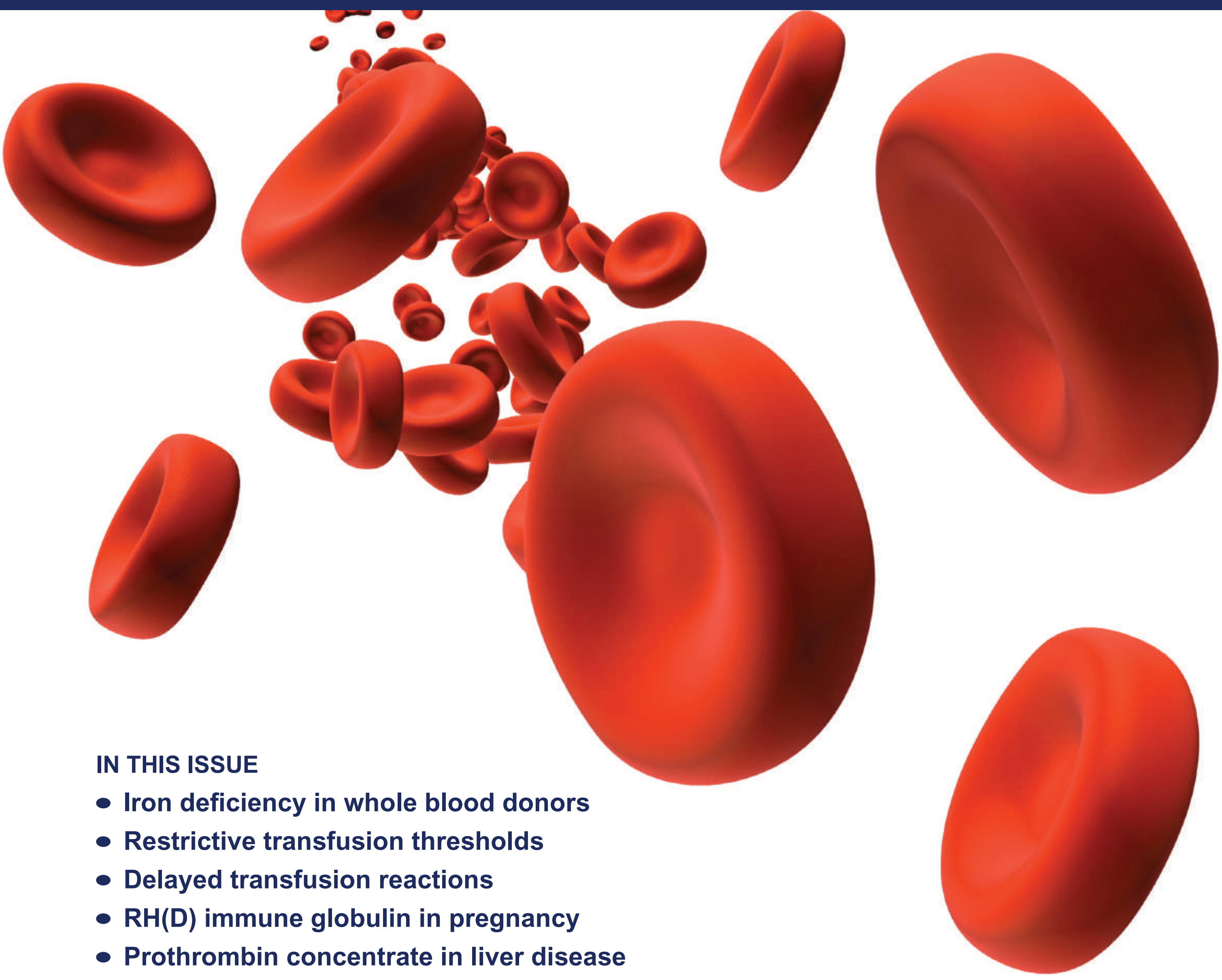
Volume 33 Number 3 • June 2023

TRANSFUSION MEDICINE

Official Journal of the British Blood Transfusion Society and the Network for the Advancement of Patient Blood Management, Haemostasis and Thrombosis



IN THIS ISSUE

- Iron deficiency in whole blood donors
- Restrictive transfusion thresholds
- Delayed transfusion reactions
- RH(D) immune globulin in pregnancy
- Prothrombin concentrate in liver disease

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An international journal published for the British Blood Transfusion Society

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Periodical ID Statement

Transfusion Medicine (ISSN 0958-7578), is published bimonthly. US mailing agent: Mercury Media Processing, LLC 1850 Elizabeth Avenue, Suite #C, Rahway, NJ 07065 USA. Periodical postage paid at Rahway, NJ. Postmaster: Send all address changes to *Transfusion Medicine*, John Wiley & Sons Inc., C/O The Sheridan Press, PO Box 465, Hanover, PA 17331.

Publisher

Transfusion Medicine is published by John Wiley & Sons Ltd, 9600 Garsington Road, Oxford, OX4 2DQ, UK. Tel: +44 1865 776868; Fax: +44 1865 714591.
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ISSN 0958-7578 (Print)
ISSN 1365-3148 (Online)

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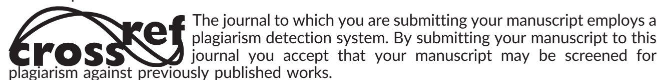
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Transfusion Medicine

Volume 33, Number 3 June 2023

COMMENTARY

197 Critical appraisal of knowledge, attitude and practice studies for blood donation in India
R. CHAURASIA, G. K. PATIDAR, H. C. PANDEY, S. PALANISAMY, L. NASEER, S. CHOPRA AND V. GUPTA

REVIEW

205 The use of prothrombin complex concentrate in chronic liver disease: A review of the literature
M.-A. VAN DIEVOET, X. STEPHENNE, M. ROUSSEAUX, T. LISMAN, C. HERMANS AND V. DENEYS

ORIGINAL ARTICLES

213 Iron deficiency in whole blood donors in a resource-poor setting: A cross-sectional study in Uganda
A. DHABANGI, R. SSENYONGA, G. SIU, S. A. ELABOROT, D. KYEYUNE AND I. BATES

221 Evaluation of two temperature storage conditions for full blood count samples from Lifeblood's donors
G. JACKO, A. GREEN, L. LYCETT, S. ISMAY, J. PEBERDY AND J. DALY

227 A second look at the mortality in thrombotic thrombocytopenic purpura score: An external validation study
H. ANDANAMALA, N. BRUNTON, R. PAI AND O. SOSTIN

232 Improvement of platelet preservation by inhibition of TRPC6
Y. TAN, W. LU, X. YI, H. CAI, Y. YUAN AND S. ZHANG

244 Rh(D) immune globulin administration in pregnancy: Retrospective audit of patient safety events followed by targeted educational intervention with Bayesian analysis
D. HAGE, K. PYRA, C. MCCUDDEN AND R. PADMORE

SHORT COMMUNICATIONS

254 Real world reduction in red cell transfusion with restrictive transfusion threshold in haematology inpatients
O. PERVAIZ, J. DHANAPAL, L. PILLAI, S. PAVORD, H. LEARY, T. EYRE, A. PENIKET, J. STAVES, P. POLZELLA AND M. J. R. DESBOROUGH

257 Cultured CD71^{+} erythroid cells modulate the host immune response
A. ALSHALANI, B. M. BEUGER, R. VAN BRUGGEN, J. P. ACKER AND N. P. JUFFERMANS

263 Using trends and outliers in managing delayed transfusions
B. BAHAR, E. A. GEHRIE, Y. D. MO, