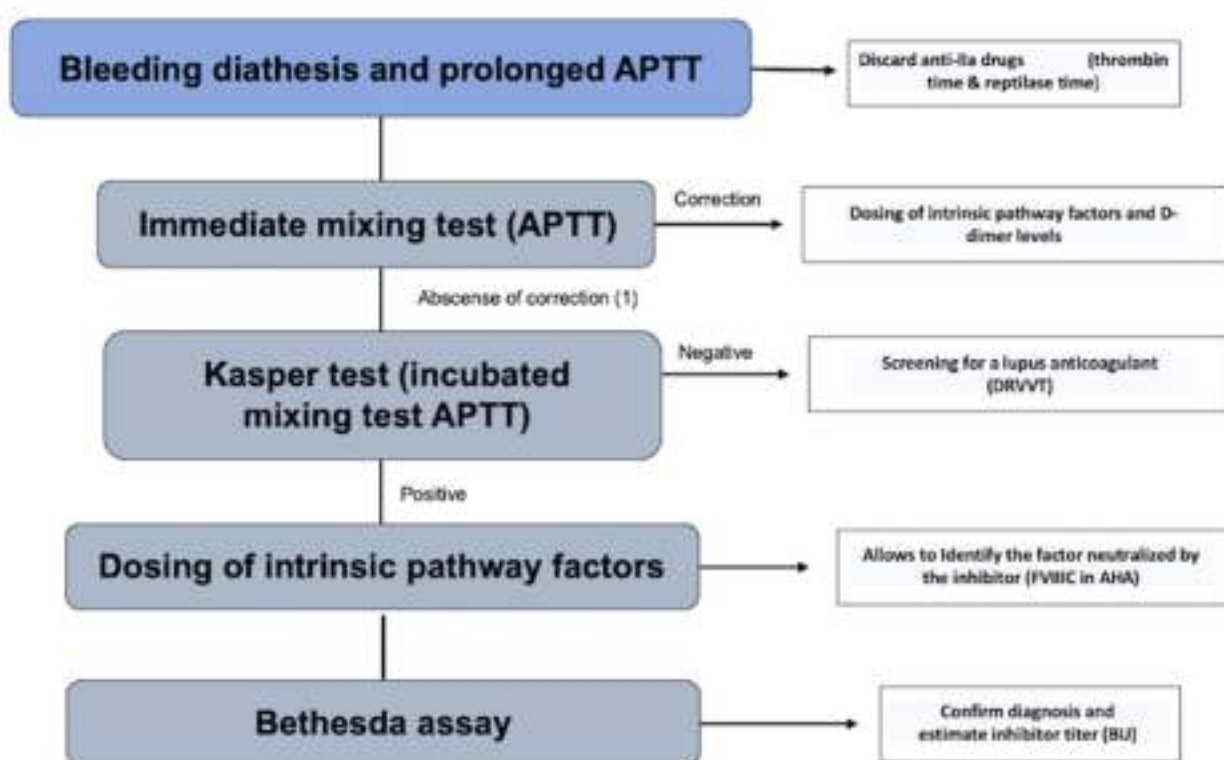


Figure 4 Diagnostic algorithm for a prolonged APTT. Kasper test and Bethesda assay requires incubation 2 hours at 37°C of the mixing samples. (1) No evidence of correction is defined as a test time 8 seconds longer than the control time.

Abbreviations: BU, Bethesda units; DRVVT, dilute Russell viper venom test.

- Given the potential severity of the bleeding and the complexity of the haemostatic and immunosuppressive treatment, whenever the patient's conditions allow it, patient should be referred to an experienced center or at least have their advice until transfer is possible.
- To avoid the use of anticoagulants or antiplatelets. Restart these therapies in the subjects for whom they were indicated when the FVIII is greater than 50% and no bleed.
- Do not perform arterial punctures, intramuscular injections or invasive procedures without appropriate haemostatic treatment and always within the framework of adequate justification.
- In case of major or minor surgery, delay the procedure until the inhibitor is eradicated and if it is not possible to perform the most effective haemostatic coverage, under the supervision of experienced health personnel.
- Patients can have an outpatient follow-up except in the case of uncontrollable bleeding in these circumstances or associated with important comorbidities or environment that compromise their follow-up.
- The patient should be trained in recognizing the warning signs and symptoms that require consultation, with guaranteed access to said consultation 24 hours a day.

Regarding the follow-up of the evolution of the hemorrhagic symptoms, a detailed clinical assessment together with the strict control of the hemoglobin and ferritin levels can be valid parameters in both mild and severe cases. FVIII and aPTT are valuable tools to follow patient evolution every 7 days or less, performing inhibitor quantification every 7 to 15 days according to FVIII evolution.^{1,84} Imaging tests such as CT or MRI are very useful for diagnosis, but they are not always available. For this reason, a valid and cost-effective option is ultrasound in the diagnosis and, above all, in the follow-up of hemorrhagic symptoms without the cumulative effect of radiation.^{1,84}

We must emphasize that the indication of complementary tests, to exclude associated pathology or make differential diagnoses, must be carefully assessed based on the acute and future clinical situation of the patient. The purpose of this is to avoid blood tests that increase the risk of bleeding or those that, despite facilitating a diagnosis, cannot be

Table 2 Goals of AHA Treatment and Studies Recommended to Rule Out Underlying Conditions in Patients Suffering from AHA

Goals of AHA Treatment	Recommended Studies
• Treat bleeding symptoms considering cardiovascular risk • Treat underlying condition as soon as possible • Individualize immunosuppressive treatment to prevent adverse events	• Cervical and thoraco-abdominal CT • Blood count • Ferric metabolism and maturational factors • Peripheral blood smear • Biochemistry: basic, hepatic, renal and lipid profile • Lactate dehydrogenase (LDH) • Proteinogram • Viral serology (herpes-virus, HIV, hepatotropic viruses) • Thyroid tests • Antinuclear antibodies and anti-DNA. • Rheumatoid factor, RPC

Abbreviations: CT, computed tomography; DNA, deoxyribonucleic acid; HIV, human immunodeficiency virus; RPC, reactive protein C, TP, trough/peak ratio.

accompanied by the treatment and management indicated for it due to the patient's comorbidities.¹ Table 2 describes some of the studies recommended to rule out underlying conditions, able to be treated.

Finally, it must be taken into account that 75% of the adverse events (bleeding and infections) in these patients occur in the first 100 days of diagnosis and 50% of mortality in the first 2 months of diagnosis.^{5,85} For this reason, the follow-up of these patients should be weekly until the elimination of the inhibitor and monthly until 6 months later.¹ From then on, the frequency of evaluations could be between 3 and 6 months depending on patient's necessities.

With regard to AHA and pregnancy, the probability of recurrence is 22%, so follow-up should be at least monthly during pregnancy.⁴² Fetus and neonate follow-up should similar to persons with haemophilia during pregnancy and childbirth, since the autoantibody against FVIII is usually an IgG and crosses the placental barrier, increasing the risk of severe hemorrhagic complications in the fetus. This risk will disappear after delivery with progressive decrease in IgG levels transferred from the mother to the child in 1 to 4 months.^{36,86–88} The recommendations are to avoid invasive procedures, instrumentalized deliveries and close monitoring with transfontanelle ultrasound in the first 24–48 hours after birth to identify possible intracranial hemorrhages early.⁸⁹

Haemostatic Management

Haemostatic treatment of AHA is based on the use of so-called by-pass agents like recombinant factor VIIa (rFVIIa, eptacog alfa activated, NovoSeven[®])⁹⁰ and activated prothrombin concentrate complex (aPCC),⁹¹ and on recombinant porcine factor VIII (rpFVIII, susoctocog alfa, Obizur[®]).⁹² Factor VIII concentrates and desmopressin (DDAVP) are not very effective, even at low inhibitor titers (<5 BU),^{8,93} so they should only be used if no other option is available.^{1–3} In the Chinese registry, FVIII concentrates effectiveness was 34%, compared to 84–100% of bypass agents.⁸ In the EACH2, by-pass agents also demonstrated their superiority with haemostatic effectiveness of 93% compared to 71% of the FVIII factors in multivariable analysis adjusted by age, gender, FVIII level, inhibitor titer, hemoglobin level, site, severity and cause of bleeding.⁹³

Time to start haemostatic treatment and its intensity will depend on location and severity of bleeding symptoms. If haemostatic treatment is indicated, it should be started early to avoid the progression of a hemorrhagic event and its complications.^{1,15,84} In EACH2 registry, the difference between patients who presented, or not, good haemostatic response was the delay in its administration.⁹³ The selection of the haemostatic drug is based on the experience of the center, availability and previous patient response, if it is known.^{1,84,85}

It is recommended to treat in case of uncontrolled bleeding, WHO grade 2 or higher hemorrhage or in case of need for surgery to prevent bleeding^{1,15,82,84} (see Table 3). Approximately 70% of the patients with AHA require haemostatic treatment.^{5,10,93} Spanish experience described 77.4% of the patients with AHA and anticoagulant or antiplatelet treatment and diagnosis versus 68.5% of the rest, but this difference is not statistically significant.⁵ In case of bleed with no indication for haemostatic treatment, close monitoring is necessary because serious and fatal bleeding events could occur

Table 3 Suggested Surgical Prophylaxis in Adults with Acquired haemophilia⁹⁴

Dental Extractions
APCC, 50 to 75 IU/kg/12 h, 2 to 3 doses, starting just before surgery rFVIIa, 90 to 120 µg/kg/2h, 3 to 4 doses, starting just before surgery minor surgery
Minor Surgery
APCC, 50 to 100 IU/kg/8–12 h, without exceeding 200 IU/kg/day. Start just before surgery. Treatment 3–7 days depending on evolution rFVIIa, 90 to 120 µg/kg/2–3 hours for 24 hours. Start just before surgery. Repeat dose every 4 to 6 hours for 3 to 7 days depending on evolution
Major Surgery
APCC, 75 to 100 IU/kg/8–12 hours on days 1 to 6, then every 12 hours on days 7 to 15, not to exceed 200 IU/kg/day. Start just before surgery. Adjust according to evolution rFVIIa, 90 to 120 µg/kg/2 hours for 24 hours, every 2 to 3 hours on day 2, every 4 hours on days 3 to 5, every 6 hours on days 6 to 15. Start just before surgery. Adjust according to evolution

up to 5 months after AHA diagnosis in patients with a persistent inhibitor titer.¹⁵ Despite the lack of correlation between residual plasma FVIII activity or inhibitor titer and bleeding severity described in the majority of series,^{3,9,10,80} patients with inhibitor titer >100 BU/mL seemed to require more and more frequently haemostatic treatment than those with levels <10 BU/mL⁵.

rFVIIa is one of the most widely used haemostatic drugs in AHA. In a recent review, a total of 12 studies were analyzed in a systematic review, including 671 patients and 1063 bleeding episodes treated with rFVIIa with dosing describing an interval of 2–3 hours between doses, and a median total number of doses of 10–28.⁹⁵ In the Spanish registry, rFVIIa was used mainly with a median duration of 7 days (3–13) and number of doses of 15 (5–48).⁵ The recommended dose is 90 µg/kg every 2–3 hours until control of the bleeding episode.¹⁵

In the case of aPCC, the recommended dose is 50–100 U/kg every 8–12 hours, up to a maximum of 200 U/kg/day¹. In the EACH2 registry, efficacy was similar compared to rFVIIa, with bleeding control rates greater than 90% when used as first-line therapy.⁹³

Repeated rVIIa or aPCC infusions could increase risk of thrombotic events. Fortunately, these events seem rare. In EACH2, 2.9% (5/174) and 4.8% (3/63) of patients with AHA who received rVIIa or aPCC, respectively, presented thrombotic events.⁹³ These data are similar to those of other series; rFVIIa 0–5%, rFVIIa and antifibrinolytics 10%, and FEIBA 0–2.9%.⁸² There are 13 cases, 8 arterial and 5 venous, the majority in subjects with vascular risk factors (atrial fibrillation, catheters, neoplasms, autoimmune diseases, arterial disease, etc). No infection by emerging pathogens has been reported in any of the bypass products discussed.

In subjects without an optimal response, we must rule out non-haemostatic causes that justify the bleeding and increase the dose and frequency of the chosen agent. If there is no improvement, switch to the unused bypass agent, and if control is not achieved with the new drug, increase its dose and frequency. In literature, there are published cases of sequential therapy alternating FEIBA and rFVIIa in patients with AHA and uncontrolled vital bleeding, with an effectiveness of 40%.^{96–98} The doses used are disparate and the intervals between agents range between 6 and 12 hours, without communication of associated thromboembolic events. In any case, this will always be an exceptional option since it is not included in the summary technical of any of the two bypass agents. It would be indicated by professionals with experience in the management of coagulopathies, under hospitalization.

Recombinant porcine factor VIII, susoctocog alfa, was evaluated in a prospective clinical study that included patients with a major bleeding event, excluding those with an rpFVIII inhibitor titer >20 BU. The initial dose was 200UI/kg and the subsequent ones and the administration interval were variable at the discretion of the investigator, monitoring levels of FVIII in the laboratory. The objective was to maintain an FVIII activity of 80% in severe episodes and 50% in the rest. Effectiveness of rpFVIII was 86% of the patients.⁹⁹ Although the approved starting dose for rpFVIII is 200 U/kg, some studies suggest that starting doses of 100 U/kg appear to be sufficient in many patients.¹⁰⁰ It is recommended to know the

inhibitor titer against rpFVIII prior to rpFVIII indication, but if this is not feasible, a dose can be administered performing a FVIII recovery 30 minutes later to assess the response.¹⁰¹ If there is an inadequate recovery, it should be considered the use of bypass agents or close monitoring depending on the severity of the bleeding and its evolution.

Regarding rpFVIII safety, in the pivotal trial, no thromboembolic events were communicated by investigators as related to the study drug. Regarding the ability of the autoantibodies to neutralize rpFVIII, in the pivotal study, 10 of the 28 patients had antibodies against rpFVIII at the beginning and 5 developed it during the study (8–85 days). However, only in two patients, the treatment was interrupted due to lack of efficacy. In another serie, the incidence of these antibodies was 44%, the majority low titer.^{102,103} When rpFVIII is used, it is recommended to monitor the activity of FVIII, using tests based on aPTT in order to adjust the dosage and identify patients with cross-reacting antibodies.¹⁵ Turkantoz et al have published that 97% of the subjects with AHA and inhibitor titers higher than 100BU and 90% of those with FVIII lower than 3.7% will present cross-reactivity with rpFVIII.¹⁰² In most cases, the inhibitor is directed against C1 domain.

There are guidelines and case series supporting the use of tranexamic acid in combination with rFVIIa or aPCC particularly for mucocutaneous bleeds or as a single agent for minor bleeds.^{104,105} However, combined therapy may increase thrombotic risk, and the risks and benefits must be considered individually.¹⁴

There are no clear recommendations on the definition of bleeding response in AHA. Tiede et al⁸³ describe by Delphi consensus that the time to achieve an adequate haemostatic response should be between 6 and 24 hours depending on the location and severity. Complete haemostatic response is considered to be the absence of bleeding or rebleeding in the same location 48 hours after discontinuation of haemostatic treatment. Signs of poor evolution of bleeding are persistence or worsening of pain, persistence of anemia or non-stabilization of hemoglobin, need for transfusion, extension of muscular or subcutaneous bleed or persistence of externalization of it.

The concept of prophylaxis in patients with AHA has been published.¹⁰⁶ Zanon et al describe their experience in the use of aPCC at low doses as prophylaxis in subjects with AHA and a high frequency of bleeding, with a 50% reduction in bleeding.¹⁰⁶ The mean number of days in prophylaxis after major bleeding was 12.7 ± 5.7 days, mean units/kg administered was 1246.8 ± 952.1 (30–60IU/kg/2–3 doses per week). Anyway, there is no clear evidence to establish its general indication.

Emicizumab is a recombinant, humanized and bispecific monoclonal antibody with FVIII mimetic activity, recently authorized for the prophylaxis of patients with haemophilia A.¹⁰⁷ It is being used off-label in AHA.^{108,109} In a recent publication, 24 patients with AHA treated with emicizumab were collected with a decrease in serious bleeding rate from 58% to 13%. One patient experienced a thrombotic event, and there were two deaths not classified as related with the drug.¹¹⁰ In another study, 12 patients with AHA were treated with emicizumab, observing a rapid improvement in bleeding symptoms with a median of 3 days (range 2–15 days).¹¹¹ However, the safety and efficacy of emicizumab in AHA is unknown, so it only should be used within clinical trials. Emicizumab doses in AHA clinical trials are 6mg/kg on day 1 and 3mg/kg on day 2, then 1.5mg/kg weekly in the American group (NCT05345197)¹¹² and once weekly in the German group (NCT04188639, dose not described).¹¹²

Immunosuppressive Treatment to Eradicate Neutralizing Autoantibodies

Immunosuppressive treatment (IST) in AHA is, together with the haemostatic treatment, the cornerstone to achieve the best clinical result. The elimination of the autoantibody should be established as soon as the diagnosis is confirmed.^{3,113,114} Complete remission is defined as reaching a normal FVIII level, without evidence of inhibitor or bleeding related to AHA in the absence of immunosuppressive treatment.^{1,15,82} The German group has coined the term partial remission (PR), such as reaching VIII levels greater than 50% and no bleeding after discontinuing haemostatic treatment for 24 hours.¹⁵ While there is detected inhibitor, patients are on a risk of bleeding, but the justification of PR definition could be the dramatic reduction of this risk when FVIII is greater than 50% in patients with AHA.⁸⁰ In any case, it is important to keep in mind that after inhibitor eradication, FVIII levels are upper normal, with level higher than 100% during the first weeks after response. If this does not happen in a patient, it could be suggested close monitoring to keep from a possible relapse.

The guidelines in IST vary according to national experiences. Immunosuppression with steroids and cyclophosphamide have been the most used protocol.^{2–6,113–115} EACH2 with a total of 331 patients, the longest registry,¹¹³ and

other national registries,^{4–8} confirm that steroids alone or in combination with cyclophosphamide are the most used scheme, with good clinical results. In EACH2,¹¹³ German group⁴ and Spanish group,⁵ they also include patients who did not respond adequately with the combination of steroids and cyclophosphamide, and who were treated with rituximab, alone or associated with corticosteroids. Cases treated first-line with rituximab alone or in combination have also been published.^{5,82,113} Table 4 describes the effectiveness data of the different schemes used by the longest published registries. These three immunosuppressant drugs are the ones used universally; however, other drugs have been used in published case series like cyclosporine, syrolimus, azathioprine, bortezomid, iv Ig, etc, as second-line IST.^{116,117}

Doses of steroids (in prednisone equivalents) start with 1 mg/kg/24h for 3 weeks, followed by gradual decrees. In general, it is accepted that, if there is no response to steroids after 3 weeks, cyclophosphamide or rituximab should be associated, if they have not been used previously. Recommended cyclophosphamide dose is 1–2 mg/kg/24h and rituximab dose is 375 mg/m²/weekly, for a total of 4 doses.^{4,113,115–117} However, there are published experiences with rituximab in AHA at doses of 100mg/m²/weekly with good results.^{118,119}

The results of IST from EACH2¹¹³ show that the association of steroids and cyclophosphamide achieved a CR rate of 77%, steroids in monotherapy 48%, rituximab and other immunosuppressive drug 59% and rituximab in monotherapy scheme dropped to 42%. In addition, to avoid bias derived from the different clinical characteristics of the patients, they perform a statistical analysis using the “propensity score”, to control age, gender, factor VIII levels, inhibitor titer and underlying pathologies, concluding that the combination of steroids with cyclophosphamide achieves a significantly higher CR rate ($p < 0.003$).

In the German-Austrian group,⁴ the authors note that FVIII < 1%, inhibitor titer >20BU, ECOG >2 or IgA antibody titer higher than 1:20 are related to a lower rate of CR, and such patients should be candidates for dual IST with steroids and cyclophosphamide or steroids and rituximab. The results with the different combinations showed CR rate of 80% versus 47% in case of steroids alone. In the steroids group, the mean of days to achieve remission was 32 days, while dual therapy with steroids and cyclophosphamide achieved CR in a median of 40 days. However, patients treated with steroids plus rituximab needed a median of 65 days to achieve remission.

In Dutch serie,⁶ there was no difference in effectiveness between steroids plus cyclophosphamide or plus rituximab (80% and 63% respectively), but both schemes of treatment were superior to steroids in monotherapy with an effectiveness of 35%. It took more than 10 weeks to achieve remission. They describe inhibitor titer higher than 20 BU, severe bleeding, and steroid monotherapy as adverse prognostic factors for remission.

Spanish experience⁵ confirms the same data on the superiority of combined immunosuppression regimens over steroids in monotherapy (Table 4). It validates inhibitor titers greater than 20 BU and steroid treatment as monotherapy as poor prognosis factor for response. It does not confirm the influence on remission rate of FVIII <1% or age (ECOG or severity of bleeding not included in analysis). Other published prognostic factor for remission could be the specificity of autoantibodies with regard to FVIII epitopes. Those directed against light chain FVIII could be related to poor response.^{55,120}

Patients who did not respond to the first-line IST and need a second line present a remission rate of 79% with steroids plus cyclophosphamide, and 65% with rituximab and steroids.¹¹³

Recently, the international recommendations for AHA diagnosis and treatment have been reviewed.¹⁵ They recommend/suggest in patients with favorable prognostic factors the use of steroids in monotherapy, and in those with unfavorable prognostic factors use as first-line cyclophosphamide or rituximab associated with steroids. In the case of rituximab, they recommend the dose of 375mg/m², weekly maximum 4 cycles. High doses of Factor VIII and high-dose immunoglobulins are not recommended. Although in the recommendations,¹⁵ FVIII <1% and an inhibitor titer >20BU are considered poor prognosis factor to get CR, only inhibitor titer >20BU has been validated for the majority of series.^{4–7} In contrast, steroid monotherapy is persistently a poor prognostic factor for achieving remission,^{5,6,113} and reduction in mortality due to infections in patients treated with steroids alone has been validated by some authors,^{4,6,9} but not by others like EACH2,^{5,7,113} the longest published registry. Perhaps, the recommendation should be restricted to the use of steroids in monotherapy in patients with inhibitor titer lower than 20BU and no fit, who may have a higher risk of infections and death. Other considerations in special populations are

Table 4 Outcomes of Immunosuppressive Treatment of Longest AHA Registries.^{4-8,113}

	Steroids		Steroids Plus Cyclophosphamide		Rituximab Plus Other Immunosuppressive Drug		Global Data (All Immunosuppressive Schemes)			
	Complete Response	Relapse	Complete Response	Relapse	Complete Response	Relapse	Complete Response	Time to Complete Response (Median)	Relapse	Time to Relapse
Collis et al¹¹³	48%	19%	77%	14%	59%	4%	65%	5 weeks	11%	137 days
Borg et al⁷	–	–	–	–	40%	–	87%	20 weeks	0% (12 months follow-up)	–
Tiede et al⁴	47% 32 days	–	80% 40 days	–	80% 65 days	–	61%	14 weeks (79 days)	–	–
Sun et al⁸	62%	22%	88%	7%	91%	5%	68%	7–10 weeks	9.4% (7 months follow up)	–
Mingot-Castellano et al⁵	68%	27%	89%	3.7%	88%	3.4%	84%	5–7 weeks	7.1% (13 months follow-up)	–
Schep et al⁶	35%	27%	80%	10%	63%	23%	46% with first line	10.7 weeks	25%	102 days

- Neoplasms: Treatment of underlying neoplasm will facilitate the elimination of the inhibitor, although it does not substitute immunosuppressive treatment. In this group, immunosuppressive treatment should be individualized according to age, patient comorbidities and the type and prognosis of the tumor. Napolitano et al have reported response rates of 88% (79% patients treated with steroids and cyclophosphamide, 20% steroids and rituximab).²³
- Pregnancy/postpartum: Mortality rates are lower, between 1.7% and 3%.⁴² Despite this, IST should be started as soon as possible to prevent from severe or vital bleeding always exists. The first line of treatment recommended is steroids.
- AHA secondary to drugs: It may be a useful IST, but taken into account that this AHA usually regresses spontaneously when the drug is discontinued.^{14,43}
- Children: They present a high rate of spontaneous remissions. The recommended first-line treatment is steroids.¹⁵ In neonates as a consequence of the transplacental transference of autoantibodies from mother, symptoms have been solved without IST.⁴²

Relapse

In AHA, relapse rate is 7.1% and 20% according to the most recent series.^{4-8,10} Collins et al¹⁰ describe a relapse rate of 20% out of a total of 90 patients who eliminated the inhibitor. Median time to relapse was 7.5 months, with a follow-up after remission of 13 months (range 0–37 months). A second remission was achieved in 10 of the relapsed patients. In EACH2, with a median follow-up of 262 days (range 30–603 days), relapse rates after treatment with steroids, steroids plus cyclophosphamide and rituximab-containing regimens were 18%, 12%, and 4%, respectively, with a median time to relapse of 139 days.¹¹³ In Table 4, there are described relapse data from recent series.^{4-8,10,113} In postpartum AHA, relapse rates reach 29% with a median time to relapse close to 8 months.⁴²

In case of relapse, there are no recommendations on immunosuppressive schemes of treatment. In general, the choice would depend on patient's characteristics at that time and the possible associated causes, the duration of the response and tolerance to previous regimens.^{1,15,84}

Mortality and Prognostic Factors

Morbidity

The mortality rate associated with AHA ranges between 7% and 38%.^{3,10,116,121} Overall survival in EACH2 registry, with a median follow-up of the series of 262 days (range 30–603 days), were 67% for steroids, 62% for steroids plus cyclophosphamide and 71% for rituximab plus other immunosuppressive agent.¹¹³

The GTH-AH 01/2010 study found that the main negative prognostic factors for survival in AHA were FVIII levels equal to or less than 1%, a score on the World Health Organization performance status (WHO-PS) functional scale greater than 2, the presence of underlying neoplasia, and not achieving CR.⁴ This group, in a post hoc analysis, described that the presence of isotype IgA autoantibodies against FVIII implies a higher rate of recurrences and therefore represents an unfavorable prognostic factor that would increase mortality.

Previous meta-analyses have associated greater overall survival and disease-free survival with achieving CR, no underlying neoplasm, and age at diagnosis under 65 years.^{9,121} Patients older than 65 years have a higher mortality, with an odds ratio of 2.4. Regarding the associated pathology, in subjects with AHA and neoplasia, rates of 44% are described, compared to 19% of the idiopathic ones or 4% of those associated with postpartum.^{3,121} Other factors related to higher mortality are lack of response to eradication treatment and low hemoglobin levels at diagnosis.^{3,9} The Dutch registry⁶ described age above 75 years, neoplasms and admission to Intensive Care Unit are poor prognostic factors for survival. Spanish registry found age superior to 65 years as the only poor prognostic factor for survival, non-validated neoplasia as underlying disease, inhibitor titer, FVIII level, get CR, scheme of immunosuppressive treatment or anticoagulant/antiplatelet secondary prophylaxis before AHA diagnosis.⁵

In AHA, bleeds are usually the cause of early mortality due to delays in diagnosis and, therefore, in the treatment of severe hemorrhages that usually motivate the initial consultation. The iatrogenic nature of immunosuppressive treatment is the protagonist in late mortality.^{3,9,121} It should not be forgotten that most of these patients are elderly and suffer from

many comorbidities. This, together with treatment with immunosuppressive drugs such as cyclophosphamide or steroids, turn this population into a “fragile” group in which the benefit of treatment must always be assessed on the basis of the morbidity it could induce. These recommendations are based on clear facts such as the incidence of infections in 30%⁶ of these patients, which contributes to 15–47% of mortality linked to AHA.^{3,4,9,10} The main causes of death today are infectious conditions in the registries of countries with adequate access to bypass agents^{4–7,113} and hemorrhagic events in those that do not have them⁸ (Table 5).

In the current registries, there are contradictory data regarding the increased risk of infections with steroid regimens in combination^{6,9} or not.^{5,113} There are no clear recommendations for the prevention of these infections. In benign hematological pathology, it could be suggested:¹²²

- Pneumocystis: TMP/SMX, 80 mg TMP and 400 mg SMX daily or double dose 3 times a week, discontinue when steroids are discontinued. If TMP/SMX intolerance, use dapsone or nebulized pentamidine once a month. These schemes could be useful in patients under prednisone ≥ 30 mg daily ≥ 4 weeks; 15 mg to 30 mg daily ≥ 8 weeks or ≥ 10 mg daily and 2 or more of the following age > 65 years, lung disease, association with another immunosuppressant
- Herpes Virus: In case of a history of previous infections, acyclovir 400mg/day could be used.
- Tuberculosis: If prednisone would be used at doses higher than 10mg for more than 4 weeks, a tuberculin test should be performed. In case of positivity, it should be consulted with Infectious Diseases Department.
- Hepatitis B virus (HBV): In patients with positive HBVc Ab and positive HBV Ag, if prednisone were used at higher doses or 20mg for 4 weeks or more; or 10–20mg daily for 8 weeks or more, prophylaxis with entecavir or tenofovir should be performed. If HBVc Ab positive, but antigen negative, close monitoring should be used.

Table 5 Cause of Mortality in Longest AHA Registries.^{4–8,113}

Study Dead Patients/Total Patients Percentage of Death	Infections	Bleeds	Underlying Disease	Cardiac or Thrombosis
Collis et al ¹¹³ 89/287 31%	12.4%	3.3%	11.2%	–
Borg et al ⁷ 27/82 33%	37%	11%	–	15%
Tiede et al ⁴ 34/102 33%	47%	9%	9%	18%
Sun et al ⁸ 11/165 6.7%	27%	55%	18%	–
Mingot-Castellano et al ⁵ 36/151 23.8%	41.7%	13.9%	14%	
Schep et al ⁶ 52/136 38%	19.2%	7.7%	13.5%	9.6%

Other measures to put into practice in case of steroid treatment beyond 4 weeks would be the prevention of osteopenia since up to 30% may present pathological fractures. Prophylaxis treatment should start from first dose of steroids with oral vitamin D at a dose of 800–1000 IU/day and calcium at a dose of 1000–1200 mg/day.¹²³

Another morbidity to assess is the high cardiovascular risk of patients with AHA given their advanced age in most cases.^{4–8,10,113} Elevation of FVIII levels to levels above normal after the eradication of the inhibitor has been clearly described.¹ Elevated levels of FVIII have been described as a cardiovascular risk factor,¹²⁴ which adds to the baseline elevated cardiovascular risk of most patients with AHA given their advanced age and comorbidity in most cases. For this reason, strict monitoring of thromboembolic disease prophylaxis is recommended in cases where it is indicated by the medical-surgical situation of the patient in these circumstances.¹ Other adverse effects are hyperglycemia, psychiatric disorders, or mucosal ulcers secondary to cytotoxic agents.^{1,15,84}

Conclusions

In conclusion, acquired haemophilia A is a rare condition but in many cases supposes a hematological emergency, and the hematologist intervention is essential to achieve a rapid diagnosis, and to introduce the treatment as soon as possible. Since most of the publications represent national registries, there are no properly randomized studies that allow conclusions to be drawn with scientific evidence. But the available data provide us the best knowledge of this disease and to prescribe the optimal treatment for acquired haemophilia A. This treatment should be based on bypassing agents of recombinant porcine FVIII and immunosuppressive drugs according to patient comorbidities.

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.

Disclosure

The authors declare no competing financial interests for this paper.

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Assessment of Blood Donation Practice and Its Associated Factors Among Wollega University Undergraduate Students, Ethiopia

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Introduction: Blood donation is the process of collecting blood from donors who are at low risk for infection and unlikely to jeopardize their own health. Blood donation addresses maternal and child mortality and contributes to saving millions of lives. But many African countries including Ethiopia are far below the minimum blood collection rate. Furthermore, the blood donation practice is not well studied among young university students in the study area. Hence, this study was done to assess the blood donation practice and associated factors among Wollega University students, Ethiopia.

Methods: A cross-sectional study was conducted among 387 students of Wollega University. The students were selected by systematic random sampling. Self-administered questionnaire was used to collect the data. The data were entered into Epidata V.3.1 and exported to SPSSV.25 for analysis. Both bivariate and multivariate logistic regressions were used to identify the associated factors. Adjusted odds ratio with 95% confidence interval and P-value <0.05 were used to declare the statistically significant associations in the multivariable regression. The analyzed result was presented using tables, graphs, and text.

Results: Of the 360 respondents, 147 (40.8%; 95% CI: 35.7–46.1%) ever donated blood. Blood donation practice was significantly associated with college of the students (AOR = 3.247; 95% CI: 1.348–7.820), not taking part in blood donation campaigns (AOR = 0.285; 95% CI: 0.161–0.503), knowledge of blood bank location (AOR = 5.297; 95% CI: 3.081–9.110), knowledge about blood donation (AOR = 2.035; 95% CI: 1.123–3.686) and attitude toward blood donation (AOR = 2.266; 95% CI: 1.122–4.577).

Conclusion: The magnitude of blood donation in this study was found to be less than the recommended level by the World Health Organization. Absence of the blood donation campaigns, college of study, lack of knowledge, and poor attitude toward blood donation were the factors that influence the blood donation practice. All health and related institutions including the Wollega University must work in coordination to improve the blood donation practice focusing on the regular voluntary blood donors.

Keywords: blood, blood donation, voluntary blood donation, practice, associated factors, students, Ethiopia

Introduction

Blood is one of the vital body fluids containing special components that deliver essential substances like oxygen and nutrients to the body's cells and is used to remove unwanted metabolic wastes from the cells.¹ It consists primarily of plasma and blood cells (white blood cells, red blood cells, and platelets), and blood donation is the only source of blood.^{2,3}

Blood donation is the process of collecting blood from donors who are at low risk for infection and unlikely to jeopardize their health by blood donation.⁴ It is a lifesaving practice for people who lost ample volumes of blood as a result of accidents, obstetric and gynecological bleedings, severe anemia, and cancers.⁵ There are three main types of blood donors: voluntary non-remunerated, replacement, and paid donors. Voluntary non-remunerated donation is giving one's own blood, plasma or other blood components by free will and without receiving any form of payment either in cash or in-kind. Family/replacement blood donation is giving the blood when it is required by a member of the patient's family or community. Paid donation is another

category of blood donation by which a person donates blood in exchange of money or other form of payment. This last category of blood donation is also known as professional blood donation.⁶ Among the categories, voluntary non-remunerated blood donation or voluntary blood donation in short is the safest form of blood donation because the transfusion-transmitted infection is lowest among these donors.⁷ In Ethiopia, a replacement donor system was applied before 2011, while voluntary blood collection system is used since 2011.⁸

The World Health Organization (WHO) recommends 10 donations per 1000 populations to satisfy the blood needs of a given country.⁷ Despite the World Health Organization's suggestion for countries to focus on young people to achieve 100% non-remunerated voluntary blood donation,⁹ there is insufficiency in blood donation to meet the demand in different corners of the world.¹⁰ Globally, over 80 million units of blood are donated each year; however, only two million units are donated in low-income countries where the need is very high.¹¹ In sub-Saharan Africa (SSA), where over 12% of world's populations live,¹² only 38% was collected out of the yearly 18 million estimated units of safe blood need.³ In Ethiopia, adequate and safe blood supply has remained a challenge where there has been great inadequacy and inequity in access to blood. The national blood requirement in Ethiopia is 18,000 units per day, while only 1100 units are being collected.¹³

The previously conducted studies in different parts showed that the magnitude of blood donation practice range from 10% to 35.69% in Asia^{14–16} and 10.64% to 61.69% in Africa^{11,17,18} which was relatively incomparable with high-income countries. It was found to be 29% in Saudi Arabia,¹⁹ 26.7% in India,²⁰ 27.1% in Ghana,²¹ 15.3% in Nigeria²² and 22.6%–47.8%,^{5, 23–28} in different regions of Ethiopia. On top of this, various factors are identified as the factors that hinder blood donation practice. These factors are a perception of being unfit to donate blood, fear of anemia, fear of health risks, and lack of information about blood donation.²⁹ Furthermore, age, gender, religion, knowledge, attitude, educational status, self-perceived health status, and family education are also factors that can affect the blood donation practice.^{5,19,21,23,24,26,28}

Insufficient blood donation and shortage of blood contribute to preventable deaths, which result from major surgery, trauma, cancer, anemia, and pregnancy-related complications.^{30,31} In developing countries, about half a million mothers die each year during pregnancy and childbirth with hemorrhage.³² Children are particularly vulnerable to a shortage of blood in the malarious area because of severe anemia.³³

The youngsters in University contribute in life-saving not only through blood donation, but also they can serve as long-term donors, less risky blood donors, role models, and motivators to increase the number of donors as they pursue their career.³⁴ The students provide the safest blood, and the country's blood supply can be improved by recruiting and retaining the student donors.²⁸ However, the practice of blood donation among these populations was not well studied. Therefore, this study was aimed to assess the magnitude and factors associated with blood donation practice among Wollega University students in western Ethiopia.

Materials and Methods

Study Area

The study was conducted from Dec 13, 2021 to Dec 28, 2021 at Wollega University Nekemte Campus, which is located 331km from Addis Ababa. The University runs 60 undergraduate and 17 graduate programs on three different campuses in Nekemte, Gimbi, and Shambu towns. Four thousand forty-eight (4048) students were attending undergraduate programs in all departments.⁶

Study Design and Period

An institutional-based cross-sectional study design was employed.

Source and Study Population

All undergraduate students who were attending their class at Wollega University, Nekemte campus were the source population. All regular students who were from the three randomly selected colleges of Wollega University were the study population.

Inclusion and Exclusion Criteria

Inclusion Criteria

All regular students found in Wollega University, Nekemte campus.

Exclusion Criteria

Students who were not present in the compound during the data collection period were excluded.

Sample Size Determination

The sample size for the study was calculated using a single population proportion formula

$n = (Z\alpha/2)^2 \frac{p(1-p)}{d^2}$ where n = the calculated sample size, z = level of significance, p = blood donation proportion, d = margin of error. The assumptions like 95% confidence interval, 5% margin of error, $\alpha = 0.05$, and 10% non-response rate were considered. The proportion (p) of blood donation used to calculate the sample size for this study was taken from the study conducted among college students in Mizan-Aman, Southwest Ethiopia, and it was 35.5%.²⁴ Accordingly, the sample size was calculated as

$$n = \frac{(1.96)(1.96)(0.355)(0.645)}{(0.05)(0.05)} \approx 352$$

By adding 10% for non-response rate, the final sample size of the study was 387.

Sampling Technique

Initially, one-third of the colleges/institutes were considered from the total nine colleges/institutes in the Wollega University, Nekemte campus. Accordingly, three colleges/institutes (College of Business and Economics, Institute of Health Sciences, and Institute of Language Studies and Journalism) were selected randomly by lottery method. The calculated sample size was proportionally allocated to colleges based on their size. List of the students was taken from the colleges to prepare the sampling frame, while the systematic random sampling technique was used to select the final study participants. The sampling interval ($K = 4$) for the systematic random sampling was calculated using the formula, $K = \frac{N}{n}$, where N is the total number of students in the selected college/institute, and n is the calculated sample size. The first participant was selected by lottery method and continued every 4th student in each selected institute/college until the allocated sample size was achieved (Figure 1).

Data Collection Tool, Techniques and Data Collectors

The data were collected by using a self-administered questionnaire, which was adapted from different literatures based on the objectives of the study.^{28,35,36} Socio-demographic factors, knowledge, attitude, and practice-related questions were the components of the questionnaire. It was developed and distributed in English language (see [Supplementary File 1](#)). Five master of public health students collected the data, while 2 master of public health holder lecturers supervised the data collection procedures. Two days of training were given for both data collectors and supervisors on the data collection tools and procedures, objectives of the study, ethical issues and data collection period.

Study Variables

Dependent Variable

The practice of blood donation.

Independent Variables

Socio-demographic and economic factors, knowledge of blood donation, attitude toward blood donation, and other behavioral factors (smoking, alcohol consumption, khat chewing, and social media usage).

Operational Definitions

Attitude towards blood donation was assessed by using twelve questions and those students who scored more than the mean value were considered as having a favorable attitude and vice versa.

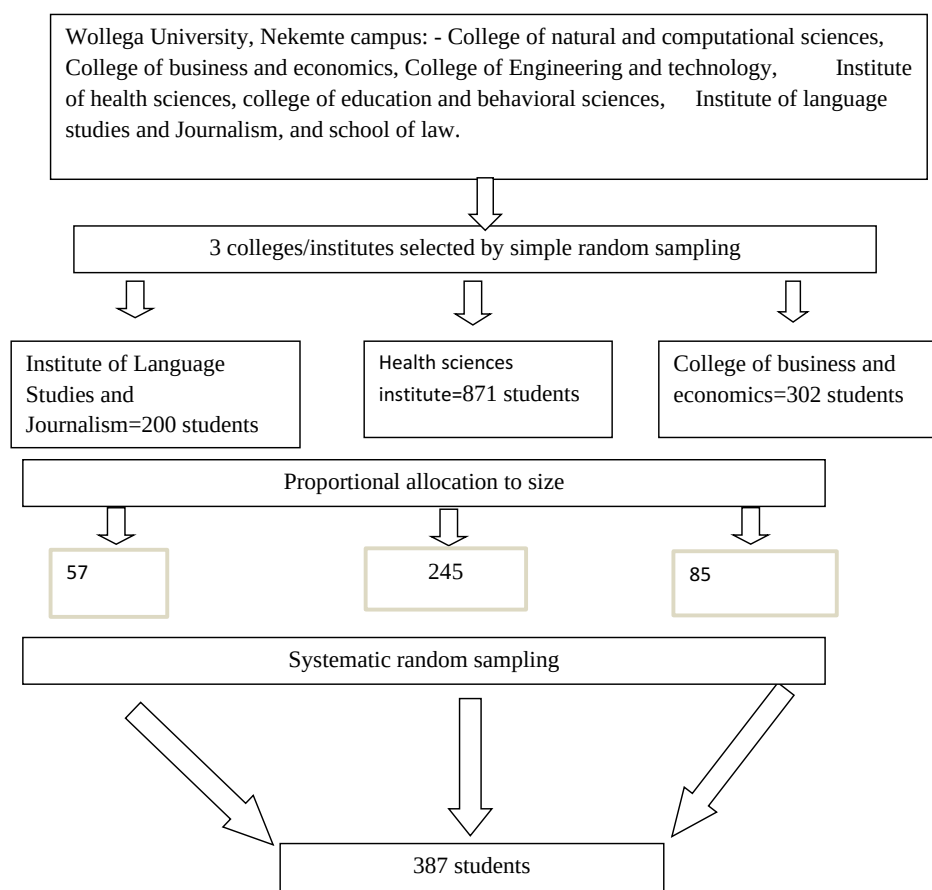


Figure 1 Sampling techniques for the study of blood donation practice and associated factors among Wollega University undergraduate regular students, 2021.

Knowledge of blood donation was assessed by using fourteen knowledge-related questions, whereas the mean value was considered to classify the students as having good knowledge or poor knowledge.

Remunerated/paid donors: Those individuals who give blood in favor of money or other payment.

Practice of Blood Donation

Those students who donated blood (voluntarily, replacement, and paid) at least once in a lifetime were categorized as “ever donated” whereas who never donated blood at all in a lifetime were categorized as “never donated”.³⁷

Data Quality Control

The data collection tool was pretested on 5% (19) of the sample size on students in Gimbi campus which was out of the study setting. Besides, two-day training was given to data collectors and supervisors. During the data collection period, the supervisors supervised the data collectors frequently and the collected data was checked for consistency and completeness on a daily basis. In case the quality problem raised, it was brought for discussion and necessary corrections were made through returning the questionnaire for respective data collector. After data collection, the collected data were entered into Epi-data software version 3.1. (the EpiData Association, Odense Denmark) twice by different individuals and checked for consistency.

Data Processing and Analysis

The data were entered into epi-data version 3.1. (the EpiData Association, Odense Denmark) and then exported into SPSS version 25 (IBM, Armonk, New York, US) for data coding and further analysis. The data were summarized using percentages, mean, and presented by texts, figures and frequency tables. The dependent variable (blood donation

practice) was recoded as practiced or not practiced. The bivariable binary logistic regression analysis was run and the variables that showed association at p -value <0.25 were considered as candidates for multivariable logistic regression. The multicollinearity was checked, and none of the independent variables had the collinearity problem at variance inflation factor >10 . The multivariable logistic regression was done. Adjusted odds ratio with their respective 95% confidence interval was used to express the magnitude of association and p -value <0.05 to declare the statistically significant association. Model fitness was checked using Hosmer and Lemeshow's test with a p -value of 0.283. Being above 0.05, the value indicated that the model fitted the data.

Results

Socio-Demographic Characteristics of the Students

Out of 387, 360 students participated with a response rate of 93.02%. The respondents' mean age was (22.45±2.096SD) years old and it ranges from 18 to 30 years. The majority of the participants were males (227 (63.1%)). Regarding religion, 194 (53.9%), 102 (28.3%) and 49 (13.6%) of the respondents were Protestant, Orthodox and Muslim, respectively. About 263 (73.1%) and 219 (60.8%) of the study participants reported that their fathers and mothers ever attended education, respectively. Two-third (239 (66.4%)) of the students get monthly pocket money of less than/equal to 500 Ethiopian birr/ETB (Table 1).

Table 1 Socio-Demographic and Economic Characteristics of Wollega University Regular Undergraduate Students, Ethiopia, 2021 (n = 360)

Characteristics	Category	Frequency (%)
Age	16–20	62(17.2%)
	21–25	269(74.7%)
	26–30	29(8.1%)
Gender	Female	133(36.9%)
	Male	227(63.1%)
Religion	Orthodox	102(28.3%)
	Muslim	49(13.6%)
	Protestant	194(53.9%)
	Catholic	5(1.4%)
	Other ^a	10(2.8%)
Ethnicity	Amhara	85(23.6%)
	Oromo	202(56.1%)
	Other ^b	73(20.3%)
Marital status	Single	300(83.3%)
	Married	27(7.5%)
	In relationship	33(9.2%)
Previous area of Residence	Urban	188(52.2%)
	Rural	172(47.8%)
College	College of Business and Economics	76(21.1%)
	Health Science Institute/HIS	239(66.4%)
	Institute of Language Studies and Journalism/ILSJ	45(12.5%)
Academic year	1st year	61(16.9%)
	2nd year	100(27.8%)
	3rd year	155(43.1%)
	4th year	44(12.2%)
Father ever attended school	Yes	263(73.1%)
	No	97(26.9%)

(Continued)

Table I (Continued).

Characteristics	Category	Frequency (%)
Father's level of education (n=263)	Primary	78 (21.7%)
	Secondary	64 (17.8%)
	Technical/Vocational	14 (3.9%)
	Higher (College/University)	107 (29.7%)
Mother ever attended school	Yes	219 (60.8%)
	No	141 (39.2%)
Mother's level of education (n=219)	Primary	103 (28.6%)
	Secondary	43 (11.9%)
	Technical/Vocational	12 (3.3%)
	Higher (College/University)	61 (16.9%)
Monthly pocket money in Ethiopia birr	<=500	239(66.4%)
	501–1000	86(23.9%)
	1001–1500	14(3.9%)
	>1501	21(5.8%)

Notes: ^aAdventist, waqefata, Apostles. ^bAfar, Agaw, Nuer, Shimasha, Sidama, Siltea, Somali, South Sudanese, Tigraway, Wolayita, Berta, Dawro, Gamo, Gedio, Gofa, Gurage, Hadiya, Kambata, Konso, Konta.

Behavioral Factors of the Students

One fifth (20.4%) of those students who have a habit of alcohol consumption consume alcohol more than five days per month, while 8 (33.3%) of the students who chew khat chew more than five days/month. Among the cigarette smokers, 8 (80%) of them smoke more than five sticks per event (Figure 2).

Knowledge About Blood Donations and Blood Bank Locations

Overall, 189 (52.5%) of the students had good knowledge about blood donation. On the other hand, 135 (37.5%) of the students have knowledge of blood bank location in their current or previous area of residence (Table 2).

Regarding the sources of information about blood donation, majority (221 (61.4%)) reported that school/training is their primary source of information, while mass media is reported as primary source of information for 188 (52.2%) of the students (Figure 3).

Attitude Toward Blood Donation

Majority (325 (90.3%)) of the study participants thought that donating blood is a good idea, while about three quarters (274 (76.1%)) are willing to donate if called upon next time. About 210 (58.3%) perceive that blood should be donated

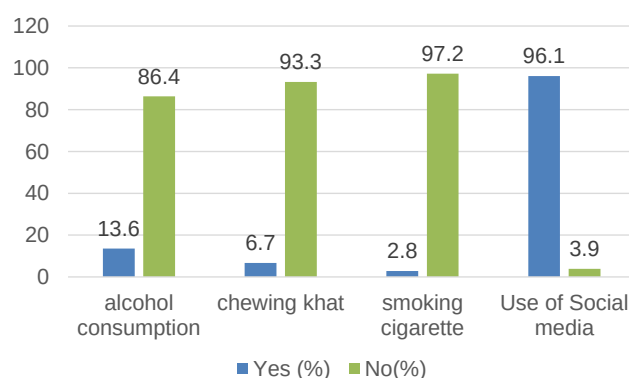


Figure 2 Behavioral characteristics of the students participated in the study of blood donations and associated factors among Wollega University undergraduate regular students, 2021 (n = 360).

Table 2 Level of Blood Donation-Related Knowledge Among Wollega University Students, Ethiopia, 2021 (n = 360)

Characteristics	Category	Frequency (%)
Knowledge/hearing or seeing blood donation	Yes*I	308(85.6%)
	No	52(14.4%)
Minimum age required to donate blood	<18 years	57(15.8%)
	18 years*I	68(18.9%)
	>18	126(35.0%)
	I do not know	109(30.3%)
Maximum age required for donating blood	45–55 years	107 (29.7%)
	56–64 years	26 (7.2%)
	65 years*I	49 (13.6%)
	Do not know	178 (49.4%)
Minimum weight required to donate blood	<45kg	54 (15.0%)
	45kg*I	48 (13.3%)
	>45 kg	135 (37.5%)
	Do not know	123 (34.2%)
Time gap required to donate again	3 months*I	142 (39.4%)
	6 months	36 (10.0%)
	Year	53 (14.7%)
	Do not know	129 (35.8%)
Amount of blood taken from a single person in one event	<500mL*I	131 (36.4%)
	500–1000mL	52 (14.4%)
	Do not know	177 (49.2%)
Time taken to donate for a single voluntary blood donor	<= 20 minutes*I	82 (22.8%)
	20–60 minutes	91 (25.3%)
	I do not know	187 (51.9%)
Site/body part where blood is drawn	Arm*I	256 (71.1%)
	Buttock	9 (2.5%)
	Another site	11 (3.1%)
	Do not know	84 (23.3%)
Know place where blood can be donated	Health facility*I	181(50.3%)
	Blood bank*I	249(69.2%)
	Campaign* I	62(17.2%)
	Do not know	48(13.3%)
Number of persons benefited from a unit of blood	One	72 (20.0%)
	Two	22 (6.1%)
	Three*I	24 (6.7%)
	Four and above	58 (16.1%)
	Do not know	184 (51.1%)
Awareness of Health benefits of donating blood for donor	Yes*I	256 (71.1%)
	No	36 (10.0%)
	Do not know	68 (18.9%)
Awareness of Transmission of Infection by blood transfusion	Yes*I	317 (88.1%)
	No	43 (11.9%)
Knowledge of common blood group types	Yes*I	286 (79.4%)
	No	74(20.6%)
Knowledge of respondents about their blood group type	Yes*I	210(58.3%)
	No	150(41.7%)
Overall knowledge about blood donation	Good	189(52.5%)
	Poor	171(47.5%)
Know blood bank location in current or previous resident	Yes	135(37.5%)
	No	225(62.5%)

Note: *I Correct answer for knowledge related questions.

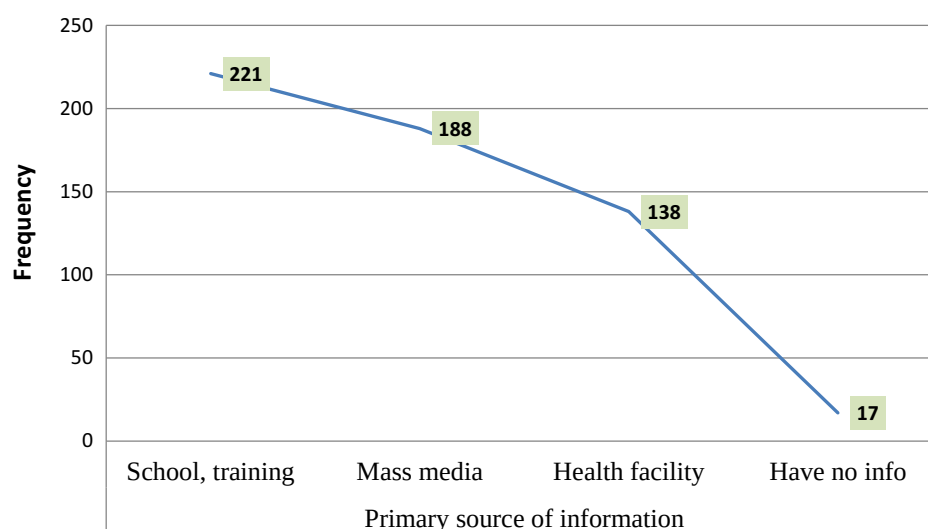


Figure 3 Primary sources of information about blood donation among Wollega University students, Ethiopia, 2021 (n = 360; some students cited more than one source of information).

for anyone in need. About 189 (52.5%) of the students perceive that something harmful could happen to a donor during/ after blood donation. Among the total respondents, 284 (78.9%) had a positive attitude towards voluntary blood donation (Table 3).

Practice of Blood Donation

Among the 360 participants, only 147 (40.8%; 95% CI: 35.7–46.1%) have ever donated blood in their lifetime. Voluntary blood donation was found to be 105 (29%), while it was reported to be 71.4% among those donated. Of those who had donated, majority of them 80 (54.4%) have donated once. One third (118 (32.8%)) of the students ever participated in blood donation campaigns. Some of the reasons hindering blood donation were family or relatives never been in need of blood transfusion (12.7%), lack of knowledge/courage (8.6%), and fear of anemia (17.8%) (Table 4).

Table 3 Attitude Towards Blood Donation Among Wollega University Regular Students, Ethiopia, 2021 (n = 360)

Characteristics	Category	Frequency (%)
Thought about blood donation	It is a good idea	325(90.3%)
	It is a bad idea	7(1.9%)
	Have no idea	28(7.8%)
Persons who should receive blood donation	No one	25(6.9%)
	Paid	6(1.7%)
	Relatives	52(14.4%)
	Anyone in need	210(58.3%)
	Do not know	67(18.6%)
Willingness to donate blood next time if get the chance or by their schedule	Yes	274(76.1%)
	No	86(23.9%)
Willingness to encourage others to donate blood	Yes	292(81.1%)
	No	68(18.9%)
Ever encountered a disease or emergency on yourself or a family where by you needed a blood	Yes	123(34.2%)
	No	237(65.8%)
Perception about Something harmful happening to a blood donor during or after blood donation	Yes	189(52.5%)
	No	171(47.5%)
Overall attitude	Favorable	284(78.9%)
	Unfavorable	76(21.1%)

Table 4 Practice of Blood Donation Among Wollega University Regular Students, Ethiopia, 2021 (n = 360)

Variable	Category	Frequency (%)
Time since the recent blood donation (n=147)	Within this year	44(29.9%)
	1–2 years ago	48(32.7%)
	≥3 years ago	55(37.4%)
Place where the recent blood donated (n=147)	In health institution	48(32.7%)
	Blood bank	57(38.8%)
	Event organized in the campus	42(28.5%)
Frequency of blood donation practice (n=147)	Once	80(54.4%)
	2–3 times	42(28.5%)
	More than 3 times	25(17%)
Reason for donating blood (n=147)	A relative/friend	27(18.4%)
	Voluntarily	105(71.4%)
	Remunerated (paid)	8(5.4%)
Reason for not donating blood (n=213)	To know own status	7(4.7%)
	Unfit to donate	28(13%)
	Fear of anemia	38(17.8%)
	Family or relative never been in need of transfusion	27(12.7%)
	Lack of knowledge/courage	31(8.6%)
	Religion forbids	8(3.8%)
	Have no specific reasons	81(38%)
Taking part in blood donation campaigns	Yes	118(32.8%)
	No	242(67.2%)

Factors Associated with Blood Donation Practice

In bivariable binary logistic regression, eleven factors, namely, age, college of the students, year of study, father's educational status, monthly pocket money, khat chewing, cigarette smoking, participating in blood donation campaigns, knowing the location of blood bank in the previous and current residence, knowledge about blood donations and attitude toward blood donation, showed significant associations with blood donation practice. However, only five factors showed significant association with blood donation practice in the multivariable logistic regression analysis. Accordingly, college of the students (AOR = 3.247; 95% CI: 1.348–7.820), not taking part in blood donation campaigns (AOR = 0.285; 95% CI: 0.161–0.503), knowledge of blood bank location (AOR = 5.297; 95% CI: 3.081–9.110), knowledge about blood donation (AOR = 2.035; 95% CI: 1.123–3.686) and attitude toward blood donation (AOR = 2.266; 95% CI: 1.122–4.577) were the independent factors of blood donation practice. The odds of blood donation were 3.2 times more likely for the students in Institute of Language Studies and Journalism than health science students. Those students who did not take part in blood donation campaigns were 71.5% less likely to donate blood as compared to their counterparts. Students who know the blood bank location in the current or previous residential areas had 5 times more odds of blood donation than their counterparts. Similarly, those students who had good knowledge about blood donation had practiced blood donation 2 times more likely than those who had poor knowledge of blood donation. In addition, the students who had favorable attitude regarding blood donation had 2.2 times more odds of blood donation compared to their counterparts (Table 5).

Discussion

This study was aimed to assess the magnitude of blood donation practice and associated factors among university students. The study showed that 147 (40.8%) of the students have ever donated blood in their lifetime. This result is greater than the findings of the study in Debre Markos (16.1%),³⁵ Mizan-Aman Health Sciences College (35.5%),²⁴ Aman sub city (26.4%),³⁸ Madda Walabu (18.4%),³⁹ Ambo University (23.6%),²⁸ and Gondar University students

Table 5 Factors Associated with Voluntary Blood Donation Practice Among Wollega University Regular Students, Ethiopia, 2021 (n = 360)

Variables	Category	Ever Donated		Bivariable		Multivariable	
		No=N (%)	Yes=N (%)	P-value	COR with 95% CI	P-value	AOR with 95% CI
Age	16–20	45(72.6%)	17(27.4%)	Ref	Ref	Ref	
	21–25	156(58.0%)	113(42.0%)	0.005*	0.267(0.106–0.673)	0.155	1.741(0.810–3.738)
	26–30	12(41.4%)	17(58.6%)	0.091	0.511(0.235–1.113)	0.143	2.524(0.731–8.714)
Gender	Female	78(58.6%)	55(41.4%)	0.878	1.035(0.670–1.599)		
	Male	135(59.5%)	92(40.5%)	Ref	Ref		
Marital status	Single	176(58.7%)	124(41.3%)	Ref	Ref		
	Married	16(59.3%)	11(40.7%)	0.952	0.976(0.43–2.175)		
	In relationship	21(63.6%)	12(36.4%)	0.582	0.811(0.385–1.709)		
Previous area of residence	Urban	112(59.6%)	76(40.4%)	Ref	Ref		
	Rural	101(58.7%)	71(41.3%)	0.869	1.036(0.680–1.578)		
College	Health science	143(59.8%)	96(40.2%)	Ref	Ref		
	Business and economics	50(65.8%)	26(34.2%)	0.354	0.775(0.451–1.329)	0.188	1.638(0.785–3.420)
	Institute of language studies and journalism	20(44.4%)	25(55.6%)	0.058*	1.862(0.980–3.540)	0.009**	3.247(1.348–7.820)
Year of study	1st year	42(68.9%)	19(31.1%)	Ref	Ref		
	2nd year	61(61.0%)	39(39.0%)	0.315	1.413(0.720–2.775)	0.544	0.770(0.330–1.792)
	3rd year	91(58.7%)	64(41.3%)	0.169	1.555(0.829–2.917)	0.417	0.721(0.327–1.590)
	4th year	19(43.2%)	25(56.8)	0.009*	2.909(1.299–6.512)	0.466	1.477(0.518–4.210)
Religion	Orthodox	62(60.8%)	40(39.2%)	Ref	Ref		
	Muslim	32(65.3%)	17(34.7%)	0.592	0.823(0.405–1.675)		
	Protestant	110(56.7%)	84(43.3%)	0.499	1.184(0.726–1.929)		
	Catholic	3(60.0%)	2(40.0%)	0.972	1.033(0.165–6.460)		
	Other	6(60.0%)	4(40.0%)	0.961	1.033(0.274–3.892)		
Father's ever attended school	No	50(51.5%)	47(48.5%)	Ref	Ref	Ref	Ref
	Yes	163(62.0%)	100(38.0%)	0.075*	0.653(0.408–1.044)	0.476	0.807(0.448–1.455)
Mother's ever attended school	No	80(56.7%)	61(43.3%)	0.452	1.179(0.767–1.812)		
	Yes	133(60.7%)	86(39.3%)	Ref	Ref		
Monthly pocket money	0–500	150(62.8%)	89(37.2%)	Ref	Ref		
	501–1000	48(55.8%)	38(44.2%)	0.258	1.334(0.809–2.200)	0.179	1.530(0.822–2.847)
	>1000	15(42.9%)	20(57.1%)	0.027*	2.247(1.095–4.612)	0.072	2.397(0.926–6.204)
Alcohol consumption	Yes	30(61.2%)	19(38.8%)	0.753	0.905(0.488–1.679)		
	No	183(58.8%)	128(41.2%)	Ref	Ref		
Chewing khat	Yes	11(45.8%)	13(54.2%)	0.174*	1.782(0.775–4.094)	0.920	0.938(0.266–3.305)
	No	202(60.1%)	134(39.9%)	Ref	Ref	Ref	Ref
Smoking cigarette	Yes	4(40.0%)	6(60.0%)	0.222*	2.223(0.616–8.021)	0.324	2.517(0.403–15.728)
	No	209(59.7%)	141(40.3%)	Ref	Ref		
Social media usage	Yes	206(59.5%)	140(40.5%)	Ref	Ref		
	No	7(50.0%)	7(50.0%)	0.479	1.471(0.505–4.287)		
Heard or seen about blood donation	Yes	181(58.8%)	127(41.2%)	Ref	Ref		
	No	32(61.5%)	20(38.5%)	0.707	0.891(0.487–1.628)		
Taken part in blood donation campaign	Yes	38(32.2%)	80(67.8%)	Ref	Ref	Ref	
	No	175(72.3%)	67(27.7%)	0.000*	0.182(0.113–0.293)	0.000**	0.285(0.161–0.503)
Know location of Blood bank	No	169(75.1%)	56(24.9%)	Ref	Ref	Ref	Ref
	Yes	44(32.6%)	91(67.4%)	0.000*	6.241(3.901–9.986)	0.000**	5.297(3.081–9.110)
Knowledge about blood donation	Poor	119(69.6%)	52(30.4%)	Ref	Ref		
	Good	94(49.7%)	95(50.3%)	0.000*	2.313(1.500–3.566)	0.019**	2.035(1.123–3.686)
Attitude toward blood donation	Unfavorable	55(72.4%)	21(27.6%)	Ref	Ref	Ref	
	Favorable	158(55.6%)	126(44.4%)	0.009*	2.089(1.200–3.637)	0.023**	2.266(1.122–4.577)

Note: *p-value<0.25, **p-value<0.051.

Abbreviations: COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval; Ref, reference; N, number.

(12.5%),¹⁷ Nigeria (13.3%),²² Tanzania (30%).⁴⁰ However, this finding is lower than the one from the study conducted in Tigray (47.8%).²⁵ This might be due to the difference in study population as the study in Tigray was among the health professionals.

Regarding the practice of voluntary blood donation, 105 (71.4% and 29% among those donated and among the total participants respectively) were volunteers, which is comparable with the study conducted in Bale.^{27,39} However, this result is higher than 10.6% voluntary blood donation practice in Birbir town,¹⁸ 16.8% voluntary practice in Bahir Dar,³⁷ and 63.7% in Debre Markos' study.³⁵ The increment in this study could be due to the awareness creation and mobilization activities done in the University in collaboration with Nekemte blood bank. The observed difference might be also attributed to the difference in the study period and populations as social mobilization and awareness creation activities are increasing from time to time.

Of the 147 ever blood donors, only 17% donated the blood more than three times. This result is greater than the study in Debre Markos town (7.4%),³⁵ while it is consistent with the finding in Mizan Aman health science college (13%).²⁴ However, it is low when compared to the recommended regular (every three months) non-remunerated voluntary blood donation in sustaining the blood supply and overcome the shortage of blood.⁷ On top of this, majority of the blood donors (103 (70%)) were elapsed donors and it was greater than one year since they donated the blood. This value is far greater than the result in the study of Mizan Aman Health College.²⁴ This indicates that besides the improving status of regular blood donation from time to time, there is a need to work for the achievement of the country's blood need.

Among the non-blood donors, fear of anemia, being unfit to donate, and absence of transfusion need by relative or family members were some of the main reasons that hindered the blood donation in this study. Similar to this study, 16% of non-donors in the study of Mizan Aman health college mentioned that they were unfit to donate the blood.²⁴ Besides, significant proportion (38%) had no specific reason to not donate the blood in our study. This later group would donate the blood if someone approach and convince them.

Nearly half (52.5%) of the students in this study were knowledgeable about blood donation. The result is comparable with the study conducted in Assosa University (48.5%)⁴¹ but higher than the finding from Ambo University (40.4%).²⁸ The variation in the level of knowledge could be from the difference in the study period, and blood donation campaigns as about 1/3rd of the students in this study also witnessed blood donation campaigns.

In the current study, more than three-quarters (78.9%) of the respondents had favorable attitude towards blood donation. The finding was better when compared to the previous studies conducted in Assosa University (68.9%),⁴¹ Ambo University (47.6%),²⁸ and India (57.8%).⁴² The observed difference could be due to the difference in the study period as the attitude may change when time goes.

The odds of blood donation practice were 5 times more likely for those students who know the blood bank location in the current or previous residential area as compared to their counterparts. This is in line with the study conducted in Kampala that the availability and accessibility of blood donation centers have association with blood donation practice.⁴³ This could be due to the fact that knowledge of blood bank location will make the students become aware of where they should donate blood, and lacking the opportunity will also decrease motivation to donate blood. In another way, the practice of blood donation could lead the students to know the location of blood bank. Hence, it might be difficult to say whether this association is causal as this study is limited due to its cross-sectional nature of study design.

The odds of blood donation practice were 2 times more likely for those students who were knowledgeable compared to their counterparts. Besides, students having favorable attitude toward blood donation were also found to practice blood donation 2 times more likely than students who have unfavorable attitude. This finding was supported by the studies conducted in other parts of Ethiopia,^{3,24,25,39} and Kenya.⁴⁴ This could be explained by the fact that both knowledge and attitude are important precursors for the behavioral change and improved practice.

The odds of blood donation among students who did not participate in blood donation campaign were 71.5% less likely as compared to those participated in the campaign. This is in agreement with the study conducted in Bale Robe town.²⁷ The possible reason to this association is that taking part in blood donation campaign can make the students aware about the importance of blood donation. In turn, it might increase the students' motivation to donate blood.

Surprisingly, the likelihood of practicing blood donation was more likely for students from the Institute of Language Studies and Journalism than health science students. The possible justification could be that health science students stay frequently out of the campus for apparent ships. They could miss the blood collection campaign in the campus. Besides, the students in the Institute of Language Studies and Journalism might donate for relatives/replacement since this study

did not differentiate the blood donation practice into its categories (voluntary non-remunerated, replacement/relatives and paid donors). The association was checked for overall ever blood donation practice.

Limitation

The cross-sectional nature of the study could not ascertain the cause–effect relationship. Although the sample size was predetermined, it might be small and could affect the generalizability of this finding. Besides, the risk of recall bias would not be ruled out.

Conclusion

The 40.8% lifetime blood donation practice in this study was low. Particularly, the voluntary non-remunerated blood donation (29%) is less than the WHO recommendations of 100% non-remunerated voluntary blood donation practice by youngsters.³⁴ This low level of blood donation could result from lack of awareness regarding blood donation benefits, poor understanding of the impact of blood scarcity, and inadequate mobilization. College/institute of the students, lack of knowledge, poor attitude, and blood donation campaigns were found to be the factors influencing blood donation practice.

All health institutions whether private or governmental including the Wollega University Referral Hospital, Nekemte Blood Bank, Red Cross Society Nekemte branch, health bureaus in Nekemte town, and the university must work in coordination to increase the level of knowledge and to shift the attitudes towards positive regarding blood donation. On top of this, it is better if blood donation club is established in the campus in order to facilitate the awareness creation activities and to increase the number of regular voluntary blood donors. Periodic campaigns for community mobilization need to be strengthened to improve the low blood donation practice particularly to increase and sustain the number of regular blood donors. It would also be better if incentives like t-shirts and certificates were given to regular donors. To make willful contribution for life saving activities, it is better if the students engage actively in the blood donation campaigns and also donate their blood voluntarily.

Data Sharing Statement

Data are available on reasonable request from corresponding author.

Ethics Approval and Informed Consent

The study was conducted in accordance with the Helsinki declaration, and ethical approval was obtained from the Institutional Review Board (IRB) of Wollega University. Cooperation letter was written from Wollega University, Institute of Health Sciences. The purpose of the study was clearly explained to the respondents, and they were told that the confidentiality is kept strictly. The respondents were also told that they have the right to involve or not at all in the study. Finally, informed, voluntary, written and signed consent was secured from the study participants and no personal detail was needed to record on the questionnaire.

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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.

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Markers of Renal Complications in Beta Thalassaemia Patients with Iron Overload Receiving Chelation Agent Therapy: A Systematic Review

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Markers of Renal Complications in Beta Thalassemia Patients with Iron Overload Receiving Chelation Agent Therapy: A Systematic Review

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Objective: The emerging renal complications in beta-thalassemia patients have raised the global exchange of views. Despite better survival due to blood transfusion and iron chelation therapy, the previously unrecognized renal complication remain a burden of disease affecting this population—the primary concern on how iron overload and chelation therapy correlated with renal impairment is still controversial. Early detection and diagnosis is crucial in preventing further kidney damage. Therefore, a systematic review was performed to identify markers of kidney complications in beta thalassemia patients with iron overload receiving chelation therapy.

Methods: Searches of PubMed, Scopus, Science Direct, and Web of Science were conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) to identify studies of literature reporting renal outcome in β -TM patients with iron overload and receiving chelation therapy. The eligible 17 studies were obtained.

Results: uNGAL/NGAL, uNAG/NAG, uKIM-1 are markers that can be used as predictor of renal tubular damage in early renal complications, while Cystatin C and u β 2MG showed further damage at the glomerular level.

Discussion and Conclusion: The renal complication in beta-thalassemia patients with iron overload receiving chelating agent therapy may progress to kidney disease. Early detection using accurate biological markers is a substantial issue that deserves further evaluation to determine prevention and management.

Keywords: thalassemia, iron overload, chelating agent, kidney, health

Introduction

Beta thalassemia is one of the most frequent haemoglobin disorders inherited in an autosomal recessive manner bringing about worldwide health problems, especially in the tropical belt. Southeast Asia accounts for about 50% of thalassemia carriers in the world. Beta-thalassemia results from reduced or lost-globin chain synthesis due to mutations in the beta-globin gene.^{1,2} Indonesia, with more than 200 million people, has a beta thalassemia carrier frequency of 6–10%, becoming an issue to anticipate by the government. The high demand for blood transfusions and chelating therapy marks their linear effect on the improved prognosis of beta-thalassemia patients.²

Despite a better survival rate due to supportive therapy, the beta-thalassemia population still have an increased risk for various complications and thus remains a challenge in affecting their quality of life. The four most common complications are cardiac, endocrine, hepatic, and renal.^{3–7} The emerging renal complications reported in beta-thalassemia patients have been correlated with the nature of the disease (eg, chronic anemia, hypoxia), iron overload due to regular blood transfusions, and chelating therapy. Altered vascular resistance and increased renal plasma flow, hyperfiltration, and renal tubular dysfunction were some common mechanisms in diminished renal function of beta-thalassemia patients. Iron overload beta-thalassemia patients were found to have distinct markers of kidney injury such as serum beta2-M, urinary calcium/creatinine, urine 2-M/creatinine, urinary NAG, urinary NAGL, urinary α 1-microglobulin, and urinary RBP.^{8–11} Moreover, renal complications also occurred significantly in those who received chelating agent therapy.^{3,10,12–14} This study aims to identify markers of renal complications in beta-thalassemia patients with iron overload and chelation agent therapy.

Methods

This study was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Literature Search

A comprehensive search of the electronic literature was done using the online databases PubMed, Scopus, Science Direct, and Web of Science. We used the following search term: ([MeSH]beta-Thalassemia] OR (thalassemia) OR (thalassemia beta major) AND (renal outcome*) OR (renal complication*) OR (renal function*) OR (renal damage*) OR (kidney injury*) OR (kidney function*) from the Cochrane Collaboration's search strategy for randomized control trials. Our search approach does not use a filter and has no year restrictions. The first search on each database was done in March, and the final search was done on May 29, 2022. Figure 1 contains the PRISMA flow for literature search.

Eligibility and Study Selection

All randomized control trials, cohort studies, case-control studies, and cross-sectional studies reporting renal outcome in patients with TM connected to chelation therapy were considered. We only list publications written in English. Unrecoverable full text studies and duplicate research were eliminated. Authors EP and EDA carried out the search. The full text of any articles that satisfied the inclusion criteria was retrieved by three writers (PZR, SDS, and AEP) after they separately screened the title and abstracts. The eligibility of full text articles was examined by both writers. The Mendeley program, a free online tool for managing references, was used during the selection process to eliminate duplicate studies and analyze the abstract and full text.

Data Extraction

Data extraction was carried out by two reviewers (BAM, CW), and any differences were settled by team consensus. Using an excel predesign table, the studies that satisfied the relevance and eligibility of our aforementioned criteria were extracted. All of collected data were: 1) A summary of the studies that were included, including methodological information about the site, sample, interventions, outcomes, and results. 2) Baseline features of the studies. 3) Renal outcome or function related to the previously described use of chelation therapy.

Results

We finally screened and obtained 17 studies of literature comparing β -TM with iron overload and with chelation therapy. We summarize them in Tables 1 and 2. From this table, 3 studies stated that there were significant differences in uNGAL/NGAL,^{10,11,15} and 5 studies stated that there were significant differences in uNAG/NAG,^{11,16–19} 2 studies revealed significant differences in uKIM-1,^{3,10} 5 studies stated significant differences in Cystatin C,^{20–24} 3 studies showed

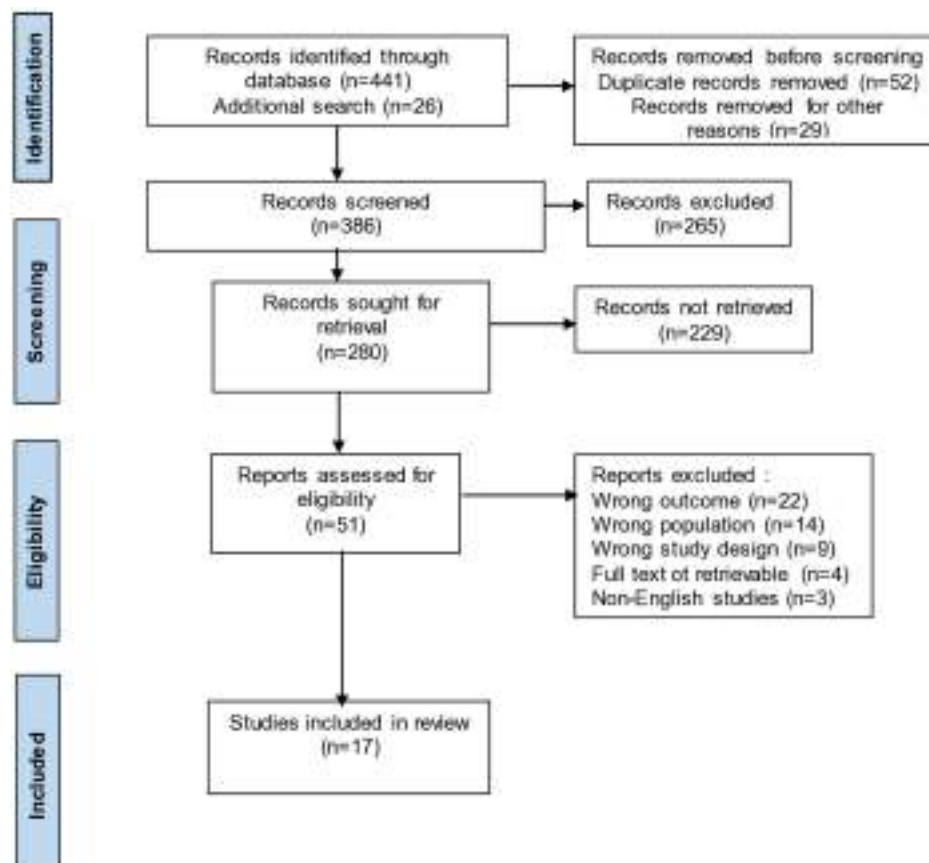


Figure 1 PRISMA flow chart.

a significant difference in $u\beta_2MG^{20,23,25}$ in beta thalassemia patients with iron overload and receiving chelating agents. Increase in $uNGAL/NGAL$; $uNAG/NAG$; $uKIM-1$; Cystatin C; This $u\beta_2MG/\beta_2MG$ is associated with early tubular and glomerular dysfunction. Three studies suggest that deferasirox is a chelating agent that causes an increase in $uKIM-1$, $uNGAL$, and $u\beta_2MG$.^{3,10,23}

Table 1 Chelation Therapy and Marker Reported from Study of Beta Thalassemia

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
I	Aldudak 2000 ¹⁷	2000	Cross-sectional	9.6 ± 5.0 ; 10 ± 4.7	Deferoxamine (70)	BUN
						S Cr
						S Na
						S K
						S Ua
						S Phosp
						Ccr

(Continued)

Table I (Continued).

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
						TRP
						uVolume
						uOsmolality
						uP/Cr
						uNAG/Cr
						uMDA/Cr
2	Smolkin 2008 ¹⁶	2008	Cross-sectional	10.8 ± 6.2; 11.2 ± 3.4; 10.2±4.5	Deferoxamine (n/a)	BUN
						Serum creatinine
						Serum uric acid
						GFR
						FENa
						FEK
						Urine Ca/Cr
						Uric acid excretion
						Tubular phosphorus reab
						Urine osmolality
						uNAG
						uNAG/Cr
3	Economou 2010 ²⁵	2010	Case-control	13.66 ± 5.11; 12.66 ± 4.2	Deferasirox (28) group A	Serum creatinine
					Deferiprone + deferoxamine (14) group B	Urea
						Cystatin-C
						U Protein
						U Ca
						U FeNa
						u Phospor, rabsorbtion
						uβ2 MG
						CrC
						eGFR

(Continued)

Table I (Continued).

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
4	Hamed 2010 ⁵	2010	Case-control	8.72±3.7; 8.4±4.1	Deferoxamine (34);	Cystatin-C
					w/o chelation (35)	Creatinine
						eGFRSchwartz
						Uric acid
						Calcium
						Inorganic phosphate
						Total antioxidant capacity
						Albumin/Cr
						NAG/Cr
						β2MG/Cr
						Inorganic phosphorus
						MDA/Cr
5	Mansi, 2013 (belum ada)	2013	Case-control		Deferoxamine (42)	Glucose
						Urea
						Creatinine
						Uric acid
						Sodium
						Potassium
						Chloride
6	Ali 2014 ²¹	2014	Cross-sectional	9.67± 1.35; n/a	Deferoxamine (62) group IA	Cystatin-C
					No chelating (38) group IB	Serum creatinine
					Control (50) group II	eGFRSchwartz
						CrC
						Albumin/Cr
7	Sen 2015 ¹¹	2015	Cross-sectional	9.14 ± 4.4; 8.8 ± 4.0	Deferasirox (59)	U Na/Cr
						U K/Cr
						U Ca/Cr
						U P/Cr
						U Mg/Cr
						U Protein/Cr

(Continued)

Table I (Continued).

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
						U Uric acid/Cr
						uNAG/Cr
						uNGAL/Cr
						uKIM1/Cr
						uL-FABP/Cr
8	Behairy 2017 ²⁴	2017	Case-control	10.41±3.86, 8.6±3.47	Deferoxamine (60%)	Urea
					Deferasirox (32.9%)	Creatinine
					Hydra (7.1%)	Urea/Cr
						Urine volume
						CrC
						Cystatin-C
						β2MG
						uAlbumin/Cr
						eGFRSchwartz
9	Bekhit 2017 ¹⁹	2017	Case-control	8.5 ± 3.5; n/a	Deferasirox (21)	S Urea
					Deferiprone (8)	S Cr
					Deferoxamine (3)	U Creatinine
						U Ca/Cr
						U uric acid
						GFR
						uNAG
10	Annayev 2018 ²³	2018	Case-control	18.4 ± 11.8; n/a	Deferasirox (38)	FENa
					Deferiprone (8)	Na
					None (4)	K
						Ca
						P
						Mg
						U Ca/Cr
						Urea
						Creatinine

(Continued)

Table 1 (Continued).

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
						Albumin
						GFR based on age
						SCystatin-C
						uβ2 MG
11	ElAlfy 2018 ²⁰	2018	Cross-sectional	12.8 ± 3.2	Deferoxamine (2)	Urea
					Deferasirox (9)	Uric acid
					Deferiprone (3)	Serum creatinine
					Combined (36)	Cystatin-C
						Urinary β2 microglobulin
						uAlbumin/Cr
						uβ2 M/albumin
12	Badelli 2019 ¹⁰	2019	Case-control	n/a	Deferoxamine (19)	GFR
					Deferasirox (21)	BUN
						Creatinine
						Cystatin-C
						GFR based on Cystatin
						uNGAL
						uKIMI
						uL-18
						uCreatinine
						uAlbumin
						uNGAL/Cr
						uL-18/Cr
						uKIMI/Cr
						uAlbumin/Cr
13	Fouad 2019 ¹⁵	2019	Case-control	29±9; 28±5	Deferasirox (11),	Albumin/Cr
					Deferiprone(3)	eGFR
						NGAL
						uNGAL/Cr
14	Nafea 2019 ³	2019	Cross-sectional	14.7 ± 4.3	Deferasirox (26)	Serum K
					Desferoxamine (14)	Serum Na
					Deferiprone(26)	Serum Ca total

(Continued)

Table I (Continued).

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
						Seurm P
						Serum Mg
						Serum creatinine
						U K/Cr
						U Na/Cr
						U P/Cr
						U Mg/Cr
						Urinary creatinine
						eGFR
						U KIM-1/Cr
15	Bilir 2020 ²²	2020	Cross-sectional	12.63 ± 4.58; 11.44 ± 4.37	Deferasirox (47)	Creatinin
						S Sodium
						S Potassium
						S Calcium
						S Phosphorus
						eGFR
						Cystatin-C
						Total antioxidant capacity
						Total oxidant capacity
						U Ca/Cr
						U Protein/Cr
						uβ2MG/Cr
						uRBP/Cr
						uNAG/Cr
						MDA/Cr
16	Capolongo 2020 ⁴⁵	2020	Case-control	34 ± 12; 33 ± 14	Deferoxamine (40)	Serum creatinine
						eGFR
						S Na
						S K
						S Ca
						S Chlorine
						S Bicarbonate

(Continued)

Table 1 (Continued).

No.	Authors	Year of Publication	Study Type	Age (Years)	Chelation of Therapy (Number of Patients)	Markers Reported
						FENa
						U Ca
						U Phosp
						uAlbumin/Cr
						uOsmolarity
						uOsmolality
						pH
17	Mahmoud 2021 ⁴²	2021	Case-control	9.58±4.07	Deferasirox (100)	Urea
						Creatinine
						eGFR
						U protein/Cr
						UNa/Cr
						UK/Cr
						UCa/Cr
						Uuric acid/Cr
						uNAG
						uNAG/Cr
						uKIMI
						uKIMI/Cr
						SCystatin-C
18	Tanous 2021 ¹⁸	2021	Cross-sectional	20.92± 9.7	Deferasirox (26)	Serum creatinine
					Deferiprone + deferoxamine (6)	Serum Na
					Deferoxamine (4)	Serum K
						Serum uric acid
						eGFR
						uNAG <12
						Abnormal uNAG (>12)
						Abnormal urine Ca/Cr (>0.14)
						Urine osmolality

Table 2 Result and conclusion from Study of Beta Thalassemia with Iron Overload and Receiving Chelation Agent Therapy

No	Authors	Results-Conclusions
1	Aldudak 2000	Administration of chelating agents in beta thalassemia patients actuates a damage to the proximal tubular kidney.
2	Smolkin 2008	In beta thalassemia patients, UNAG and UNAG/creatinine ratio markers can be used as markers to determine the frequency of transfusion and the effect of chelating agent therapy on ferritin levels.
3	Economou 2010	Glomerular and tubular dysfunction in iron overload beta thalassemia patients receiving chelating agents was observed through the elevation of cystatin C, proteinuria, hypercalciuria and beta2-microglobulin.
4	Hamed 2010	Chelating agents in beta thalassemia patients have been shown stimulate glomerular and tubular dysfunction.
5	Smolkin 2008	In beta thalassemia patients, UNAG and UNAG/creatinine ratio markers can be used as markers to determine the frequency of transfusion and the effect of chelating agent therapy on ferritin levels.
6	Mansi 2013	Kidney damage occurs in beta thalassemia patients receiving chelating agents characterized by increased urea, creatinine, uric acid, urinary sodium and potassium.
7	Ali 2014	Elevated cystatin C, serum creatinine, and serum ferritin and high uACR indicate kidney damage in beta thalassemia
8	Sen 2015	NAG and NGAL can be used as markers of renal injury in beta thalassemia patients experiencing iron overload and receive therapeutic chelating agents.
9	Behairy 2017	Cystatin-C and beta-2 microglobulin are specific and sensitive early biomarkers for monitoring glomerular and tubular dysfunction in children with beta-TM.
10	Bekhit 2017	NAG is excreted following renal damage, hence used as marker of the toxicity index in beta thalassemia patients receiving chelating agents.
11	Anayev 2018	Kidney damage was concluded after an increase in Cystatin C and beta microglobulin in beta thalassemia patients receiving iron chelating agents.
12	ElAlfy 2018	Iron overload in beta thalassemia patients with long-term use of chelating agents revealed differences in serum cystatin C, uACR, and urinary B2 microglobulin, and B2 microglobulin/albumin ratio values.
13	Badeli 2019	Administration of deferasirox led to kidney damage, characterized by an increase in uNGAL, inflammatory factors, sort of IL18, in beta thalassemia patients.
14	Fouad 2019	uNGAL levels, uNGAL/creatinine ratio, eGFR and urinary albumin creatinine ratio were distinctive between group with iron overload and added chelating agents.
15	Nafea 2019	UKIM-I is a biomarker used to detect worsening eGFR in beta-thalassemia patients with iron overload and routine deferasirox.
16	Bilir 2020	Iron overload marked by high serum ferritin, is associated with proteinuria, increased cystatin C, and urinary protein/Cr in beta thalassemia patients routinely receiving chelating agents.
17	Capolongo 2020	The administration of chelating agents in patients with beta thalassemia iron overload induced renal tubular damage and required early detection to prevent further kidney damage.
18	Mahmoud 2021	Serum cystatin-C is a good predictive marker in the evaluation of glomerular dysfunction. Urine concentrations of NAG and KIM-I represent sensitive, specific, and highly predictive early biomarkers for acute renal injury in patients with beta TM when subclinical kidney injury or dysfunction is expected before serum creatinine increases.
19.	Tanous 2021	The use of deferasirox for approximately 10 years induced the decline of eGFR and was negatively correlated with uNAG in beta thalassemia patients with iron overload.

Discussion

uNGAL/NGAL

The proximal tubule, distal tubule, and loop of Henle segments all contain epithelial cells that express the 25-kDa lipocalin iron-carrying protein known as neutrophil gelatinase-associated lipocalin (NGAL), which is released by active neutrophils.

Detection of NGAL can be done through serum or urine withdrawal. However, in renal tubular damage, NGAL is often upregulated.²⁶ NGAL is a low-concentration protein that binds iron-siderophore complexes and is found in a variety of cell types. NGAL is discharged in urine when proximal tubular damage inhibits the reabsorption or increase the synthesis. An abrupt increase in NGAL is brought on by acute kidney injury (AKI). High NGAL levels are also present in patients with other abnormalities, such as lupus nephritis, immunoglobulin A nephropathy, and urinary tract infections.^{27,28} Significant relationships between urinary NGAL (uNGAL) and proteinuria were documented in chronic kidney disease.²⁹

Recent case-control studies revealed that β -TM patients receiving deferasirox and regular blood transfusions had considerably greater levels of uNGAL and NGAL.⁹ It is interesting to note that in β -TM patients, combined values of albumin/creatinin ratio and the uNGAL/creatinin ratio may be a more accurate predictor of kidney impairment and taken into account as potential indicators of renal failure. Additionally, as previously demonstrated, uNGAL somehow can predict renal function since it can estimate eGFR, evaluate the course of CKD, and serve as a surrogate measure for baseline eGFR.³⁰

NGAL is highly associated with early renal complication regardless of the creatinine level, both in major and intermediate thalassemia.³¹ In both young and adult beta-thalassemia patients, NGAL level elevates due to iron overload and prolonged use of chelating agents. Since renal injury might elevate NGAL to a significant level, it is wisely advised to routinely monitor the NGAL level. Another highlighted feature of NGAL is that it immediately elevates right after kidney injury, even before serum creatinine, urinary N-acetyl glucosaminidase, and 2-microglobulin levels are detected.

uNAG/NAG

Tubular damage is indicated by an increase in the level of N-acetyl-beta-d-glucosamine (NAG), a well-known biomarker for proximal tubular injury.³² According to Aldudak et al, people with beta thalassemia had higher levels of the tubular damage indicators, such as NAG, malondialdehyde, and b2-microglobulin excreted in their urine.¹⁷ In addition, Jalali et al showed that β -TM patients had significantly higher urine NAG levels than controls, with high NAG levels being the norm in most cases.³³ Besides, participants in Sen et al's study who had renal proximal tubular damage also showed significantly elevated levels of uNAG/Cr and uNGAL/Cr.¹¹

The proximal tubular dysfunction that results from thalassemia itself may be caused by chronic hypoxia brought on by persistent anemia, by iron deposition, or by iron chelation.^{4,19,23,34} According to some studies, the absence of a link between urine indicators and hemoglobin, haematocrit and ferritin levels may be due to very early tubular dysfunction, as seen by the rise of only NAG and NGAL, but not KIM-1, L-FABP, or urinary electrolytes.^{11,19,34,35}

uKIM-1

A transmembrane protein called urinary human kidney injury molecule-1 (uKIM-1) is found in the proximal renal tubules within 24 hours of acute tubular necrosis after renal ischemia. Even while serum creatinine concentrations are unaffected by exposure to certain nephrotoxic drugs, urinary KIM-1 may still be detected.³⁶

According to the findings of the Nafea et al study, young thalassemia patients receiving deferasirox therapy had evident subclinical nephrotoxicity when compared to patients receiving other chelation therapies. This was demonstrated by the statistically significantly higher levels of serum creatinine, eGFR, and UKIM-1/Cr. The serum levels of phosphorus, magnesium, creatinine, and blood sugar all showed a significant positive association with UKIM-1/Cr, however the blood hemoglobin level showed a significant negative correlation.³

According to Badeli et al's study results, the deferasirox group had significantly greater levels of uIL-18, uNGAL, uNGAL/CREA, uKIM-1/CREA, and BUN than the control group.¹⁰ Deferasirox treatment led to partial necrosis in the renal tubules and increased urinary NGAL, Cystatin C, KIM-1, protein, and glucose production, as demonstrated by Sánchez González et al in an animal study.³⁷

Cystatin C and u β 2MG

Cystatin C, not excreted by the renal tubules or reabsorbed into the serum, is a sensitive biomarker for glomerular filtration rate (GFR). All cells in the body continuously produce cystatin C and its production is unaffected by changes in age, sex, gender, or muscle mass. Cystatin C is a reliable and early marker of glomerular dysfunction in the pediatric

population.^{22,36,38} A low-molecular-weight protein called urinary $\beta 2$ microglobulin (u $\beta 2$ MG) is freely filtered by glomeruli, reabsorbed by renal tubules, and then eliminated. Due to its continual production, both are thought to be a more reliable endogenous measure of early glomerular filtration rate (GFR) affection than creatinine.³⁹ For monitoring glomerular and tubular dysfunction in kids with β -TM, $\beta 2$ MG is a sensitive early biomarker.^{23,24} $\beta 2$ MG levels are very low in healthy people; it rises in inflammatory, immunologic, and cancerous conditions.³⁶ Other than that, Cystatin C and $\beta 2$ MG have strong correlation with age and creatinine clearance.⁴⁰ Early detection of glomerular disease will decrease the rate of renal failure and mortality.

Referring to a study by ElAlfy et al, thalassemia patients had significantly greater serum levels of cystatin C than healthy controls.²⁰ Positive correlations were found between serum cystatin C and indirect bilirubin, LDH and serum ferritin. Additionally, there was no connection between any of these indicators and the kidney function tests (serum creatinine, urea, and uACR) or between serum cystatin C and u2MG.²⁰ Elbedewy et al⁴¹ and Mahmoud et al⁴² showed a significant positive correlation between cystatin-C and serum ferritin and negative correlation with eGFR.

Behairy et al discovered that serum cystatin C and u $\beta 2$ MG were negatively correlated with creatinine clearance, hemoglobin, and estimated GFR in children with β -TM while both markers were positively correlated with urea, creatinine, serum ferritin, UACR, duration of chelation therapy, and frequency of blood transfusion per year. As indicators for glomerular and tubular dysfunction, cystatin C and $\beta 2$ MG have good sensitivity and good specificity.²⁴

Cystatin C and $\beta 2$ M, as well as serum ferritin and liver iron deposition were found to be significantly positively correlated, according to Kacar et al.⁴³ Serum ferritin levels were discovered to be associated with cystatin C and $\beta 2$ M levels by Uzun et al⁴⁴ found that serum ferritin is correlated with level of cystatin C and $\beta 2$ M. The risk of glomerular and tubular dysfunction may increase with iron buildup in the body.

Conclusion

The renal complication in beta-thalassemia patients with iron overload receiving chelating agent therapy may progress to kidney disease. Early detection using accurate biological markers is a substantial issue that deserves further evaluation to determine prevention and management.

Ethics

This study was approved by Airlangga Hospital's ethical board with certificate number 024/KEP/2022. All analyses for the present study were based on previous published research, thus no patient consent was required.

Disclosure

The authors report no financial or other conflicts of interest in this review article.

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Practical Guidance for the Use of Voxelotor in the Management of Sickle Cell Disease

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Abstract: Sickle cell disease (SCD) is one of the most common inherited blood disorders. Deoxygenated hemoglobin S (HbS) polymerizes and causes anemia and various end organ effects. Voxelotor acts by increasing HbS oxygen affinity, decreasing anemia and hemolysis. Voxelotor is approved for use in individuals with SCD age 4 years and older. Phase 3 trials demonstrated an increase in hemoglobin levels and a decrease in markers of hemolysis; however, data on benefits related to clinical and quality of life outcomes are relatively limited and varied across different studies. This review summarizes the published clinical trials and research studies focused on the use of voxelotor in SCD to provide an evidence-based practical guide for hematology providers on its utilization in clinical settings, including physicians and independent licensed practitioners.

Keywords: sickle cell, sickle cell anemia, sickle cell disease, hemoglobinopathy, pediatric, children, adolescents, young adults, adult, voxelotor, oxbryta, disease-modifying therapy

Introduction

Sickle cell disease (SCD) is one of the most common inherited blood disorders affecting 100,000 individuals in the United States and millions worldwide.¹ A mutation in the beta globin chain of the hemoglobin creates hemoglobin S (HbS) and leads to a complex pathophysiology in SCD, including polymerization of HbS in the deoxygenated state. There are several clinical complications in SCD, such as anemia, vaso-occlusive pain episodes, acute chest syndrome, and various end organ damage, leading to decreased life expectancy.² Given that curative approaches, namely hematopoietic stem cell transplant and gene therapy or editing, though promising, are still not yet widely available, disease modifying therapies are and will likely remain critical for optimizing health outcomes in SCD.^{3,4}

With recent research advancements in the last decade, there are now four Federal Drug Administration (FDA) approved medications for SCD: hydroxyurea, L-glutamine, crizanlizumab, and voxelotor.³ In particular, voxelotor targets anemia and hemolysis in SCD, which plays a key role in the pathogenesis.² Voxelotor received initial FDA approval in November 2019 for individuals with SCD age 12 years and older, and recently received FDA approval for children with SCD age 4 years and older.⁵ Voxelotor binds to hemoglobin resulting in a decrease in HbS polymerization.^{6,7} Clinical trials demonstrated that voxelotor can effectively increase hemoglobin by 1 g/dL in approximately 50% of patients at 24 weeks with improvement in markers of hemolysis.^{8,9} With the recent expansion of FDA approval, practical guidance for use of voxelotor in SCD is essential to aid SCD providers considering this disease modifying therapy for their patients.

Methods

A literature search was conducted January 2017 to June 2022 through the PubMed database using key search terms: voxelotor, oxbryta, and GBT 440. Inclusion criteria were phase 3 clinical trials, cross-sectional studies, retrospective studies, and case series focused on the use of voxelotor in children, adolescents, and adults with SCD in English language. Exclusion criteria included non-English language, and phase 1 clinical trials or pre-clinical studies.

Results

Efficacy

HOPE and HOPE KIDS-1 Trials

Expected clinical efficacy is a key consideration in prescribing voxelotor for SCD. Clinical trials demonstrated statistically significant benefits related to hemoglobin levels and markers of hemolysis, while impact on clinical outcomes varied. The HOPE trial was a multicenter phase 3 randomized clinical trial of individuals with SCD age 12–64 years, mostly HbSS or HbS β^0 Thal. In the HOPE trial, voxelotor demonstrated a significant increase in hemoglobin by 1 g/dL in 51% of patients along with a decrease in markers of hemolysis, such as indirect bilirubin and reticulocyte.^{9,10} Similarly, the HOPE KIDS-1 trial showed a significant increase in hemoglobin by 1 g/dL in 47% of patients as well as decreased markers of hemolysis among children with HbSS or HbS β^0 Thal.⁸ Neither trial showed significant reduction in vaso-occlusive pain episodes or improvement in health-related quality of life outcomes.^{8–10} However, long-term follow up of the HOPE trial at 72 weeks showed less frequent vaso-occlusive episodes among individuals with SCD on voxelotor with higher hemoglobin response above 10 g/dL with an annualized incidence rate of vaso-occlusive crisis of 2.8 in the voxelotor 1500 mg group compared to 3.2 in placebo.¹¹ Post hoc analysis of the HOPE trial demonstrated voxelotor 1500 mg caused improvement or resolution of leg ulcers in all (5/5, 100%) patients with active leg ulcers.¹² Data for efficacy are primarily for individuals with severe phenotypes (ie, HbSS and HbS β^0 Thal), while other genotypes, such as HbSC and HbS β^+ Thal, were present in small numbers (combined approximately 10% of participants) in the HOPE trial and excluded in the HOPE KIDS-1 trial.^{8,10}

Real-World Evidence

A retrospective study of national claims data of 3128 individuals with SCD age 12 years and older demonstrated voxelotor impacted clinical outcomes, though these potential clinical benefits must be interpreted within the context of the inherent methodological limitations in retrospective studies.^{13,14} Voxelotor significantly decreased the mean annualized vaso-occlusive episode rate from 10.9 to 8.4 ($p<0.001$).¹³ The mean annualized rate of VOC hospitalizations and all-cause hospitalizations decreased from 10.9 to 8.4 ($p<0.001$), and from 7.4 to 4.6 ($p<0.001$), respectively. Voxelotor decreased blood transfusion requirements with a decrease in the mean annualized transfusion rate from 7.0 to 3.3 ($p<0.001$).^{13,14} The study also demonstrated a decrease in mean number of outpatient visits for individuals on voxelotor from 24.8 to 22.5 visits ($p=0.006$), and a decrease in the mean days supply of prescribed opioids from 312 to 272 days ($p<0.001$).¹³ This study suggested possible real-world evidence for clinical benefits related to the use of voxelotor beyond increasing hemoglobin levels.

Other reported beneficial effects of voxelotor include improvements in well-being, and symptoms of depression.^{15,16} A single center review of 77 individuals with SCD age 12 years and older demonstrated 67% of pediatric patients and 54% of adult patients on voxelotor had improved quality of life measured by the Patient Global Impression of Change and Clinical Global Impression of Change questionnaires.¹⁶ A case series of compassionate use of voxelotor in 7 individuals with SCD demonstrated that 2 of 2 patients with depression had improved affect measured by the Patient Health Questionnaire-9.¹⁵ Potential concerns about impact on exercise capacity due to decreased oxygen delivery to tissues was not seen in a pilot study of 9 individuals with SCD ages 12–20 years who had no significant difference in cardiopulmonary exercise testing.¹⁸ Research studies evaluating the impact of voxelotor on transcranial Doppler ultrasound velocities (HOPE KIDS-2) and other quality of life outcomes are ongoing.¹⁷

Adverse Events

HOPE and HOPE KIDS-1 Trials

Overall, voxelotor is well tolerated, and most side effects are minor, grade 1, or grade 2 as defined by the Common Terminology Criteria for Adverse Events (CTCAE). In the HOPE trial in individuals age 12–64 years, 34/88 (39%) of participants receiving voxelotor 1500 mg had 1 or more treatment related adverse events. Headache and diarrhea were the most common adverse events, occurring in at least 20% of patients receiving voxelotor 1500 mg daily.¹⁰ Other common side effects included nausea, abdominal pain, and rash.^{10,19} Dose modifications occurred in 41% (36/88) of individuals receiving voxelotor. Per protocol, dose reduction was indicated for grade 2 or higher adverse event. The dose was reduced by one 300 mg capsule/tablet and could be resumed to the original dose once the grade 2 adverse event resolved to $\leq$ grade 1.

The dose was held for up to five days for grade 3 or higher adverse event and could be resumed at the original dose when adverse event $\leq$ grade 1.¹⁰

The HOPE KIDS-1 trial demonstrated similar findings with the most common drug related adverse events including diarrhea, rash, and vomiting with grade 1 adverse events most common.⁸ Four patients required dose modification due to drug related adverse events. Dose reduction occurred in 1/45 (2%) of patients and was due to a grade 2 rash. Dose was reduced by one 300 mg tablet. Dose interruption without dose reduction occurred in 3/45 (7%) of patients in the HOPE KIDS-1 trial, one for grade 2 hypersensitivity, one for grade 2 liver enzyme elevation, and one for grade 3 liver enzyme elevation and bilirubin increase.¹⁰

Real-World Evidence

Case series of use of voxelotor in individuals with SCD demonstrate adverse events similar to those reported in the HOPE and HOPE KIDS-1 trials. A single center review of 77 individuals with SCD age 12 years and older demonstrated that 4 of 77 (5%) of individuals had adverse events that resulted in dose modification, including 2 diarrhea, 1 rash, and 1 fever.¹⁶ A case study reported rebound hemolysis and multi-organ dysfunction after abrupt discontinuation of voxelotor in a HbSS patient with pulmonary hypertension and renal disease.¹⁷ Although a rare complication, there are limited data on implications of abrupt cessation of voxelotor. This is relevant given voxelotor is not currently widely available in hospital formularies and medication continuation during hospital admission relies on patient supply of the medication from home. There are limited long-term follow up data as well as limited publications on use of voxelotor in certain subpopulations of individuals with SCD including those with multi-organ dysfunction or pregnant.

Initiating Voxelotor

Eligibility

Individuals with SCD age 4 years and older with severe phenotypes (HbSS and HbS β^0 Thal) are eligible for use of voxelotor. It can be considered as an added therapy to hydroxyurea or alternative if hydroxyurea is ineffective, not tolerated, or preferred by patients or parents. Individuals age 12 years and older with milder genotypes, such as HbSC or HbS β +Thal with baseline hemoglobin <10 g/dL and/or episodes of vaso-occlusive episodes may be considered for voxelotor, consistent with the HOPE trial, but data are limited.¹⁰ Patients on concomitant hydroxyurea may be started on voxelotor with data supporting use in patients on stable hydroxyurea dose for at least 3 months.^{8,10} Combination therapy with voxelotor and other SCD disease modifying agents may provide the ability to target the complex pathophysiology of SCD via different pathways, but data on its use with crizanlizumab and L-glutamate are limited.¹³ Further, patients' pill or medication burden should be considered to ensure optimal adherence level over time to one or more therapies, which can be a challenge in clinical practice. In a retrospective study of 3128 individuals with SCD age 12 years and older on voxelotor, patients were also treated with other therapies, including hydroxyurea (57%), 7% L-glutamine (7%), and crizanlizumab (1%).¹³

Figure 1 illustrates a suggested algorithm for practical considerations for use of voxelotor in SCD. Potential candidates for voxelotor include SCD individuals with hemoglobin ≤ 10.5 g/dL and those with continued anemia despite maximum tolerated dose of HU, especially those with symptomatic anemia.^{8,10} Individuals who are non-responsive or have adverse side effects to hydroxyurea or other disease modifying sickle cell therapy may also be considered. SCD patients with Hb <10.5 g/dL and red blood cell alloimmunization may also be considered as an increase in hemoglobin may be desired, particularly if there is difficulty locating compatible blood for transfusion needs.

Ineligible patients for voxelotor include individuals with SCD less than age 4 years. Some clinical conditions may represent a challenge, such as short gut syndrome or other complex gastrointestinal history, leading to potential inability to absorb the medication, given the medication is given orally and a parental method of administration is not available. Individuals with liver or kidney impairment require special consideration and may require dose adjustments given the role of the kidneys in drug excretion and potential for liver toxicity.

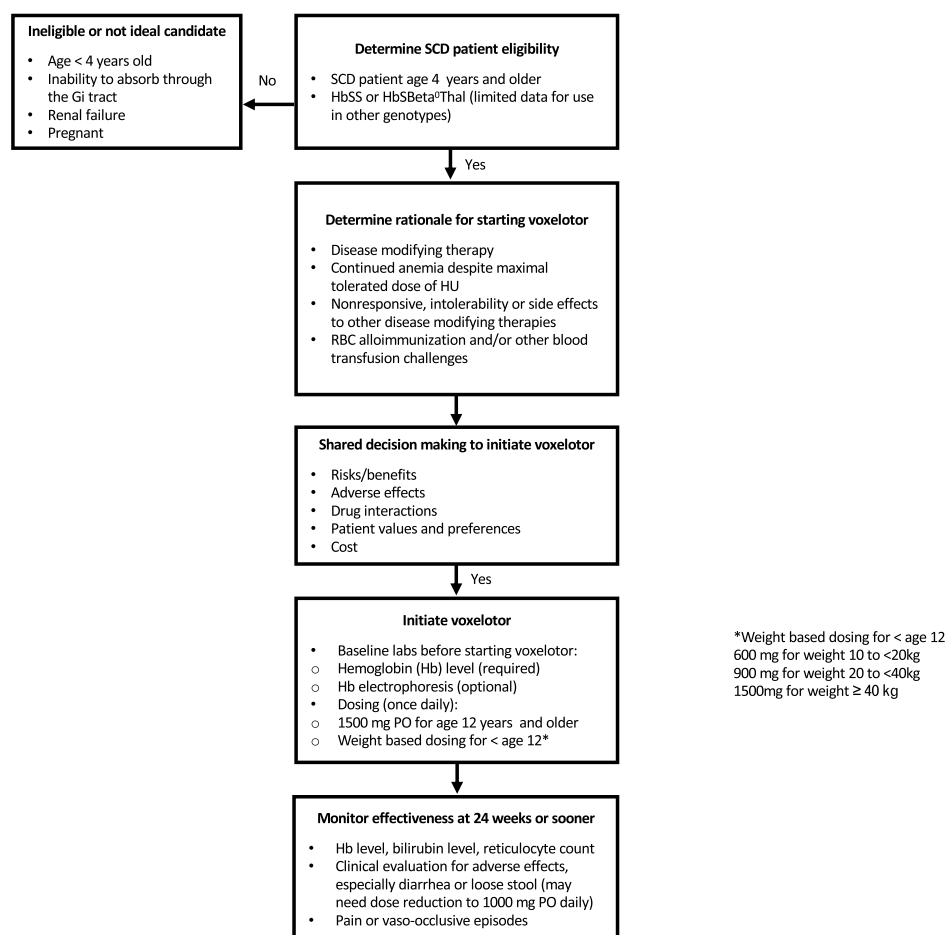


Figure 1 Suggested decision algorithm for considering initiation of voxelotor in SCD.

Medication Administration, Dosage, and Monitoring

Voxelotor is a once daily oral medication available as tablets and tablets for oral suspension. The starting dose for individuals age 12 years and older is 1500 mg once daily. Children age 4–12 years use weight based dosing including: 600 mg for weight 10 to <20 kg, 900 mg for weight 20 to <40 kg and 1500 mg for weight ≥40 kg.^{8,20} For oral suspension, the tablet can be mixed with a room temperature clear drink as detailed by the manufacturer.²⁰ Medication can be taken with or without food. Dose adjustments are indicated for patients with diarrhea or loose stools, severe hepatic impairment, or those on concomitant CYP3A4 inducers. Voxelotor has a half-life of 35.5 hours and reaches steady state in eight days.^{21,22} Time to response in the phase 3 clinical trials was 24 weeks but response may be observed earlier and response has been documented as early as 2 weeks in clinical trials.⁸

Dosage information for certain medical conditions and comorbidities in SCD is limited. Voxelotor has not been extensively studied in individuals with SCD and renal failure. The HOPE trial excluded patients with severe renal impairment and individuals on dialysis.¹⁰ A small retrospective study showed a decrease in albuminuria in individuals with SCD and chronic kidney disease stage 1–3 but was not statistically significant.²¹ Voxelotor has not been studied in pregnant women and there are no dosage recommendations in this population.

Laboratory evaluation of hemoglobin, reticulocyte count, and liver function are recommended to assess for treatment response and adverse effects.²³ Voxelotor may interfere with hemoglobin electrophoresis and discussion with a hematopathologist about the potential impact on hemoglobin electrophoresis results in these patients is recommended.^{24,25} Supporting laboratory results for voxelotor efficacy include improved hemoglobin and a decrease in markers of hemolysis, such as indirect bilirubin and reticulocyte count.⁹ Moreover, evaluating hemoglobin response or

increase with trend over time can be helpful for patients to reinforce adherence behavior and for insurance to maintain coverage.

Dose modification is generally recommended for treatment related adverse event $\geq$ grade 2 per the HOPE and HOPE KIDS-1 trial as well as recommendations from GBT.^{8,10} In individuals age 12 years and older, there is temporary dose reduction by one 500 mg tablet to 1000 mg daily or dose interruption based on the severity of the adverse effect. Dose reduction in the HOPE KIDS-1 trial was a decrease in dose by one 300 mg tablet.

Special Considerations

There are reported concerns that voxelotor may limit oxygen delivery due to the increase in oxygen affinity. A theoretical model by Hebbel and Hedlund details how increased oxygen affinity may decrease oxygen delivery and functional oxygen content in the brain. Decreased cerebral oxygen delivery may increase risk for adverse cerebrovascular consequences in SCD.²⁶ This model has not been replicated in vivo and there were no reported cerebrovascular events in the clinical trials. Further research on the impact on cerebral hemodynamics and oxygen delivery is warranted.

Cost

Medication cost is important to consider when initiating voxelotor. As of November 2022, the cost is \$138.89 USD per 500 mg tablet and \$208.34 USD per 300 mg soluble tablet. A cost effective analysis by the Institute for Economic and Clinical Review found that voxelotor was not cost effective at the current price.²⁷ Further research regarding cost effectiveness of voxelotor is needed. Insurance coverage and financial assistance from the manufacturer Global Blood Therapeutics (GBT) may help address financial barriers.

Counseling Patients and Shared Decision Making

Shared decision making (SDM) can aid discussions on the initiation of voxelotor. Key components of SDM include a collaborative approach between the patient and clinicians resulting in a partnership to make medical decisions supported by evidence and aligned with the patient's preferences, values, and treatment goals.^{28,29} Published strategies on the use of SDM for voxelotor in SCD are limited. Clinical teams may consider referencing shared decision making practices for other sickle cell disease modifying therapies, such as hydroxyurea when utilizing SDM for voxelotor.³⁰ Adapting these strategies for voxelotor has not been validated but may help facilitate open communication about the decision to initiate therapy.²⁹ A well informed discussion can be facilitated by a clinical team that is familiar with the clinical evidence as well as the expected benefit and risks of voxelotor. Key topics that should be discussed in SCD SDM aids include clinical efficacy, side effects, costs, and overall benefits while weighing the pros and cons of different potential disease modifying therapies.²⁹

Conclusion

Voxelotor is a well-tolerated oral agent in the era of multiple disease modifying agents for SCD. It can be considered as a component of multimodal treatment strategy to optimize outcomes in SCD. There are limited data to support its use as monotherapy. Randomized controlled trials demonstrated efficacy in raising the hemoglobin level and improving markers of hemolysis as well as a favorable safety profile. Data on the impact of voxelotor on clinical outcomes, such as pain episodes and quality of life, are less robust but suggest potential benefits related to reduction in pain episodes, hospitalizations, and blood transfusion requirements. Most data on the impact on clinical outcomes are limited to retrospective studies or case series which have limitations and are at risk for bias including confounding and selection bias. Further research is needed to assess the impact on clinical outcomes. Theoretical risks to oxygen delivery exist and warrant consideration and further research.³¹ Cost of the medication, insurance coverage, and limited current availability on hospital formularies warrant consideration.

Several clinical questions related to voxelotor remain unanswered. Efficacy in children with SCD less than 4 years old is currently being studied. Long-term data on the impact on end organ dysfunction are not yet available, and efficacy on neurovascular outcomes, including TCD velocities, is currently being studied. Further research on clinical outcomes is essential, needed to better support counseling of patients and families on voxelotor. Additional research on SDM tools

and resources for voxelotor in SCD may aid practical guidance and facilitate discussion with patients and families as they make their decisions among different disease modifying therapies.

Abbreviations

CTCAE, Common Terminology Criteria for Adverse Events; GBT, Global Blood Therapeutics; SCD, Sickle cell disease; SDM, Shared decision making; FDA, Food and Drug Administration; HbS β^0 Thal, Hemoglobin S Beta Zero thalassemia; HbS β^+ Thal, Hemoglobin S Beta Plus thalassemia.

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Disclosure

The authors report no relevant conflict or competing interests for this work.

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Alloimmunization to Rh Antigen (D, C, E, C, E) Among Pregnant Women Attending Antenatal Care in South Western Uganda

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Introduction: Maternal red cell alloimmunization to Rh antigen in pregnant women occurs when the immune system is sensitized by foreign red blood cell surface antigens, in this case fetal red blood cells, inducing an immune response. Various antigens of blood group systems may cause alloimmunization, especially the Rh, Kel, Fy, JK, and MNS systems. This study aimed to determine alloimmunization to the different frequencies of Rh antigen among pregnant women in South Western Uganda.

Methods: A total of 1369 pregnant women consented and were recruited into a cross-sectional study during their regular antenatal visits during the period August 2020 to July 2021. Samples (4 mL) of anticoagulated and coagulated blood were obtained, and Rh blood grouping including Rh antigen and the indirect antiglobulin test (IAT) was carried out using the agglutination technology of the LISS ID-Card technique in the Ortho Biovue ID-Micro Typing System.

Results: Out of 1369 participants recruited to the study, 78 (5.7%) were D⁻, 1291 were D⁺, and 134 (9.8%) had alloantibodies. Among those with alloantibodies, 115 (85.8%) were D⁺ and 19 (14.2%) D⁻. The percentage alloimmunization according to the Rh antigens was highest in e (9.72%), c (2.48%), C (2.34%) and E (0.94%) antigens. With the ABO system, alloimmunization was highest in blood group B (10.7%), followed by A (10.6%), O (9.2%) and then AB (7.1%). Alloimmunization was more prevalent in D⁻ (24%) than in D⁺ participants (8.9%). Rhesus antigen e was the most prevalent antigen (99.8%), followed by c. The alloimmunization rate of 9.8% among these participants is high, and appears in both D⁺ and D⁻ women. The other Rhesus antigens are seen to cause alloimmunization, with antigen e causing the highest prevalence. In conclusion, there is a need to identify antibodies and study the outcome for clinical significance, especially in D⁺ women, to facilitate proper pregnancy management.

Keywords: Rh alloimmunization, Rh antigens, hemolytic disease of the fetus and newborn, pregnant women, Western Uganda

Introduction

Antibodies of blood groups are immunoglobulins that are produced to react with corresponding antigens, mainly on the surface of red blood cells. These may be acquired naturally or through immunization in response to antigens present on red cells that are foreign for the individual.¹ The ABO blood group system was first described by Karl Landsteiner in 1900; since then, more than 60 different red blood cell antigens have been discovered and linked to eliciting an antibody response, which is commonly referred to as alloimmunization, with anti-D being the most commonly reported antigen.²

Alloimmunization is known to result from the genetic difference in red blood cell antigens between the fetus and the mother, or in patients with multiple transfusions. This alloimmunization occurs due to exposure of foreign red cell antigens inherited by the fetus from father to the antenatal immune system and subsequent sensitization. The Rhesus (Rh) antigen is the most common cause of this antibody response (alloimmunization), followed by ABO, while the Kidd, Kell,

and Duffy systems are less common causes.^{3,4} Exposure during gestation occurs as a result of the transplacental crossing of fetal red blood cells during delivery, miscarriage, or ectopic pregnancy, or when an invasive procedure such as amniocentesis is carried out.¹ Age, gravidity/parity, and poor obstetric/gynecological history are other factors that are linked to alloimmunization in pregnant women.

Rh alloimmunization and hemolytic disease of the fetus and newborn are closely linked; this was first described by Levine and later by Louis.⁵ The antigens that are commonly linked with perinatal hemolytic disease of the fetus and newborn are mainly of the Rh system, and include D, C, E, c, e, f, and Cw, among others. ABO antibodies are mainly of the IgM type and do not commonly cause hemolytic disease of the fetus and newborn, as they cannot cross the placenta, but ABO-related hemolytic disease of the fetus and newborn has been reported to occur in group O mothers with high titers of naturally occurring IgA anti-A or B. Hemolytic disease of the fetus and newborn commonly follows antigenic exposure by transfusion, or fetomaternal hemorrhage or leakage.^{1,6}

In South Western Uganda, the distribution of the ABO and Rh antigens has been determined, with O (49.4%) being the highest in the ABO system and e (99.8%) antigen the most common in the Rh antigen system, but the prevalence of Rh alloimmunization is not known.⁷ The prevalence of alloantibodies and related hemolytic disease of the fetus and newborn varies from population to population, and there are very limited data on this subject in this part of the country. Determination of the prevalence of maternal alloimmunization to Rh antigens in both the D⁺ and the D⁻ populations of pregnant women during the antenatal period is key in the control of hemolytic disease of the newborn.

During antenatal visits, Rh alloimmunization is not routinely screened for and antigen typing is restricted to D phenotype screening only. Young women of child-bearing age with recurrent pregnancies in South Western Uganda are at higher risk of developing hemolytic disease of the newborn as a result of antibodies against non-screened Rh antigens, which can be fatal.⁸ This study aimed to obtain information on the prevalence of alloimmunization and how it is distributed within the different Rh blood phenotypes among pregnant women, to provide a baseline for policy information and future planning towards pregnancy management, as a way of preventing hemolytic disease of the newborn.⁹⁻¹¹

Materials and Methods

Between August 2020 and July 2021, a cross-sectional, descriptive study was conducted in which a total of 1396 pregnant women attending an antenatal clinic were recruited after providing their written informed consent. Samples (4 mL) of anticoagulated and coagulated blood were collected. Screening for Rh antigen and detection of the presence of alloantibodies was carried out using the direct antigen grouping and indirect antiglobulin test (IAT), both employing the agglutination technique, in which glass beads and reagents are contained in a column of gel to capture agglutinates in a semi-solid medium. These tests were performed using the Ortho Biovue ID-Micro Typing System (Ortho Clinical Diagnostics, Raritan, NJ, USA).

Internal quality control was achieved by including both known positive and negative controls, in accordance with the relevant guidelines for quality assurance. The study identification number and individual initials were used to identify ID-Cards. The aluminum foil was removed, then 50 µL cell suspension of 0.8% concentration was added, followed by 40 µL of the participant's sample, to the respective microtubes, and incubated at 37°C for 10 minutes. ID-Cards were then centrifuged for 15 minutes and visually inspected for agglutination.

Results

In total, 1369 pregnant women fulfilling the eligibility criteria were recruited into the study. The maternal age ranged from 16 to 45 years, with a mean age of 26 years. Most of the study population (74.7%) were less than 30 years of age. There were 592 primigravida (G0) women (43.8%) and the rest were multigravida (G2 to G5+) (Table 1).

Out of the 1369 pregnant women enrolled in the study, 1291 (94.6%) were D⁺, of whom 387 (29.8%) were group A individuals, 214 (16.5%) group B, 54 (4.1%) group AB, and 664 (49.6%) group O (Table 2).

Of the total number enrolled, 134 (9.8%) pregnant women had Rh alloantibodies. The distribution of alloimmunization was 115 (8.9%) among the D⁺ pregnant women, as shown in Figure 1. Among the D⁻ pregnant women, 19 (24.4%) had the alloantibodies (Table 3). Regarding the Rh antigen distribution, alloimmunization was highest in the C, 32 (12.2%), and lowest in the E antigens, 13 (6.1%) (Table 4).

Table 1 Demographic and Clinical Characteristics of the Participants (N=1369)

Variable	Category	Frequency	%
Age (n=1364)	10–20 years	338	24.8
	21–30 years	681	49.9
	31–40 years	328	24.1
	41–50 years	17	1.2
Parity (n=1349)	0–2	826	61.2
	3–5	418	31.0
	6–8	99	7.3
	9–11	6	0.4
Gravidity (n=1353)	0	592	43.8
	2–4	540	39.9
	5+	196	14.5
Trimester (n=1327)	First	88	6.6
	Second	862	65.0
	Third	377	28.4
Blood transfusion (n=1069)	No	1040	97.3
	Yes	29	2.7
Transfusion times (n=24)	Once	19	79.2
	Twice	2	8.3
	More than three times	3	12.5
Indication for transfusions (n=12)	Anemia	7	58.3
	Hemorrhage	5	41.7

Table 2 Distribution of D Antigens by Blood Group (N=1369)

Blood Group	D	
	Positive (%)	Negative (%)
A	385 (29.8)	19 (24.3)
B	212 (16.5)	21 (27.1)
AB	52 (4.1)	4 (2.9)
O	642 (49.6)	34 (45.7)

Of the five major Rh antigens, c (99.3%) and e (99.8%) antigens were found to be the most common antigens, with antigen e (9.72%) having the highest alloimmunization, followed by D (8.77%), while E (0.94%) was the lowest (Table 4). The distribution of alloimmunization among ABO blood groups showed the highest level in blood group B, 25 (10.7%), and the lowest in AB, 4 (7.1%) (Table 5).

RhD+ IAT Test (Alloimmunization)

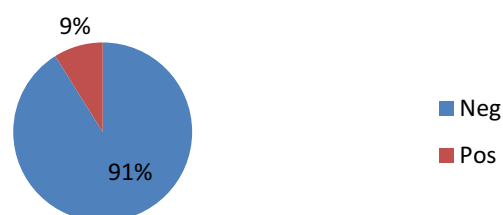
**Figure 1** Percentage distribution of Rh alloimmunization among the Rh D⁺ pregnant women.

Table 3 Distribution of IAT-Positive Results by Rh Blood Group (N=1369)

Rh D	IAT		
	Negative	Positive	% Positive
Positive	1176	115	8.9
Negative	59	19	24.4
Total		134	

Table 4 Rh Antigen and IAT Distribution (N=1369)

Antigen	Total	IAT		
		%	Positive	% Positive
Rh antigen D	1299	94.9	114	8.77
Rh antigen E	218	15.92	12	0.94
Rh antigen C	263	19.21	32	2.34
Rh antigen e	1366	99.78	133	9.72
Rh antigen c	1360	99.34	34	2.48

Table 5 Distribution of IAT-Positive Results by ABO Blood Group (N=1369)

Blood Group	Total	IAT		
		Negative	Positive	% Positive
A	404	361	43	10.6
B	233	208	25	10.7
AB	56	52	4	7.1
O	676	614	62	9.2

Discussion

The distribution of maternal alloimmunization varies in different populations in different countries. The alloimmunization rate in this study was 9.8%, with that among the D⁻ participants being 24.4%, which is much higher than reported by Das et al⁴ (2.27%), Sahoo et al¹² (3.6%), and Kahar¹³ (1.02%) in studies conducted in India and Europe, where the prevalence was in the range of 0.5–2.57%. The higher rate of alloimmunization in this study could be due to the variations in the population ethnicity. This rate is much lower than that reported by Li¹⁴ (45.5%) in China, and this could be due to the recruitment criteria, which emphasized alloimmunized participants.

The alloimmunization prevalence is higher in this population when compared with developed countries such as the Netherlands, as reported by Koelewijn et al¹⁵ (1.2%), and Sweden (0.5%), studied by Filbey et al.¹⁶ It is also found to be higher in comparison with other developing countries, such as a frequency value of 4.8% in Nigeria in the study by Jeremiah et al.¹⁰ The overall alloimmunization rate was also high compared with studies conducted in India by Mangwana et al¹⁷ (1.3%) in New Delhi and by Dholakiya et al¹⁸ (1.5%) in South India. The reduced rates of alloimmunization in developed countries could mainly be due to well-developed antenatal services and anti-D prophylaxis programs,^{19–21} but in developing countries such as India and in African countries, there is a significant risk due to a lack of anti-D prophylaxis.^{18,22} The variations may also be due to differences in race, environment, and the socio-economic status of a given area.

In this study, 115 (8.9%) of the D⁺ pregnancies were alloimmunized, which indicates the presence of alloantibodies in the D⁺ participants, as seen in other studies such as those by Das et al⁴ (2.27%) and Ghesquière et al.^{4,18,23} This prevalence has also been reported by Lurie et al,²⁴ Adeniji et al,²⁵ Mbalibulha et al,²⁶ and Pahuja et al²⁷ as 0.2%, 0.15%, 10.3%, and 0.12%, respectively. This requires special follow-up and intervention for the management of hemolytic disease of the fetus and newborn. This will require the formulation of guidelines for universal antibody screening for pregnant women in this area.

Rhesus antigen e was observed to be the most prevalent antigen, at 99.8%, but with less alloimmunization stimulation (9.72%); this is similar to a study conducted in Ivory Coast (99.9%), but different from results obtained in Nigeria by Gwaram et al (96.1%)²⁸ and Jeremiah et al (95.6%)¹⁰ This disparity may be due to ethnic variations originating from the heterogeneous nature of the different populations. Antigens of the Rhesus blood group have been recorded to vary among races. All of the Rh antigens (D, C, E, e, and c) have been found to cause hemolytic reactions, although with different capabilities, and particularly delayed reactions resulting in hemolytic disease of the newborn.

Rh D alloimmunization is high in this population, and the study clearly shows that alloimmunization is also present among D⁺ women, at a level of 8.9%. This suggests that irregular antibodies were detected in both the D⁻ and D⁺ pregnant women. There is need to determine the titer of these antibodies and to establish the clinical significance at that certain critical level, so that the clinical course be followed up to enable proper management. It is important to note that universal antenatal screening of all pregnant women is key, since in D⁺ women, just as in D⁻ women, alloantibodies can be formed. There is a need to consider universal antenatal screening and to formulate pregnancy management guidelines accordingly.

Ethical Approval

All the participants were taken through the consent procedure and gave their written informed consent to join the study, and parental written informed consent (assent) was obtained for participants under the age of 18 years. The participant protection procedures were followed in conformity with the Declaration of Helsinki. Ethical clearance was sought and obtained from the ethical review board of the School of Medicine as part of a large study at the College of Health Science, Makerere University (REC Ref No 2019-114), and was registered under number HS508ES by the Uganda National Council for Science and Technology.

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Disclosure

All authors declare no competing conflicts of interest relevant to the work presented in this article.

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Efficacy of Oxymetholone in Severe and Nonsevere Acquired Aplastic Anemia: A Propensity Score Matching Analysis

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Background: Bone marrow transplantation, antithymocyte globulin/cyclosporine and eltrombopag are recommended as first-line therapy of severe aplastic anemia (SAA). However, androgens could be considered as front-line treatment among any patients ineligible for better methods although unsatisfactory efficacy is presented.

Objective: This retrospective study aimed to evaluate response and survival rate of practical-based treatment with oxymetholone.

Patients and Methods: This constituted an analysis of patients receiving a diagnosis of acquired aplastic anemia (AA) at the age of 15 or over and receiving oxymetholone between January 2004 and December 2018. Propensity Score Analysis (PSA) 1:1 matching was performed, according to sex, age and interval from first symptom to treatment. The primary outcome was one-year overall response (OR).

Results: Seventy-four patients were successfully matched by PSA. The 1-year OR of oxymetholone in the nonsevere AA (nSAA) and SAA/very severe AA (vSAA) groups was 54.1 and 13.5%, respectively ($P < 0.001$). With median follow-up 2.7 years, the overall survival was 59.5% in nSAA and 37.8% in SAA/vSAA ($P = 0.051$). Median survival in nSAA and SAA/vSAA were 7.0 years and 1.8 years, respectively ($P = 0.045$). However, the responders of SAA/vSAA had longer survival than nonresponders of the nSAA group.

Conclusion: These results revealed longer survival among the responders of patients with AA, even in the SAA/vSAA group. However, close monitoring of therapeutic responses is still performed. Switching therapy is necessary when remission is undetected after 6 months of oxymetholone treatment.

Keywords: androgen, hematologic disease, real world practice, survival

Introduction

Aplastic anemia (AA) is a rare, life-threatening hematologic disease characterized by hypocellular bone marrow. Its incidence in Asian populations is 2 to 3 times higher than that of western countries.¹ The severity of AA is classified as nonsevere (nSAA), severe (SAA) and very severe (vSAA) based on the peripheral blood and bone marrow of patients at diagnosis. The patients having AA with absolute neutrophil count (ANC) < 1000 cells/ μ L are at increased risk of severe bacterial and fungal infection, leading to high morbidity and mortality. Specific treatments including bone marrow transplantation (BMT), eltrombopag, immunosuppressive therapy (ISTs) and androgen in addition to supportive care play a crucial role in improving their prognosis.^{2–4} However, the first-line treatment in AA depends on patients' age, severity, availability of an HLA donor and patients' decision-making.

Androgens are an alternative treatment option in SAA.⁴ The possible mechanism of action is enhanced erythropoietin production. One in vitro study showed the telomerase activity stimulation of androgens could result in hematologic improvement.^{5,6} The overall response rate of androgens was up to 50% among patients with nSAA.⁷ On the other hand, androgens have still shown a poor response of first-line treatment among patients with SAA/vSAA.^{7,8}

In addition to some limitations of BMT, eltrombopag and ISTs including patients' characteristics, HLA matching and the high cost of these treatment, oxymetholone is currently used for all AA severities in clinical practice even though the lower response or delay of response was presented in SAA and vSAA groups. Thus, this study was undertaken to evaluate response and survival of practical-based treatment with oxymetholone.

Materials and Methods

Study Design and Population

This retrospective cohort study enrolled patients receiving a diagnosis of acquired AA at the age of 15 or over and receiving oxymetholone between January 2004 and December 2018. AA severity was defined according to the Camitta Criteria.² SAA was defined as bone marrow cellularity <25% (or 25 to 50% with <30% residual hematopoietic cells), plus at least two criteria of ANC <0.5 x 10⁹/L, platelets <20 x 10⁹/L and reticulocyte count <20 x 10⁹/L. Very severe AA was defined similar to severe AA but ANC <0.2 x 10⁹/L. Patients treated with antithymocyte globulin (ATG) or other androgens within three months before or after oxymetholone initiation were excluded. The eligible patients were followed until the end of study. When they died, were lost to follow-up or discontinued treatment, they were censored.

Response Assessment

Responding to treatment was divided in complete response (CR) and partial response (PR). CR was assessed by blood transfusion independent with all of the following conditions: hemoglobin >10 g/dL, platelet >100 x 10⁹/L and ANC >1 x 10⁹/L at least four weeks apart.⁹ PR was described as blood transfusion independent and no longer meeting the SAA criteria. The primary outcome was overall response rate (OR), which defined as a CR or PR, of oxymetholone at 12 months. The secondary outcomes included OR at 1, 3 and 6 months of treatment, relapse, 1-year overall survival (OS) and adverse events.

Statistical Analysis

In retrospective study, the possibility of selection bias due to confounding variables could occur in an estimate of treatment effects. Therefore, we performed propensity score matching to balance the distribution of covariates between nSAA and SAA/vSAA groups. The estimated propensity scores were calculated to predict the probability for each patient who received oxymetholone via a logistic regression using baseline variables including sex, age, and interval from first symptom to treatment. Patients with nSAA and SAA/vSAA were matched with one-to-one ratio.

Results were shown as frequency counts and percentages for categorical data while median and range were presented as continuous data. OR was shown as percent of responders and Fisher's exact test was used to compare response rate between severity groups. The OS was analyzed using the Kaplan-Meier method and compared by the Log rank test. Factors associated with OS were determined using Cox proportional hazard models. P-value less than 0.05 was considered statistically significant.

Ethical Consideration

This study was approved by the Human Research Ethics Committee of Songklanagarind Hospital, Prince of Songkla University, as well as the hospital director. The research protocol followed in this retrospective study used an electronic medical records and hospital databases that were collected for routine medical care. We requested a waiver of informed consent for this study. All data will be anonymized to ensure privacy and confidentiality of patients' personal information, with each participant assigned a unique identifier. This study is in line with the Declaration of Helsinki.

Results

One hundred and three patients were included in this study. Before matching, 59 patients were nSAA and 44 patients were SAA/vSAA (Figure 1). Fifty-five patients (53.4%) were male. The median age was 52.9 years, while nSAA group had a significantly higher median age ($P = 0.015$). The patients with SAA/vSAA had shorter duration from first symptom

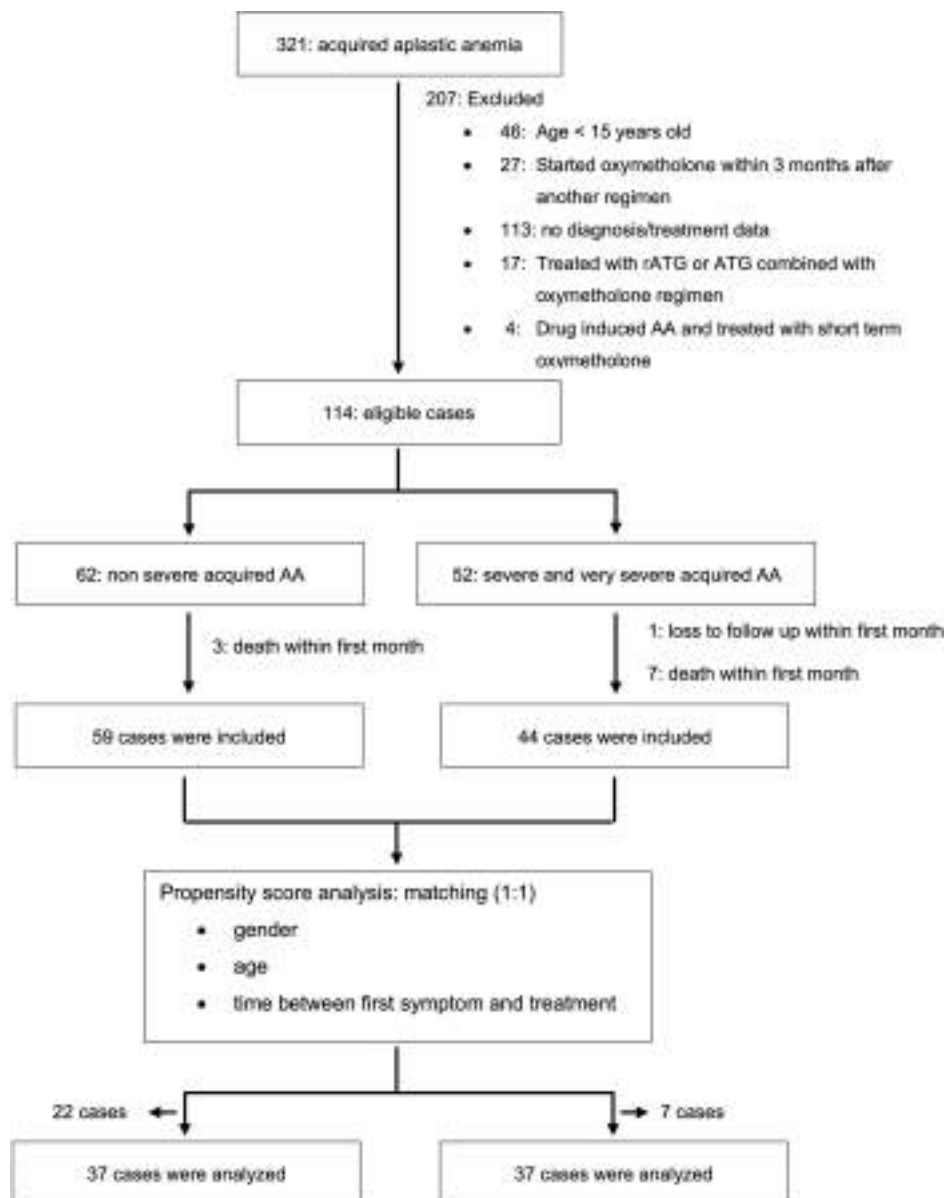


Figure 1 Flow diagram of patient selection and study design.

to treatment than those with nSAA (2.5 months vs 4.9 months, $P = 0.034$). The median dose of oxymetholone in the SAA/vSAA and nSAA groups was 150 mg/day and 100 mg/day, respectively ($P = 0.009$).

After propensity score analysis was performed, 74 patients were successfully matched. All imbalanced characteristics became well balanced without statistical differences. Baseline characteristics are shown in [Table 1](#).

The 1-Year Response Outcome

The 1-year OR of oxymetholone in the nSAA and SAA/vSAA groups were 54.1 and 13.5%, respectively ($P < 0.001$). The CR achievement was 13.5% for the nSAA group. Only one patient (2.7%) with SAA/vSAA achieved CR at eleventh month. However, this patient finally relapsed within six months after CR. Consequently, there was no CR reported at 1-year visit.

Table 1 Baseline Characteristics of Patients

Characteristic	Unadjusted Model			Propensity Score-Matched Analysis		
	nSAA (n=59)	SAA/vSAA (n=44)	P	nSAA (n=37)	SAA/vSAA (n=37)	P
Age, median (IQR)	54.2 (30.4)	39.9 (38.0)	0.015	48.2 (23.5)	52.9 (37.9)	0.750
Male, n (%)	29 (49.2)	26 (59.1)	0.212	20 (54.1)	21 (56.8)	0.500
TST (months), median (IQR)	4.9 (19.9)	2.5 (8.0)	0.021	3.3 (4.1)	2.5 (7.6)	0.552
TDT (months), median (IQR)	1.2 (6.3)	0.7 (2.1)	0.144	0.7 (1.7)	0.8 (1.7)	0.854
Marrow cellularity (%), median (IQR)	5 (20)	5 (0)	0.021	5 (15)	5 (0)	0.103
Dose of oxymetholone (mg/day), median (IQR)	100 (50)	150 (50)	0.001	150 (50)	150 (50)	0.055

Abbreviations: nSAA, non-severe aplastic anemia; SAA, severe aplastic anemia; vSAA, very severe aplastic anemia; TST, time from first symptom to treatment; TDT, time from diagnosis to treatment.

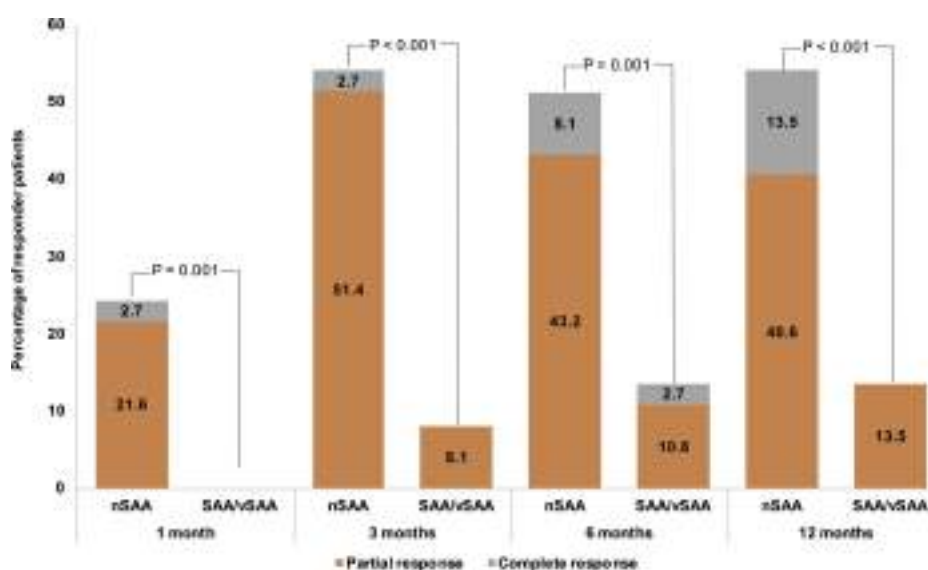
The 1-, 3-, and 6-Month Response Outcome

The percentage of responders at 1-, 3- and 6-month follow-up are shown in Figure 2. The response of oxymetholone was found within one month after treatment initiation for the nSAA group and after three months of treatment for the SAA/vSAA group. The median response time was 1.9 months and 5.8 months for nSAA and SAA/vSAA, respectively.

The Relapse Rate and Factors Associated with Response

For a total of 40 responders, relapse was observed among 16 patients. Based on the number of responders in each group, the relapse rates were 45.5% (5/11) in SAA/vSAA group and 37.9% (11/29) in nSAA group. The median duration of relapse was 5.6 and 15.6 months for the SAA/vSAA and nSAA groups, respectively. Of a total of 16 relapsing patients, 13 died (4 patients in the SAA/vSAA group and 9 patients in the nSAA group).

Univariable and multivariable analysis showed that SAA/vSAA was associated with a lower response rate (OR=0.12, 95% CI: 0.02–0.61, $P = 0.011$). Baseline ANC $\geq 0.5 \times 10^9/L$ was associated with increased one-year response by univariable analysis (OR=3.94, 95% CI: 1.17–13.20, $P = 0.026$). However, we found no statistically significant association between baseline ANC and response in multivariable analysis ($P = 0.845$).

**Figure 2** The percentage of responders at 1-month, 3-month, 6-month and 12-month follow-up.

The One-Year Overall Survival

During median follow up of 2.7 years, the OS was 48.6% with median survival of 4.8 years. The survival rate was 59.5% for the nSAA group and 37.8% for the SAA/vSAA group ($P = 0.051$). The median survival for nSAA and SAA/vSAA were 7.0 years and 1.8 years, respectively ($P = 0.045$). However, in the response subgroup, median survival for nSAA was 13.0 years compared with 11.8 years for SAA/vSAA ($P = 0.956$) (Figure 3).

Factors Associated with Overall Survival

Univariable analysis showed the patients' baseline characteristics that were significantly related to OS included baseline ANC $<0.5 \times 10^9/L$ (HR=2.23, 95% CI: 1.16–4.28, $P = 0.016$) and SAA/vSAA (HR=2.25, 95% CI: 1.17–4.33, $P = 0.049$). However, the multivariable analysis did not show any association of those variables and OS ($P = 0.478$ and $P = 0.157$, respectively). The univariable and multivariable analysis of factors associated with OS is provided in Table 2.

Regarding subgroup analysis by severity, among patients with SAA/vSAA, univariable analysis demonstrated that time from first symptoms to treatment (TST) ≤ 3 months (HR=1.41, 95% CI 1.007–5.761, $P = 0.048$) and time from diagnosis to treatment (TDT) ≤ 1 month (HR=2.54, 95% CI 1.059–6.101, $P = 0.037$) were associated with higher OS (Table 3). However, significance was not found using multivariable analysis.

Survival Curves According to Time to Treatment Initiation

For nSAA group, the patients with early treatment had a longer median survival time (13.0 vs 6.7 years for TST ≤ 3 months and >3 months, respectively, $P = 0.461$ and median not reached vs 6.7 years for TDT ≤ 1 month and >1 month, respectively, $P = 0.811$).

On the other hand, for the SAA/vSAA group, the median survival time was significant greater in the longer TST group (0.6 vs 4.8 years for TST ≤ 3 months and >3 months, respectively, $P = 0.042$). The same value of median survival was presented in the longer TDT group (0.6 vs 4.8 years for TDT ≤ 1 month and >1 month, respectively, $P = 0.031$) (Figure 4).

Safety Outcomes

The most common adverse effect (AE) of oxymetholone was infection (64.8%), follow by impaired liver function (40.5%), bleeding (13.5%), virilization (12.1%) and other AEs (8.1%). Moderate to severe impaired liver function was a leading cause of treatment discontinuation (62.5%). Moreover, one patient wanted to discontinue oxymetholone because of increased appetite and weight gain although most patients could continue treatment after virilization was observed.

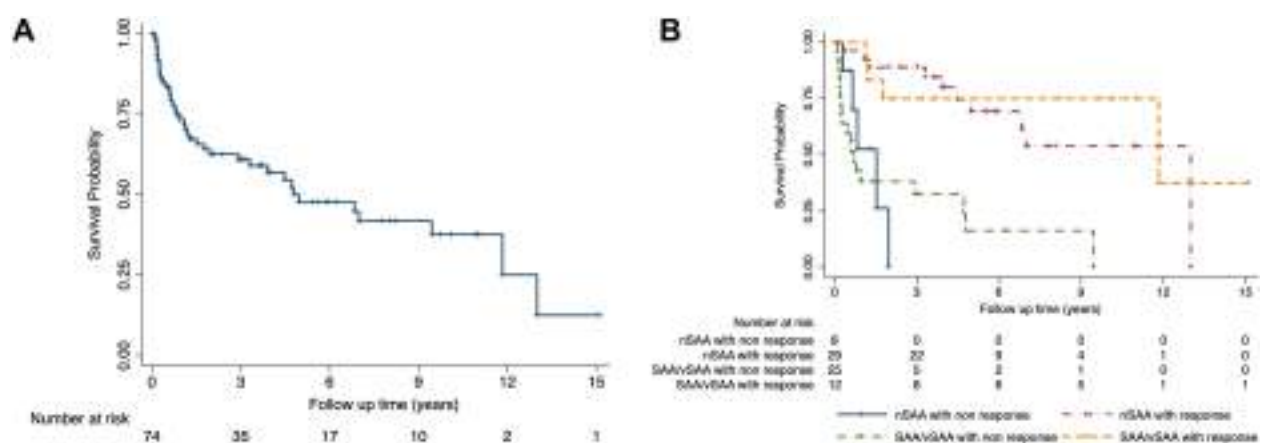


Figure 3 Kaplan-Meier survival curves of overall survival analysis (A). Overall survival in patients with different severity and response (B).

Table 2 Univariable and Multivariable Analysis for Overall Survival Among Acquired AA Patients

Factor	Univariate Analysis		Multivariate Analysis	
	Crude HR (95% CI)	P	Adjusted HR (95% CI)	P
Sex: male	1.30 (0.679–2.505)	0.425	1.36 (0.584–3.152)	0.478
Age: ≥50	1.24 (0.648–2.375)	0.516		
Severity: severe and very severe	1.93 (1.004–3.717)	0.049		
TSD: ≤1 month	1.26 (0.658–2.401)	0.488		
TST: ≤3 months	1.52 (0.793–2.896)	0.209		
TDT: ≤1 month	1.54 (0.796–2.996)	0.198	1.83 (0.792–4.231)	0.157
Baseline hemoglobin: <6.5 g/dL	1.37 (0.683–2.730)	0.378		
Baseline platelet: <10×10 ⁹ /L	1.54 (0.782–3.036)	0.211		
Baseline ANC: <0.5 ×10 ⁹ /L	2.23 (1.163–4.278)	0.016		
Baseline PRC transfusion: ≤2 units/month	1.42 (0.698–2.871)	0.335		
Baseline platelet transfusion: >4 units/month	1.28 (0.626–2.634)	0.496		

Abbreviations: TSD, time from first symptom to diagnosis; TST, time from first symptom to treatment; TDT, time from diagnosis to treatment.

Table 3 Univariable Analysis for Overall Survival in Acquired AA with nSAA and SAA/vSAA

Factor	nSAA		SAA/vSAA	
	HR (95% CI)	P	HR (95% CI)	P
Sex: female	1.95 (0.640–5.925)	0.241	1.03 (0.445–2.383)	0.946
Age: ≥50	0.83 (0.286–2.398)	0.728	1.43 (0.615–3.306)	0.408
TSD: ≤1 month	1.70 (0.570–5.086)	0.341	1.08 (0.470–2.475)	0.858
TST: ≤3 months	0.67 (0.230–1.956)	0.464	1.41 (1.007–5.761)	0.048
TDT: ≤1 month	0.88 (0.308–2.516)	0.811	2.54 (1.059–6.101)	0.037
Baseline hemoglobin: <6.5 g/dL	4.84 (1.267–18.457)	0.021	0.69 (0.291–1.626)	0.394
Baseline platelet: <10×10 ⁹ /L	0.95 (0.123–7.274)	0.958	1.07 (0.467–2.434)	0.878
Baseline ANC: <1 ×10 ⁹ /L	2.69 (0.887–8.130)	0.080	3.194 (0.734–13.799)	0.120
Baseline PRC transfusion: ≤2 unit/month	2.88 (0.374–22.208)	0.310	1.91 (0.833–4.381)	0.126
Baseline platelet transfusion: >4 unit/month	0.35 (0.069–1.733)	0.197	1.29 (0.546–3.026)	0.565

Abbreviations: nSAA, non-severe aplastic anemia; SAA, severe aplastic anemia; vSAA, very severe aplastic anemia; TSD, time from first symptom to diagnosis; TST, time from first symptom to treatment; TDT, time from diagnosis to treatment.

A total of 38 patients (51.4%) died during the study period. The most common cause of death was infection complications (42.1%). The other causes of death were malignancy (5.3%), bleeding (5.3%), nonAA-related causes (13.1%) and unknown (34.2%).

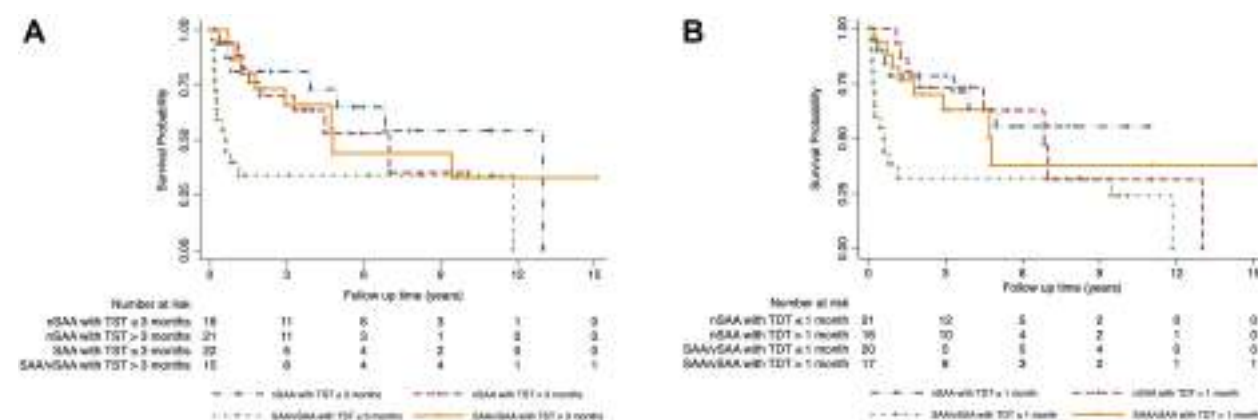


Figure 4 Kaplan–Meier survival curves of overall survival analysis. Overall survival in patients with different time from symptom to treatment (A) and with different time from diagnosis to treatment (B).

Discussion

Aplastic anemia is a serious hematologic disease. The mortality rate of disease improved dramatically after the era of BMT, eltrombopag and ISTs with ATG.^{9–12} Although ATG combined with and cyclosporine or eltrombopag is recommended as first-line treatment among patients with SAA ineligible for BMT,^{2,13} androgens are currently used for all AA severities in our setting due to financial barriers or lacking HSCT donors. This constitutes a retrospective study evaluating the efficacy of oxymetholone, one of androgens. The nSAA group showed higher one-year OR than that of the SAA/vSAA group ($P < 0.001$), as well as higher OS ($P = 0.051$). However, life expectancy could be prolonged among patients with SAA/vSAA responding to oxymetholone.

Androgens were forced to be used as monotherapy in limited resources countries and the efficacy of these agents was evaluated among patients with AA.^{8, 14–16} The response of oxymetholone was reported as 10% and 56% for SAA/vSAA and nSAA, respectively.⁸ Among responders, most patients responded to oxymetholone after six months of treatment.⁸ Furthermore, the median time to initial response was three months with danazol treatment.¹⁵ Similarly, this study revealed one-year ORs with 13.5% for SAA/vSAA and 54% for nSAA. The majority of our patients achieved response in six months after the beginning of treatment with the median time to response of 2.7 months. However, in comparison with one related study,¹⁴ the median time to PR and CR in this study was shorter (14.8 vs 3.0 months for PR, and 25.58 vs 10.0 months for CR). This difference could be affected by the severity proportion. As we know, SAA/vSAA has a longer median time response than nSAA. Accordingly, the longer median time to PR or CR was shown in the related study exhibiting the greater proportion of severe cases.

Many studies have presented OS ranging from 41 to 65% over two to five years.^{14–16} In this study, with the median follow up time of 2.7 years, 48.6% OS was observed. The SAA/vSAA group had a lower OS, and shorter median time as well. However, the responders of this group could prolong the median survival close to that of the responders of the nSAA group (11.8 years and 13.0 years, respectively). It revealed that oxymetholone could be considered for SAA on condition that BMT, ATG or eltrombopag was unaffordable. Nonetheless, the median survival time from the beginning of oxymetholone was about six months in both nSAA and SAA/vSAA groups. As a result, regimen should be changed if the response was not found within six months after initial treatment to prolong life and delay death.

Although the median survival in non-responders were 1.5 years and 0.7 years for nSAA and SAA/vSAA group, respectively. The prognosis was likely to be worse in nSAA group (Figure 3B). This finding might be a result of compliance and out-of-hospital treatment. However, this study could not evaluate these factors. Moreover, among eight non-responders in nSAA group, two of them were censored due to discontinue treatment from developing transaminitis.

Because of the high mortality among patients with SAA/vSAA receiving supportive care alone,¹⁷ the specific treatment should be started. Early treatment of AA has been associated with decreased risk of mortality. However, in the SAA/vSAA group, our study showed higher death among patients presenting shorter TST and TDT. This was probably the result of the reimbursement problem in our country. In the past, oxymetholone was considered as front-line therapy for SAA/vSAA even though poor efficacy was shown. As a result, although oxymetholone was initiated rapidly, the mortality rate remained high. In the delayed treatment group, any patients not responding to prior rabbit ATG/cyclosporine (CsA) was switched to oxymetholone. Although the study had a washout period of around three months, the survival effect of oxymetholone might also be influenced by the effects of rATG/CsA in that group. In addition to the benefit of rATG/CsA, because the rATG/CsA nonresponders died earlier, the remaining patients in the delayed-treatment group were those with better prognosis. Accordingly, lower mortality could be presented among those patients with delayed oxymetholone treatment.

The higher risk of infections, and main cause of death were related to the lower ANC of patients.¹³ $ANC < 5 \times 10^9/L$ was presented as a factor influencing mortality.¹⁶ On multivariable analysis, we did not find significant differences between $ANC < 5 \times 10^9/L$ and $ANC \geq 5 \times 10^9/L$. Undetectable association between neutrophil count and mortality could have been affected by the small sample size which was one of our limitations. The increasing number of samples might show the obvious relation of influencing factors. To confirm this finding, further study with a large sample size is required. Nevertheless, patients with lower ANC, particularly $< 5 \times 10^9/L$, should be focused on to prevent infection and reduce mortality.

In addition to the small sample size, the retrospective design was another limitation of our study. Nevertheless, we performed propensity score analysis to balance baseline characteristics and decrease confounding effects. Lastly,

compliance and out-of-hospital treatment such as supportive treatments could not be collected. The prospective study should be done to confirm the results of our study.

Conclusion

This study revealed that oxymetholone showed a better response rate among patients with nSAA. However, with the lower mortality and longer survival time among the responders of the SAA/vSAA group, it could indicate these patients were ineligible for BMT, eltrombopag or ATG/CsA. Response of treatment should be monitored and switching therapy is necessary when the occurrence of remission is undetected within six months after the beginning of treatment.

Data Sharing Statement

Data available upon reasonable request to the corresponding author.

Ethics Approval

This study was approved by the Human Research Ethics Committee of Songklanagarind Hospital, Prince of Songkla University (approval number: REC.63-226-14-1).

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Disclosure

The authors declare that there are no conflicts of interest in this work.

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Background: Endocrinopathies and metabolic complications are common in beta thalassemia major patients receiving blood transfusions. Chelation therapy has a role in preventing or delaying such complications. However, patients may face difficulties adhering to chelation therapy for several reasons.

Aim: To evaluate endocrine complications in beta thalassemia major patients (2–30 years) in the Eastern Province of Saudi Arabia and compare the onset of endocrine complications among compliant and noncompliant patients. Moreover, we assessed the barriers that hinder compliance with chelating therapy.

Methods: A cross-sectional study was conducted on 89 patients (43 males and 46 females) aged 2 to 30 years attending different hospitals in the Eastern Province of Saudi Arabia. A semi-structured questionnaire was used to collect demographic data and medical histories. The questionnaires were completed by face-to-face interviews with the patients or their caregivers, and the required laboratory data were retrieved from the medical records of patients.

Results: The most prevalent abnormality was underweight detected in (40.9%) of patients, followed by subclinical hypothyroidism (37.7%), short stature (35.2%), hypothyroidism in (17.0%) and diabetes mellitus in (13.6%). A significant difference between those who were compliant with iron chelation therapy and those who were not in terms of the prevalence of short stature (P value=0.05) and hypothyroidism (P value=0.05). The percentage of patients who were not compliant with chelation therapy was 21.6% and 9.1% of patients were not taking them at all.

Conclusion: Despite the role of chelation therapy in the management of iron overload, the risk of secondary endocrine and metabolic complications remained considerable. Subclinical hypothyroidism and short stature were the most frequent endocrine complications encountered in this study.

Keywords: iron overload, metabolic complications, hereditary blood diseases, blood transfusions

Introduction

Beta-thalassemia is a group of hereditary blood disorders characterized by anomalies in the synthesis of the beta chains of hemoglobin.¹ These anomalies result in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. Beta thalassemia (“ β -thal”) is classified into three forms depending on the severity of symptoms: thalassemia trait, thalassemia minor and thalassemia major. Among the three forms of thalassemia, thalassemia major is considered the most severe.²

The prevalence of beta-thalassemia is considered to be one of the highest among autosomal recessive disorders worldwide including in the Middle East. The prevalence of β -thal globally is approximately 1.5%³ and 0.05% in Saudi Arabia.⁴ Regarding β -thal prevalence in Saudi Arabia, there is a noticeable variance by region. The highest rates were observed in the region of Jazan, with 2% for the β -thal trait and 06% for the β -thal major. The second highest rate was reported in the eastern region with 37% for the β -thal trait and 04% for the β -thal major.⁵

Treatment options include transfusion along with chelation or stem cell transplantation with the latter being the only curative method with a success rate greater than 80.0%. However, due to its limited availability, transfusion along with chelation therapy becomes the common choice of management in most centers.⁶ Standard iron chelation therapy is based on the usage of deferoxamine. The effectiveness of this modality of treatment depends primarily on compliance with the medication.⁷ Deferiprone (Ferropro) is another oral chelator that was recently approved in many countries, including the United States.⁸ The most common post-therapeutic complication due to iron chelation is liver fibrosis.⁹

Regularly observed endocrine complications in beta thalassemia patients (TM) include hypothyroidism, osteoporosis, delayed puberty, hypogonadism, short stature and failure to develop secondary sexual characteristics, with iron toxicity as the most likely cause of these complications. Delayed puberty and hypogonadism are the most common endocrine complications of iron overload respectively.⁸

Blood transfusion in beta thalassemia patients is mandatory to overcome and eliminate anemia, but iron overload that follows the transfusion process is the primary cause of endocrine and metabolic complications. These complications are frequently faced by TM patients, and for this reason chelation therapy is a necessity.

Several studies have suggested lack of awareness about receiving chelating therapy, especially among patients' parents, and for that reason, awareness must be increased for a better outcome from chelation therapy.¹⁰

This study aims to assess the endocrine and metabolic complications in regularly transfused β thalassemia patients, and to study the relation of age, gender, and the compliance to iron chelation therapy to the prevalence of these complications. As well as the barriers, or reasons for not being compliant to iron chelation.

Despite the fact that, previous studies aimed to provide a better understanding of the prevalence of endocrine and metabolic complications, very few highlighted the efficiency of chelating agents in reducing these complications and the influence of different factors that affect patients' intake and compliance with chelation.

Research Aim

1. To evaluate endocrine complications that might occur in β thalassemia patients.
2. To compare the onset of endocrine complications among patients who are compliant with chelation therapy and those who are not.
3. To assess the barriers that hinder compliance with chelating therapy.

Materials and Methods

Type of study: Cross-sectional

Study population: Saudi patients aged 2–30 years, of either sex, diagnosed with beta-thalassemia, who have received blood transfusion and had been followed up in public hospitals or medical centers in the Eastern Province of Saudi Arabia.

Data Sources

Haematology Units in public hospitals and medical centres in the Eastern Province of Saudi Arabia, including:

1. Qatif Central Hospital
2. King Fahad University Hospital- Al Khobar
3. Hereditary Blood Disease Center- Al-Ahsa'a
4. Maternity and Children Hospital- Dammam

Inclusion Criteria

Not establishing any of the above with the study population and exclusion criteria is sufficient to have the general characteristics of this study population.

Exclusion Criteria

1. Patients with no medical history or no updates within one year.
2. Pediatric patients who are not accompanied by their parents.

Sample size

A convenience sample of 89 patients was included in the study.

Study instrument

The tool used to collect the data was a semi-structured questionnaire. It is written in English and Arabic and contains three sections. The questionnaires were completed by face-to-face interviews with the patients or their caregivers. The first section of the questionnaire included the sociodemographic data of the child. The second section is composed of six questions to explore the history of the disease and the management received. The second section includes both open and closed-ended questions. Close-ended questions include yes/no and multiple choices with open-ended options in some of them. Endocrine abnormalities were assessed by evaluating the growth parameters, hormonal level and other laboratory data, which are included in the 3rd section of the questionnaire. Growth parameters consist of the weight, height, BMI for age and Z-score. Laboratory investigation result retrieved from the electronic files included the levels of fasting blood glucose, ferritin, serum thyroxine 3 and 4, thyroid stimulating hormone and parathyroid hormone. For participants aged 16 and above, females were asked about their menstrual history and testosterone levels were retrieved from medical records for males to assess gonadal complications. The results were considered normal or abnormal according to the World Health Organization and other international laboratory reference ranges attached in [Appendix 1–3](#). Evidence for growth failure includes the assessment of height, body mass index for participants aged above 5 years and weight-height ratio for participants less than 5 years of age. All of these are abnormal in the event of a standard deviation less than -2 . ([Appendix 4–9](#)) In addition, evidence for diabetes mellitus was based on the American Diabetes Association and World Health Organization criteria. Glucose tolerance was classified into 3 categories based on fasting blood glucose: (1) fasting blood glucose < 5.5 mmol/l was considered normal; (2) fasting blood glucose of $5.5–7$ mmol/l was defined as impaired fasting glucose; and (3) fasting blood glucose > 7 mmol/l warranted the diagnosis of diabetes ([Appendix 1](#)).

Evidence for the existence of primary hypothyroidism was considered when free thyroxine was less than normal < 0.8 ng/dl and thyroid-stimulating hormone was greater than normal > 4 mIU/l. evidence for subclinical hypothyroidism was when free thyroxine was normal at $0.8–1.8$ ng/dl and thyroid stimulating hormone was greater than normal > 4 mIU/l ([Appendix 2](#) and [3](#)).

Ethical Consideration

The research proposal was approved by the research committee of Arabian Gulf University. Additionally, approval from the Saudi Ministry of Health and the assigned hospitals was obtained.

Prior to data collection, verbal and written consent was obtained from the participants. We introduced ourselves as medical students and gave the patient an overview of the research topic and aim. Participants were informed that participation was voluntary, refusal to participate would not affect the treatment or involve any penalties, and the information would be confidential.

Statistical Analysis

The data were entered and analyzed using SPSS software version 23. Frequencies and percentages were computed for categorical variables. The mean and standard deviation were computed for quantitative variables. Cross-tabulation was performed between two categorical variables. A simple bar chart and pie chart were plotted to present a categorical variable. The chi-square test was used to determine whether there was a significant association between two categorical variables. A P -value $= 0.05$ was considered statistically significant.

Results

The clinical data of 89 patients (43 males and 46 females) with thalassemia major were obtained from four hospitals in the Eastern Province of Saudi Arabia. Most participants were from the hereditary disease center in Al-Ahsa'a, accounting for 44.9% of the total study population. Participants ranged in age from 2 to 30 years. The mean age of the participants was 15.5 years [$\sigma \pm 8.3$; CI 95%: 13.7–17.2], of which 20 males and 21 females were above 16 years of age. The majority of participants were between the ages of 11 and 20 years (37.1%) (Table 1).

Table 1 Demographical Characteristics of Patients with Beta Thalassemia Major in Eastern Province of Saudi Arabia

		n (%)
Hospital	Qatif central hospital	32 (36%)
	Maternity and child hospital	8 (9%)
	Hereditary diseases in Al-Ahsa'a	40 (44.9%)
	King Fahad university hospital	9 (10.1%)
	Total	89 (100%)
Living area	Qatif	14 (15.7%)
	Dammam	18 (20.2%)
	Khobar	8 (9%)
	Jubail	2 (2.2%)
	Al-Ahsa'a	39 (43.8%)
	Others	8 (9%)
	Total	89 (100%)
Gender	Male	43 (48.3%)
	Female	46 (51.7%)
	Total	89 (100%)
Gender (Patient Age > 16 years)	Male	20 (48.8%)
	Female	21 (51.2%)
	Total	41 (100%)
Age in years	≤10 years	29 (32.6%)
	11–20 years	33 (37.1%)
	>20 years	27 (30.3%)
	Total	89 (100%)

Most of the participants were diagnosed before the first year of life with a mean age of 1.91 years [$\sigma \pm 2.50$; CI 95%: 1.34–2.48] with a minimum of 0 years and a maximum of 13 years and had started blood transfusion around the same age of diagnosis (mean age 2.12 years) [$\sigma \pm 2.72$; CI 95%: 1.49–2.76]. Sixty four percent received blood transfusions once every month (Table 2).

Information obtained from interviews with patients or their caregivers showed that 69.3% of participants were compliant with chelating agents, while 21.6% were not compliant (Figure 1).

Regarding receiving chelating agents, 21.6% of the study population were not compliant with taking chelating agents. At the same time (9.1%) of the same study population were not taking chelating agents at all. The main reason for non compliance was the fact that, it must be taken daily early in the morning on an empty stomach lifelong. On the other hand, the second most common reason was fearing side effects, which included nausea and vomiting (Figure 2).

Endocrine and metabolic complications were assessed in the study, with the most prevalent abnormalities being underweight (40.9%) followed by short stature (35.2%). Primary hypothyroidism was observed in 17.0% of participants with a mean age of 24.00 years [$\sigma \pm 7.43$; CI 95%: 18.29–29.71], subclinical hypothyroidism was present in 37.7% and diabetes mellitus was observed in 13.6% of participants with a mean age of 19.56 years [$\sigma \pm 5.92$; CI 95%: 15.01–24.10] (Table 3).

Table 2 Blood Transfusion and Chelation Therapy in the Study Population

		n (%)
Age at diagnosis in years	<1	35 (46.1%)
	1–2	24 (31.6%)
	>2	17 (22.4%)
	Total	76 (100%)
Receiving blood transfusion	Yes	86 (96.6%)
	No	3 (3.4%)
	Total	89 (100%)
Age at starting blood transfusion in years	<1	34 (46.6%)
	1–2	20 (27.4%)
	>2	19 (26%)
	Total	73 (100%)
Number of times of receiving blood transfusion	Not every month	1 (1.2%)
	Once a month	55 (64%)
	Twice a month	27 (31.4%)
	Three times a month	3 (3.5%)
	Total	86 (100%)

The results of fasting blood glucose, serum T4, serum thyroid stimulating hormone (TSH), serum parathyroid (PTH) and testosterone were retrieved from the patients' folders after interviewing the patients or their caregivers at the time of data collection, and the results showed that 25.8% of participants had impaired levels of glucose, while 10.6% had high levels. Moreover, 3.8% had T4 levels lower than normal, with 46.3% of participants having high levels of TSH. Testosterone levels for males aged 16 years or above showed that 36. %of them had low levels. Differences between the diagnosed cases and obtained serum levels could be explained by either the effect of receiving medications or failure to diagnose the cases early. Not all participants were featured in this table due to missing laboratory data from the participants' records at the time of collection (Table 4).

**Figure 1** Percentage of compliance to chelation therapy among the study participants.

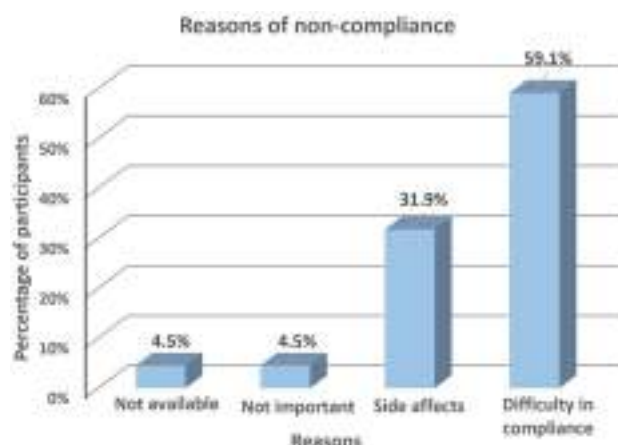


Figure 2 Reasons of non-compliance to chelation therapy among the study participants.

The relationship between receiving chelating agents and endocrine disorders among beta thalassemia major patients in Eastern Province of Saudi Arabia was studied and showed that patients who were compliant with chelation therapy had a statistically significant lower prevalence of short stature (P value=0.05). and hypothyroidism (P value=0.05). In contrast, BMI (P value <0.289) and diabetes (P value <0.521) were poorly correlated with compliance with chelation therapy (Table 5).

The relationship between gender and age with endocrine disorders among beta thalassemia major patients in the Eastern Province of Saudi Arabia showed that the prevalence of short stature was statistically significantly lower in females than in males (P value=0.05). Nonetheless, no significant difference was seen between males and females in the prevalence of BMI (P value <0.177), diabetes (P value <0.430) and Hypothyroidism (P value <0.146) (Table 6).

Table 3 Prevalence of Endocrinopathies and Menstrual Abnormalities in Beta Thalassemia Major Patients in Eastern Province of Saudi Arabia

Disorder		n (%)
Having menses for female > 16 years	Yes	14 (70%)
	No	6 (30%)
	Total	20 (100%)
Hypothyroidism	Clinical	9 (17%)
	Subclinical	20 (37.7%)
	Normal	24 (45.3%)
	Total	53 (100%)
Diabetes	Diabetic	9 (13.6%)
	Pre-Diabetic	15 (22.7%)
	Normal	42 (63.6%)
	Total	66 (100%)
Height	Normal	57 (64.8%)
	Short stature	31 (35.2%)
	Total	88 (100%)
BMI	Underweight	36 (40.9%)
	Normal	47 (53.4%)
	Overweight	5 (5.7%)
	Total	88 (100%)

Table 4 Hormonal and Biochemical Tests Related to Endocrine Disorders in Beta Thalassemia Major in Eastern Province of Saudi Arabia

Test	Results	n (%)
Level of fasting blood glucose	Normal	42 (63.6%)
	Impaired	17 (25.8%)
	High	7 (10.6%)
	Total	66 (100%)
Serum level of T4	Low	2 (3.8%)
	Normal	47 (88.7%)
	High	4 (7.5%)
	Total	53 (100%)
Serum level of Thyroid stimulating hormone (TSH)	Normal	29 (53.7%)
	High	25 (46.3%)
	Total	54 (100%)
Serum level of parathyroid hormone (PTH)	Low	2 (4.4%)
	Normal	37 (82.2%)
	High	6 (13.3%)
	Total	45 (100%)
Serum level of testosterone	Low	4 (36.4%)
	Normal	7 (63.6%)
	Total	11 (100%)

Table 5 Relationship Between Receiving Chelating Agents and Endocrine Disorders Among Beta Thalassemia Major Patients in Eastern Province of Saudi Arabia

Disorder		Receiving Chelating Agents			P-value
		Yes	No	Yes, But Non-Compliant	
		n (%)	n (%)	n (%)	
Height	Normal	45 (75%)	4 (50%)	7 (36.8%)	0.007*
	Short stature	15 (25%)	4 (50%)	12 (63.2%)	
BMI	Underweight	22 (36.7%)	3 (37.5%)	11 (57.9%)	0.289
	Normal	35 (58.3%)	5 (62.5%)	6 (31.6%)	
	Overweight	3 (5%)	0 (0)	2 (10.5%)	
Diabetes	Diabetic	4 (8.7%)	1 (25%)	4 (25%)	0.521
	Pre-Diabetic	11 (23.9%)	1 (25%)	3 (18.8%)	
	Normal	31 (67.4%)	2 (50%)	9 (56.3%)	
Hypothyroidism	Clinical	5 (15.2%)	3 (60%)	1 (6.7%)	0.024*
	Subclinical	11 (33.3%)	0 (0)	9 (60%)	
	Normal	17 (51.5%)	2 (40%)	5 (33.3%)	

Note: The text in bold indicates a p-value of <0.05, which is considered statistically significant.

Moreover, Endocrine Disorders were poorly correlated to a specific age group in the study. The proportion of patients with short stature (P value <0.366), BMI (P value <0.473), diabetes (P value <0.139) and Hypothyroidism (P value <0.239) was not significantly distinctive between different age groups (Table 7).

Table 6 Chi-Square Test for Relationship Between Gender and Each of Height, BMI, Diabetes, and Hypothyroidism

		Gender		P-value
		Male	Female	
		N (%)	N (%)	
Height	Normal	20 (47.6)	37 (80)	0.001
	Short stature	22 (52.4)	9 (19.6)	
BMI	Underweight	19 (45.2)	17 (37)	0.177
	Normal	19 (45.2)	28 (60.9)	
	Overweight	4 (9.5)	1 (2.2)	
Diabetes	Diabetic	6 (19.4)	3 (8.6)	0.430
	Pre-Diabetic	7 (22.6)	8 (22.)	
	Normal	18 (58.1)	24 (68.6)	
Hypothyroidism	Clinical	3 (9.4)	6 (28.6)	0.146
	Subclinical	12 (37.5)	8 (38.1)	
	Normal	17 (53.1)	7 (33.3)	

Note: The text in bold indicates a p-value of <0.05, which is considered statistically significant.

Table 7 Chi-Square Test for Relationship Between Age and Each of Height, BMI, Diabetes, and Hypothyroidism, Having Period

		Age			P-value
		≤10 Years	11–20 Years	>20 Years	
		N (%)	N (%)	N (%)	
Height	Normal	16 (55.2)	22 (66.7)	19 (73.1)	0.366
	Short stature	13 (44.8)	11 (33.3)	7 (26.9)	
BMI	Underweight	11 (37.9)	12 (36.4)	13 (50)	0.473
	Normal	15 (51.7)	19 (57.6)	13 (50)	
	Overweight	3 (10.3)	2 (6.1)	0 (0)	
Diabetes	Diabetic	1 (5.9)	5 (20.8)	3 (12)	0.139
	Pre-Diabetic	1 (5.9)	7 (29.2)	7 (28)	
	Normal	15 (88.2)	12 (50)	15 (60)	
Hypothyroidism	Clinical	1 (11.1)	1 (5.3)	7 (28)	0.239
	Subclinical	5 (55.6)	8 (42.1)	7 (28)	
	Normal	3 (33.3)	10 (52.6)	11 (44)	

Discussion

This study aims to evaluate endocrine complications that might occur in beta thalassemia patients, and the relationship between receiving chelation therapy, and the onset of these complications. The sample consisted of 89 patients, 2–30 years of age, attending hospitals in the Eastern Province of Saudi Arabia in 2019–2020. Endocrinopathies are common

among beta thalassemia major patients, as has been reported by several studies.^{11–13} Chelation therapy has a major role in decreasing or delaying such complications. The findings of this study might help improve the quality of healthcare services provided to these patients by exploring barriers that hinder compliance with chelating therapy.

Underweight was observed less frequently in our population (40.9%), compared to other studies including a study conducted in Tehran, where the prevalence of underweight participants reached 64.2% with a positive association between patients age and BMI (P value <0.002) as opposed to our study which no significant difference was seen between different age groups (P value <0.473). Moreover, there was no significant relationship between gender and BMI in the study (P value <0.89), along with ours (P value <0.177).¹² Participants were considered underweight according to The Growth Charts for Saudi Children and Adolescents endorsed by The Health Services Council of Saudi Arabia, where a BMI less than -2 standard deviations was identified as underweight.

In this study, the prevalence of short stature (35.2%) is lower compared to data from a study performed across 16 countries and reported that 53.0% were short, with a positive association between gender and short stature (P value <0.006) showing prevalence of 61.49% in males and 38.5% in females.¹³ While, the prevalence of short stature in our study was statistically significantly lower in females than in males (P value $=0.05$). Short stature was present in 29% of female patients based on the growth charts for Saudi children and adolescents compared to 71% of males in the study.

However, the prevalence of short stature in our study was higher than that reported from a study of endocrinopathies in beta-thalassemia major northwest Saudi Arabia, which was identified in 20.9% of their cohort. Along, with a positive association between age and short stature (P value <0.02) with a prevalence of 31.1% in those aged 10–28 years.¹¹ In contrast, there was no marked association between age and short stature in this study (P value <0.366).

On the contrary, primary hypothyroidism was observed to have a higher prevalence than that reported in other studies conducted in Tehran,¹⁴ Italy¹⁵ and Northwest Saudi Arabia.¹¹ This difference in prevalence from other countries could be explained by the difference in cut-off hormone levels and/or variation in management protocols and patient compliance between different centers. The prevalence of hypothyroidism in the study was based on participants diagnosed with hypothyroidism and participants who met the diagnostic criteria at the time of data collection.

Moreover, the rate of diabetes mellitus in this study was higher 13.6% (66.7% males, 33.3% females), than that reported in a study conducted in Tabriz, Iran (8.9%).¹⁶ In addition to that, no significant difference was seen between males and females in the prevalence of diabetes mellitus (61.1% male, 38.9% female).¹⁶

This has been suggested to be due to risk factors for diabetes, including age, increased amount of blood transfusion, serum ferritin level, compliance with iron chelating therapy, family history of diabetes and pubertal status. The prevalence of diabetes in the study was calculated based on those who had high levels of glucose at the time of data collection and those who were already diagnosed with DM.

To our knowledge, no other studies have compared receiving chelation therapy and the onset of complications. However, in this study, there was a statistically significant association between compliance with iron chelation therapy and the prevalence of some endocrine complications. Those who were compliant with chelation therapy had a lower prevalence of short stature (P value $=0.05$) and hypothyroidism (P value $=0.05$). In contrast, there was no significant difference in BMI (P value <0.289) or diabetes (P value <0.521).

Although most of the patients knew the importance of chelation therapy, some of them were non-compliant. There is a number of reasons, for patients to refuse or to not comply to iron chelation therapy, with the major reason being, that the chelation therapy is a lifelong medication that must be taken daily on an empty stomach, which is difficult to incorporate into their daily life routine. The second most common reason was fearing the side effects, which included nausea and vomiting and a minority thought it was not important or available.

When compared with a study that assessed the reasons behind patients missing chelation therapy in several countries, including Iran, India and the United States.¹⁷ It has been shown that access to the drug was the main concern in India (51.0%), which was also a common response in Iran (25.0%), which was not the major cause in our study.

In other countries, however, the most common reason for non-compliance was related to their beliefs in taking the treatment (58.0%). The second most common reason was the side effects of the treatment (42.0%).

Conclusion

Despite the role of chelation therapy in the management of iron overload, the risk of secondary endocrine and metabolic complications remains considerable. Subclinical hypothyroidism and short stature were the most frequent endocrine complications encountered in this study. Hypothyroidism, hypoparathyroidism, diabetes mellitus and hypogonadism were also detected. Evaluating patients with TM should be carried out at an early age and regularly. Patient education regarding the importance of compliance with chelation therapy since it has a role in decreasing several iron overload-related endocrine and metabolic complications is essential.

Data Sharing Statement

All relevant data are within the paper. The questionnaire used is included in ([Appendix 10](#) and [11](#)).

Recommendations

Endocrinopathies and metabolic complications are common beta thalassemia major patients receiving blood transfusions. Chelation therapy has a role in preventing or delaying such complications. Further longitudinal studies with a larger sample size are needed to assess such complications and evaluate the role of chelation therapy. Proper protocols and guidelines should be developed and implemented by a multidisciplinary team with the emphasis of endocrine and metabolic complication screening to improve the quality of care provided to this group of patients.

Limitations

There are a few limitations to be considered in relation to the findings of our study.

First, the initial estimate of the sample size was 200, and we aimed to conduct random sampling. However, the duration of data collection was limited to the summer in which most of the patients were away or traveling; hence they were not within easy reach. For that reason, we had a convenience sample of 89 patients.

Second, we could not attain approval from hospitals easily; for instance, one of the hospitals that was included in our protocol did not have the chance for data collection at all, making the sample size smaller; therefore, the statistical analysis remains of limited power.

In addition, the cohort is young which must have had an influence on the prevalence of age-related complications such as diabetes.

Another point to consider is the fact that many of the laboratory tests required to assess endocrinopathies were not conducted or documented in some of the centers, which made the assessment of complications challenging.

Data Sharing Statement

A semi-structured questionnaire was used to collect demographic data and medical histories. The questionnaires were completed by face-to-face interviews with the patients or their caregivers, and the required laboratory data were retrieved from the medical records of patients attending hematology clinics across four different centers in Eastern Province, Saudi Arabia.

Ethics Approval and Consent to Participate

This study complies with the declaration of Helsinki. The research and ethical committee of Arabian Gulf University approved the research. Signed written consent was obtained from all participants above the age of 18 and from the caregivers of participants who were below 18 ([Appendix 12](#)).

Consent for Publication

The authors affirm that the participants provided informed consent for publication.

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Disclosure

The authors have declared that no competing interests exist. The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Additionally, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

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Iron Deficiency and Blood Donation: Links, Risks and Management

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Abstract: The purpose of this review is to raise awareness about the frequently underappreciated association of blood donation with iron deficiency, and to describe methods for its prevention and management. Blood donors cannot expect any health benefits from the donation but have justified expectations of no harm. Iron deficiency without anemia (IDWA) and iron deficiency anemia (IDA) are common consequences of regular blood donation, and this activity is the most important factor affecting iron status in regular blood donors. Awareness of blood donation as a primary cause of sideropenia is surprisingly low among physicians. Blood donation screening identifies potential donors with IDA but is frequently inadequate to detect IDWA. For the assessment of body iron stores, plasma or serum ferritin, transferrin saturation (TSAT) and soluble transferrin receptors (sTfR) concentrations are the most widely used biochemical markers, although the percentage of hypochromic mature erythrocytes and the hemoglobin content of reticulocytes are also useful. IDWA can be prevented by limiting the total volume of blood collected, by iron deficiency screening and deferral of sideropenic donors, by prolonging the interdonation intervals, and by iron supplementation between donations. IDWA tends to be more prevalent in younger people, females, and high-intensity donors. A potentially effective strategy to address sideropenia in blood donors is serum ferritin testing, but this may lead to a higher rate of deferral. Most regular blood donors cannot replenish their iron deficit by an iron-rich diet alone and will benefit from low-dose oral iron administration with various commercially available products post-donation, a well-tolerated strategy. However, valid concerns exist regarding the possibility of worsening the iron overload in donors with undiagnosed hemochromatosis or masking the symptoms of a clinically important gastrointestinal hemorrhage or other underlying medical condition. Finally, educational efforts should be intensified to improve the awareness of blood donation as a primary cause of iron deficiency among physicians of all specialties.

Keywords: blood donation, iron deficiency, iron deficiency without anemia, iron deficiency anemia, iron supplementation, transferrin saturation

Introduction

Voluntary blood donation is a selfless and irreplaceable act necessary to preserve the blood supply. Volunteer blood donors cannot expect any health benefits related to donation but have the reasonable expectation of no physical harm. Hence, maintaining donor health is no less important than ensuring a safe and continuous blood supply, and blood centers should explore screening programs for donors to improve their health, recruitment, and retention.¹ In this regard, reducing and preventing possible side effects of phlebotomy without deterring current or future donors is crucial.

Considering the above, iron deficiency without anemia (IDWA) and iron deficiency anemia (IDA) are potential harms to regular blood donors, and it is the ethical duty of blood bank and other physicians (hematologists, general practitioners, etc.) to be aware of the risk of iron depletion in blood donors and evidence-based prevention measures and management of low iron stores.^{2,3} Donors' behavior and genetic factors contribute to first-time donor iron status and hemoglobin, but iron supplementation is far more critical than subjects' genetics in those donating frequently.⁴ The

purpose of this review is to raise awareness about the frequently underappreciated association of blood donation with iron deficiency and to describe methods for its avoidance and management.

Non-Anemic Iron Deficiency

Non-anemic iron deficiency or IDWA is a disease by itself that deserves prompt diagnosis and therapy.⁵ IDWA is especially frequent in women of childbearing age, who also may benefit from iron supplements. It can be suspected and diagnosed with a variety of indexes derived either from the full blood count or biochemical tests (ferritin, transferrin saturation, soluble transferrin receptors (sTfR), etc). IDWA has been associated with exercise intolerance, fatigue, reduced cognitive function, pica, and restless legs syndrome (RLS). It also impairs aerobic adaptation among previously untrained women and this is correctable with oral iron supplementation.^{6,7} In a study of 716 adolescent girls with IDWA, participants were randomly assigned to oral ferrous sulfate or placebo for 8 weeks. Post-intervention hematological measures of iron status were significantly improved in the treatment group, while girls receiving iron performed significantly better on a test of verbal learning and memory.⁷ Non-anemic women with unexplained fatigue may benefit from iron supplementation, but this effect is restricted to women with low or borderline serum ferritin.⁸ The beneficial effect of oral iron was also shown in a randomized, placebo-controlled, trial in France in 198 women aged 18–53 years, who complained of fatigue, had ferritin <50 µg/L and hemoglobin >12 g/dL, and received either oral ferrous sulfate or placebo for 12 weeks.⁹

It is surprising how low among physicians is the awareness of blood donation as a primary cause of sideropenia. Only 4 out of 50 (8%) internal medicine and family practice physicians in Austria considered blood donation as a potential cause of iron deficiency, and only 28% reported knowing if one of their patients was a regular blood donor.¹⁰ In dedicated blood donors, donation activity is the single most important factor affecting iron status, far ahead of red-meat consumption or iron supplementation.¹¹

Hemoglobin values do not necessarily reflect iron stores, and measurement of hemoglobin or hematocrit alone is insufficient for detecting blood donors with IDWA.¹² Donors who have adequate iron stores are deferred from donating blood, while others who are donating blood may have inadequate iron stores.¹³

As mentioned earlier, IDWA can be associated with pica and could aggravate or even trigger RLS. Therefore, otherwise healthy donors with symptoms of RLS should be very cautious about donating blood, and an assessment of iron stores before the donation is a rational precaution.¹⁴ Regarding pica, its presence has been associated with a high probability of iron depletion in blood donors.¹⁵ Factors associated with pica were analyzed in four US blood collection agencies. Lower ferritin, non-Asian race, higher red cell distribution width, younger age, and RLS were significantly associated with pica.¹⁶ Iron replacement in these patients is effective and reduces symptoms.

Each donation causes a loss of approximately 210 to 240 mg of iron, as 1 mL of red cells contains 1.12 mg of iron. In a study from Germany, 26% of regular donors had ferritin levels <15 µg/L and 12% suffered from IDA.¹⁷ IDWA tends to be more prevalent in younger people, females, and those with a more intensive blood donation history. A prospective national study in France of 11,258 WB donors showed that iron deficiency was more frequent in women than in men (39.5% vs 18%). Among 7200 repeated donors, women <50 years old, new female donors, and repeated donors of both sexes had the highest prevalence of iron deficiency. In these identified subgroups, the prevalence of iron deficiency was up to 72.1%.¹⁸

Current practices by blood banks identify potential donors with IDA but are inadequate to detect IDWA.¹⁹ This is particularly true for premenopausal women and teenagers. A nationally representative study in the US showed that the problem is indeed more intense among adolescents and adult females.²⁰ A stratified approach to prevention and treatment considering risk factors and serum ferritin testing is likely to be the most effective management strategy.²¹

In the US, a minimum acceptable hemoglobin concentration of 12.5 g/dL and a minimum interval between donations of 56 days is required for blood donation irrespective of the donor's sex. This is counterintuitive since iron requirements are substantially higher in women of childbearing age. Thus, it is not surprising that in the REDS-II Donor Iron Status Evaluation (RISE) study, only 1.6% of attempted donations by men were deferred due to anemia versus 17.7% of attempts by women.¹³ A recent study in 30,806 teenage donors in the US showed that 11.4% of females and 9.7% of males had inadequate iron stores at index donation. Follow-up donation iron stores were highly dependent on index

ferritin and achieving a $\geq 90\%$ prevalence of adequate ferritin at 12 months required index ferritin values >50 ng/mL. Teenagers with borderline or low iron stores should consider receiving iron supplements to permit regular donations. In addition, increasing the minimum recommended inter-donation interval to allow time for a refill of iron losses should strongly be considered.²² A large, randomized trial of 45,000 donors in the United Kingdom showed that more frequent donations than is standard practice resulted in more deferrals and IDWA.²³ In an Australian study of >3000 blood donors, the prevalence of serum ferritin <15 ng/mL in new donors was 12% in females and 1.3% in males. The prevalence of iron deficiency in female WB-only donors was 26.4%, increasing with donation frequency and young age, while in males it was 6.3% with no evident change with age or donation frequency.²⁴

Female blood donors of childbearing age who donate blood on a regular but moderate basis do not appear to be at higher risk of adverse pregnancy outcomes, such as low birth weight, preterm birth, and stillbirth.²⁶ This is reassuring knowing that IDA is associated with preterm delivery and low birth weight and that iron supplementation during pregnancy decreases these risks.

Volunteer WB donor eligibility criteria vary substantially by country due to cultural and historical reasons, body habitus changes, and other causes, and these differences likely affect the prevalence of IDWA and IDA among donors.²⁵ In the US and elsewhere, despite guidelines described in the AABB Bulletin #12-03, nearly no blood center routinely monitors donor iron status because of the increased cost and the workload related to reviewing the results and counseling the donor. A recent, revised version of these recommendations (AABB Bulletin #17-02) that strongly encourages monitoring iron status in at-risk subgroups has recently been circulated (July 2022).²⁶ In Switzerland, premenopausal women are allowed to donate only three times a year, serum ferritin is measured in all WB donors, albeit not in every visit, and iron-depleted donors are offered three possibilities, ie, to extend the donation interval, to follow a more iron-fortified diet or to receive oral iron supplements.²⁷ In Denmark, ferritin screening is performed on the first donation and every 10 subsequent donations, or more frequently depending upon prior results. Iron-depleted donors are given iron supplements. Not surprisingly, deferrals for iron deficiency have decreased in these two countries.²⁸ Moreover, many centers around the globe try to develop hemoglobin-based algorithms in order to identify the donors who will benefit from ferritin measurement.

Finally, although IDWA is mainly a problem for WB donors, it can also affect plateletpheresis donors, who have smaller iron losses per donation but are able to donate much more frequently than WB donors. An Irish study showed that male plateletpheresis donors had a statistically significant drop in serum ferritin after donation, while they had significantly lower serum ferritin levels than first-time male blood donors.²⁹ Similarly, a study from India showed that regular plateletpheresis donation may lead to depletion of iron stores and subclinical iron deficiency.³⁰

Prevention of Non-Anemic Iron Deficiency in Blood Donors

IDWA in blood donors can be prevented by limiting the volume of blood collected to 400–500 mL per donation, by iron deficiency screening and deferral of sideropenic donors, by blood donation only after hemoglobin/ hematocrit and iron stores recovery, by oral or parenteral iron therapy between donations, by lengthening the inter-donation interval, and by switching WB donors to low-frequency plasma or plateletpheresis donors.³¹ To date, many blood services do not follow all these recommendations.

Methods for Assessment of Body Iron Stores in Blood Donors

For the assessment of body iron stores, plasma or serum ferritin, transferrin saturation, and sTfR concentrations are the most widely used biochemical markers. The percentage of hypochromic mature erythrocytes and the hemoglobin content of reticulocytes (Ret-He) have also been shown to be much better screening measures for the identification of iron depletion in blood donors than hemoglobin.³² Unfortunately, measurement of capillary hemoglobin only is what is usually available in most blood centers. Regarding hepcidin, it may serve as a marker of the iron repletion needed for erythropoiesis, although it cannot serve as a predictor of response to iron treatment,³⁴ its use is almost exclusively limited to research purposes, and there is a lack of a gold standard measurement assay, pending resolution of the international efforts for harmonization.

Considering ferritin, its measurement at regular intervals is a useful indicator of iron depletion in blood donors.¹⁷ Even though the correlation between ferritin and hemoglobin is poor, donors with low ferritin have much higher odds of deferral at subsequent donation visits.³³ Gorlin et al, have shown that it is feasible to use serum ferritin for the determination of iron stores to reduce potential deferrals for low hemoglobin in regular blood donors that are offered appropriate iron replacement therapy.³⁴

One potentially effective strategy to address IDWA and IDA in blood donors is serum ferritin testing in females donating $\geq$ twice per year and men donating $\geq$ three times per year. This strategy can identify donors with low iron stores who will benefit from appropriate iron supplementation, and patients with undiagnosed hemochromatosis who must avoid taking iron supplements. It may also lead to referrals for further evaluation and treatment of anemia in first-time or infrequent blood donors with underlying diseases, such as gastrointestinal malignancies.³⁵ The population-based Danish Blood Donor Study showed that ferritin was the strongest predictor of the risk of low hemoglobin.³⁶ A recent study from Iceland included WB donors and new donors between 1997–2019. Before October 2017, ferritin was measured only for new donors and intermittently for WB donors, but thereafter for all donors at every donation visit. In January 2018, the ferritin threshold for donation increased from 14 to 16 $\mu\text{g/L}$ for new donors and from 8 to 10 $\mu\text{g/L}$ for WB donors. After the policy change, the proportion of WB donations associated with iron deficiency decreased from 6.4% to 4% for males and from 18.9% to 14.1% for females.³⁷ In a Canadian blood center that practices routine ferritin testing, the minimum hemoglobin for donation was increased to 13 g/dL for males and the minimum inter-donation interval was changed to 84 days for females based on their iron status. This led to a significant reduction in deferrals for low hemoglobin.³⁸ Donors informed of their low ferritin donated less often and had a lower return rate compared to donors with normal ferritin, but they had an increase in follow-up ferritin when tested one year after initial testing.³⁹ In conclusion, regular ferritin testing may improve donor management. However, simply providing donors with ferritin results and written information on iron deficiency is likely insufficient to improve their understanding of iron needs and may lead to an inadequate increase in iron intake over time.³⁹ France et al, have shown that providing information to blood donors regarding their risk of iron depletion enhances the overall awareness of donation-related sideropenia with no negative impact on donor retention. Combining education with an action plan may normalize the iron balance.⁴⁰

In contrast, regular ferritin measurement in all blood donors may be associated with a decrease in blood donation and additional operational costs and these issues should be considered by blood banks before its implementation. Canadian investigators developed a simulation model representing a population of blood donors tested for ferritin who may exit the blood donation system. Data were derived from operational data, donor research studies from the Canadian Blood Services, and previously published sources. They estimated that red cell collections will decline by 3.1–19.2% after ferritin testing is put in place. Therefore, blood agencies need to ensure that donors with low ferritin will modify their lifestyle and eventually return to the donor pool.⁴¹

Besides ferritin, zinc protoporphyrin (ZPP) is another marker of sideropenia used mostly in the Netherlands. Increased levels of ZPP indicate iron deficiency and elevated ZPP was predictive of deferral due to low hemoglobin in a Dutch study of 4598 WB donors.⁴²

Other markers of iron deficiency are sTfR and Ret-He. The accuracy of Ret-He in detecting latent iron deficiency was studied in 501 blood donors in India. The study used sTfR as the gold standard and showed that sTfR and Ret-He detected IDWA in 148 and 135 donors, respectively. In comparison to sTfR, Ret-He had a sensitivity of 92.7%, which was significantly higher than that of ferritin, iron, and TIBC in detecting latent iron deficiency.⁴³ Use of the sTfR-F index (ratio of sTfR to $\log_{10}$ ferritin) and a combined cell index, as described by Boulton et al, show that the iron status of men giving three donations in two years resembled that of menstruating women and that men not taking iron supplements can become markedly iron-deficient on donating six times over a two year period.⁴⁴

Increased frequency of blood donations is associated with microcytosis and hypochromia, as indicated by lower MCV and MCH, respectively. These immediately available red blood cell indices, or newer parameters measured by automated blood cell analyzers such as the percentage of hypochromic erythrocytes, should be used to monitor early onset iron depletion in regular blood donors.⁴⁵ In a Malaysian study, the percentage of hypochromic red cells with a cut-off value of 0.6% was excellent in evaluating iron status among blood donors with very high sensitivity and specificity. It also has the additional advantage of a faster turnaround time than conventional biochemical markers of sideropenia.⁴⁶ In a pilot study

of 150 Swedish blood donors versus 104 controls, donors had significantly more microcytic erythrocytes (median 0.9% vs 0.6%), lower serum ferritin concentration (median 43 vs 91 $\mu\text{g/L}$), and higher sTfR-F index (median 1.60 vs 1.27). Microcytic erythrocytes and mean cell hemoglobin of reticulocytes (MCHr) predicted iron depletion with moderate sensitivity that was improved when these two parameters were combined.⁴⁷

In a study from New Zealand, blood donation, being of Asian ethnicity, and history of iron deficiency were all stronger predictors of suboptimal iron status than differences in dietary patterns in premenopausal women.⁴⁸ In the RISE study, in a cohort of 2425 first-time/reactivated and frequent blood donors, among frequent donors, 66.1% of women and 48.7% of men had iron-deficient erythropoiesis, while absent iron stores (ferritin $<12 \mu\text{g/L}$) were seen in 16.4% and 27.1% of men and women, respectively. Increased donation intensity, female sex, premenopausal status, black race, low body weight, and younger age were important independent predictors of absent iron stores or iron-deficient erythropoiesis.^{13,49} The association of decreased iron stores with the female sex was expected since premenopausal women have higher daily iron requirements (1.5 mg vs 1 mg) due to menstrual blood loss.¹⁰ The RISE study concluded that iron supplementation may allow frequent donation without iron depletion. However, given that no intervention was performed, the basis of this conclusion and whether it should apply to all donors has been legitimately questioned.⁵⁰ Consequently, blood banks should try to better identify their own donor population at real risk of iron deficiency and tailor targeted interventions to minimize harm.

Dependence only on food intake to replenish iron stores in regular blood donors is inadequate, especially in settings where IDA is common, to begin with. For example, in Ghana, where IDA is endemic and post-blood donation iron supplementation is not widely practiced, more than three-quarters of the blood donors could not achieve pre-donation iron stores five months post-last donation.⁵¹

Studies of Iron Supplementation in Regular Blood Donors

Given the numerous oral and several parenteral available iron products, iron supplementation in regular blood donors should be individualized. For example, ferrous sulfate often causes significant gastrointestinal side effects. In this case, alternative oral iron products should be considered.⁵² Many studies have been performed to test the efficacy and tolerability of the various iron formulations in the replenishment of iron stores of blood donors. The chosen iron replacement strategy should be convenient, easily available, cheap, and with minimal complications. The most important of these studies are shown in Table 1 and briefly reviewed below.

The Hemoglobin and Iron Recovery Study that was part of the Recipient Epidemiology and Donor Evaluation Study-III (REDS-III), conducted by the National Heart, Lung, and Blood Institute in the US compared donors who did not receive iron supplementation with those who did. Using a ferritin cut-off of 26 ng/mL, recipients of iron supplementation had shortened time to 80% hemoglobin recovery in both the low- and higher-ferritin groups.⁵³ Even in iron-replete donors, the mean hemoglobin recovery was only 70% at eight weeks since absorption of dietary iron is a limiting factor for the refill of iron stores.⁵³ A study comparing placebo vs carbonyl iron in women 18 to 40 years old for 56 days after phlebotomy showed that therapy, diet, or both, was sufficient to replenish the iron stores in 85% of those receiving carbonyl iron vs only 29% of those taking placebo. Moreover, deferral rates from repeat donations were 8% and 36% respectively.⁵⁴ An Australian Red Cross Blood Service study investigated the effect of carbonyl iron daily or placebo for 8 weeks, as post-donation iron replacement therapy. Donors receiving carbonyl iron had significantly higher ferritin and mean hemoglobin at week 12. In addition, the proportion of iron-deficient donors was significantly lower in the carbonyl iron group, thus improving eligibility to re-donate at week 12. Approximately 87% of the donors receiving carbonyl iron indicated that they would continue to take it, even though significantly more donors receiving iron supplements had at least one gastrointestinal side effect.⁵⁵ Hence, short-term carbonyl iron supplementation in female blood donors seems to be effective in replacing safely the iron loss of phlebotomy.

A Swedish randomized study on 120 donors with at least five previous WB donations compared IV iron sucrose vs oral iron sulfate for 20 days, after each blood donation for 1 year. Both treatments were safe, but the IV iron sucrose group increased iron stores to a significantly greater extent than the oral iron group. Moreover, female donors, particularly <50 years of age, were more responsive to intravenous iron sucrose compared to the oral formulation.⁵⁶ In another study, routine administration of ferrous sulfate tablets for 60 days was safe and effective and prevented the

Table I Studies of Oral or Parenteral Iron Supplementation in Blood Donors

Authors	Study Design	Iron Products Used	Main Study Outcomes
Kiss JE, et al ⁵³	Randomized	Ferrous gluconate 37.5 mg/day vs no iron for 168 days post donation of 500 mL of whole blood.	Recovery of iron stores in participants who received supplements took a median of 76 days; for participants not taking iron, median recovery time was 168 days ($p < 0.001$). Without iron supplements, 67% of participants did not recover iron stores by 168 days.
Gordeuk V, et al ⁵⁴	Randomized double-blind	Carbonyl iron 100 mg orally vs no iron at bedtime for 56 days after phlebotomy	At day 56, estimated iron absorption was sufficient to replace iron in 85% of those receiving carbonyl iron but in only 29% of those taking placebo ($p < 0.001$)
Marks DC, et al ⁵⁵	Randomized, double-blind, placebo-controlled	Carbonyl iron 45 mg daily vs placebo for 8-weeks post-donation	The mean Hb level in the carbonyl iron group (134.6 ± 8.7 g/L) was significantly higher than in the placebo arm (130.0 ± 9.9 g/L; $p < 0.001$), significantly improving eligibility to donate at Week 12
Birgegård G, et al ⁵⁶	Randomized	Iron sucrose 200 mg IV versus 20×100 mg tablets of oral iron sulphate (1 tablet/day) after each blood donation for 1 year	The IV iron group increased storage iron to a greater extent than the oral iron group after 12 months ($p=0.0043$)
Bryant BJ, et al ⁵⁷	Single arm study	Deferred donors and iron-deficient control donors were given 60 tablets containing 325mg ferrous sulfate (1 tablet/day for 60 days). Another 60 tablets were dispensed at all subsequent visits	Iron-depleted or -deficient donors taking iron showed normalization of iron-related laboratory parameters, even as they continued to donate
Krayenbuehl PA, et al ⁵⁸	Randomized, double-blind, placebo-controlled	Iron sucrose IV 800 mg vs intravenous placebo	In patients with baseline ferritin ≤ 15 ng/mL, fatigue decreased by 1.8 in the iron group compared with 0.4 in the placebo group ($p = 0.005$), and 82% of iron-treated compared with 47% of placebo-treated patients reported improved fatigue ($p=0.03$)
Gybel-Brask M, et al ⁵⁹	Randomized double blind, placebo-controlled	First-time female blood donors randomized 1:1 to iron isomaltoside 1000 mg IV vs placebo	The increase in Hb was significantly higher for iron isomaltoside compared with placebo right before both the second ($p = 0.0327$) and the third blood donation (primary endpoint, $p < 0.0001$)
Cable RG, et al ⁶⁰	Randomized	Donors randomized to oral ferrous gluconate daily (37.5 mg iron) or no iron for 24 weeks after blood donation	At 24 weeks, total body iron in donors taking iron increased by 281 mg (95% CI, 223.4–338.6 mg) compared to before donation, while in donors not on iron decreased by 74.1 mg (95% CI, –112.3 to –35.9; $p < 0.0001$, iron vs no iron)
Chiamchanya N ⁶¹	Randomized, three arm study	Oral ferrous fumarate 200 mg/day (group I) vs ferrous fumarate 200 mg/day + ascorbic acid 100 mg/day (group II) vs ferrous fumarate 200mg/day+ascorbic acid 500 mg/day (group III)	The recovery times of MCV, MCH, and MCHC to before blood donation levels in groups II and III were similar (0.5 months in every value), which was more rapid than in group I ($> 3, 3$, and 1 month, respectively) ($p < 0.001$)

Drexler C, et al ⁶²	Randomized, two arm study	Donors randomized to a single dose of ferric carboxymaltose IV or oral ferrous fumarate (100 tablets of 100 mg [10 tablets per week] for 10 weeks	Median TS and ferritin were significantly higher in the IV iron group (27% vs 21%, $p < 0.001$ and 105 vs 25 ng/mL ($p < 0.001$), while median Hb levels were comparable (IV, 13.6 g/dL vs oral 13.6 g/dL)
Mast AE, et al ⁶³	Randomized, five arm study	Three double-blinded arms provided 60 once-daily pills after each donation (38 mg, 19 mg, or 0 mg of iron) and two single-blinded arms provided iron status (ferritin) information or no information letters after each donation	Prevalence of ferritin <12 or <26 ng/mL declined by more than 50% and was statistically indistinguishable in the three intervention groups (19mg or 38 mg of iron; iron status letter). Improved iron status in iron pill groups and worsening iron status in control groups (placebo; no information letter). The iron pill groups experienced a net increase of approximately 0.6 g/dL Hb compared to control groups
Radtke H, et al ⁶⁴	Randomized placebo-controlled study	Donors assigned to treatment with either 40 mg, 20 mg, or 0 mg per day of elemental iron as ferrous gluconate for a period of 6 months, during which one unit of whole blood was collected on 4 occasions in males or 3 in females	Daily doses of either 40 mg or 20 mg of ferrous gluconate adequately compensated for iron loss in males, who gave blood at 2-month intervals, but did not result in a positive iron balance or an increase in storage iron, while in females who donated at 3-month intervals, the same daily doses restored the iron balance and increased storage iron
Radtke H, et al ⁶⁶	Randomized, two arm study with placebo phase	Donors were randomly assigned to receive 100 mg of ferrous iron or placebo daily. Group A received iron capsules after the first three donations, and Group B after the second three donations	Mean serum ferritin concentration decreased after each donation in the placebo phase of both treatment groups, but it remained mostly constant during the iron phase in Group A, and even increased during the iron phase in Group B
Macher S, et al ⁶⁷	Randomized, controlled, single-centre trial	Ferric carboxymaltose 1000 mg IV as a single dose or oral iron supplementation (100 capsules of 100 mg iron fumarate, 1 capsule/day for 100 days)	Significant improvement in the severity of symptoms for RLS, fatigue and sleep quality ($p < 0.001$). Significant decrease in headaches, dyspnea, dizziness, and palpitations ($p < 0.05$) with no difference between intravenous vs oral iron and clinical outcome

Abbreviations: Hb, hemoglobin; CI, confidence intervals; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; TS, transferrin saturation; IV, intravenous; RLS, restless leg syndrome.

development of iron deficiency in blood donors.⁵⁷ Of the donors receiving ferrous sulfate, intolerance requiring a switch to ferrous gluconate developed in 21%, but only 5% of donors were intolerant to both products and discontinued iron replacement therapy.⁵⁷ In a randomized, double-blind, placebo-controlled study, premenopausal women presenting with fatigue, serum ferritin ≤ 50 ng/mL, and hemoglobin ≥ 12 g/dL were randomized to receive either 800 mg of intravenous iron sucrose or placebo. Fatigue and serum iron status were assessed at baseline and after 6 and 12 weeks. Overall, 82% of iron-treated compared with 47% of placebo-treated patients reported improved fatigue, a significant difference. Drug-associated adverse events were observed in 21% of iron-treated patients and in 7% of placebo-treated ones, but none of these events was serious.⁵⁸ A Danish study showed that IV iron isomaltoside before the second and third blood donation was well tolerated and increased the hemoglobin, improved plasma ferritin and transferrin saturation, and also improved fatigue symptoms.⁵⁹ A randomized study comparing ferrous gluconate vs placebo for 24 weeks after blood donation showed that only donors on iron supplementation replaced their iron stores.⁶⁰ A study of female regular blood donors in Thailand showed that a combination of ferrous fumarate 200 mg and ascorbic acid 500 mg daily was well tolerated, and effective to accelerate hemoglobin recovery to the pre-donation values.⁶¹ In a clinical trial of blood donors with ferritin <30 ng/mL, donors were randomized to either a single dose of intravenous ferric carboxymaltose or oral iron fumarate. A single dose of ferric carboxymaltose was highly effective to prevent IDWA/IDA, although oral iron was an acceptable alternative.⁶² A US study examined the efficacy of two different doses of elemental iron (38 mg vs 19 mg, vs placebo vs nothing) in 692 frequent blood donors. Withdrawal from the study was more common in those taking iron pills (39% vs 7%), but no difference in adverse events was seen between those receiving iron or placebo, and moreover, those taking iron had improved iron status regardless of the dose.⁶³ A German study also showed that only 20 mg of elemental iron per day as ferrous gluconate for 6 months could adequately compensate for iron loss in donors who donated WB up to four (females) or six (males) times per year.⁶⁴ The same group in a subsequent study showed that 20 mg of elemental iron as ferrous gluconate and 400 mg of ascorbic acid daily for only 30 days post-donation adequately compensated for iron loss in most WB donors.⁶⁵ The same authors also examined the effect of supplementation with 100 mg of oral ferrous iron-glycine-sulfate-complex vs placebo after each of three, two-unit red cell apheresis, and demonstrated its efficacy in preventing iron deficiency.⁶⁶ Finally, Macher et al, compared the frequency and severity of symptoms associated with iron deficiency before and after intravenous or oral iron supplementation in iron-deficient blood donors. Clinical symptoms were evaluated by a survey before and after 8 to 12 weeks of iron therapy. A significant improvement in well-being was reported with both oral and parenteral iron therapy, while there was no difference between the type of iron supplementation and clinical outcome.⁶⁷

The Recommended Dose of Oral Iron Supplementation for Regular Blood Donors

The Panel on Micronutrients of the Institute of Medicine has established the tolerable upper limit of daily elemental iron intake at 45 mg. By subtracting the approximately 11 mg/day average dietary iron intake of a western diet, this allows for approximately 34 mg of supplemental iron before gastrointestinal side effects are seen.⁶⁸ The STRIDE study of blood donors' iron supplementation supports this figure since adverse events did not differ among subjects receiving elemental iron 19 or 38 mg/day or placebo, and supplements were effective at both doses for alleviating iron deficiency in regular blood donors.⁶³

Recovery of Blood Collected from Donors with IDWA Compared to Iron-Replete Donors

An important issue is whether the recovery of transfused blood is diminished in donors with IDWA compared to iron-replete ones. A multicenter study of 13,403 donors that evaluated stored blood for osmotic and oxidative hemolysis and iron status by plasma ferritin measurement and self-reported intake of iron supplements, showed that prior donation intensity was negatively associated with in vitro oxidative hemolysis in multivariate analyses correcting for race-ethnicity, age, and sex.⁶⁹ Moreover, in healthy human volunteers, a high proportion of storage-induced microerythrocytes in long-stored red blood cell concentrates correlates with poor transfusion recovery.⁷⁰ The clinical

significance of this information is unknown and remains to be determined by future studies with carefully selected endpoints.

Potential Adverse Effects of Oral Iron Supplementation in Regular Blood Donors

Regular blood donors are healthy, and they may object to taking oral pills daily, a maneuver that is viewed as turning them into patients. The STRIDE study, which, as previously explained, randomized 692 donors into one of two educational groups, or one of three interventional groups showed that de-enrolment within 60 days was more common in the interventional group, although not more common in those receiving iron than placebo.⁷¹ In a study of 1974 blood donors, 89% of whom were between the ages of 18 and 50 years, 49% declared that they would rather visit their general practitioner and 46% would rather receive iron supplementation. A higher number of donations was significantly associated with a preference for iron supplementation, and regular female donors were even more willing to take iron supplements.⁷²

An important issue with oral iron supplementation in regular blood donors is safety, given the potential association of intravenous iron administration with infections.⁷³ In this regard, a study of 82,062 Danish blood donors, who completed a questionnaire on health-related items showed that iron supplementation was safe, as it was not associated with an increased risk of infections requiring hospitalization or oral administration of antimicrobials.⁷⁴

Another concern with providing iron to all blood donors is the possibility of worsening iron overload in donors with undiagnosed hemochromatosis, masking the symptoms of a clinically important gastrointestinal hemorrhage or other underlying medical conditions -most importantly gastrointestinal cancer, and causing side effects, mainly from the gastrointestinal tract. Finally, the potential risk of accidental iron poisoning in children of donors should be considered, since iron salts remain the leading cause of death from pediatric accidental poisoning.^{75,76}

Conclusions

As shown by numerous studies, IDWA and IDA are very common among regular blood donors. There is an ethical obligation of blood bank physicians to preserve the health of volunteer blood donors by limiting iron deficiency, but without compromising the precious blood supply. The latter may be at risk due to the shrinking blood donor base and the potentially increasing blood requirements imposed by the aging population and other reasons, eg, climate change and its associated medical disasters. The optimal strategies to achieve that are not totally clear and ongoing clinical research will clarify unresolved issues. Pending further research in this area, most regular blood donors will benefit from the systematic administration of iron supplements post-donation. The AABB bulletin #17-02 correctly advocates low-dose iron supplements (≥ 18 mg/day for 60 days after WB donation or after every fourth or fifth apheresis donation), longer inter-donation intervals to allow dietary recovery of iron stores, or ferritin testing to help donors decide on ways of improving their iron status.^{22,26} While providing donors with iron supplements is effective in preventing declines in ferritin concentration and lowers the possibility of future donation deferrals for low hemoglobin, many factors should be taken into consideration when prescribing iron supplements in blood donors, such as the cost, potential side effects, availability of blood bank physicians for providing education in preventing iron deficiency, establishing responsibility for donors prescribed iron, etc.⁷⁷ A double-blind, randomized controlled trial is underway to optimize the efficacy of oral iron supplementation while minimizing gastrointestinal complaints in WB donors (FORTE trial). Twelve hundred of them with ferritin ≤ 30 $\mu\text{g/L}$ will be randomly assigned to one of six arms, ie, to receive capsules containing 0 mg, 30 mg, or 60 mg of iron, either on alternate days or daily for 56 days. At baseline and on days 56, 122, and 182 of follow-up, ferritin and hemoglobin will be measured, along with an assessment by questionnaire of the general health, compliance, dietary iron intake, gastrointestinal side effects, and iron deficiency-related symptoms.⁷⁸ Finally, educational efforts should be intensified to improve the awareness of blood donation as a primary cause of iron deficiency among physicians of all specialties.

Abbreviations

AABB, American Association of Blood Banks; IDA, iron deficiency anemia; IDWA, iron deficiency without anemia; MCH, mean corpuscular (cell) hemoglobin; MCHr, mean cell hemoglobin of reticulocytes; MCV, mean corpuscular

(cell) volume; Ret-He, reticulocyte hemoglobin content; RLS, restless leg syndrome; sTfR, soluble transferrin receptors; TIBC, total iron binding capacity; TSAT, transferrin saturation; US, United States of America; WB, whole blood; ZPP, zinc protoporphyrin.

Consent for Publication

All authors approved the submitted text and are accountable for all aspects of this work.

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.

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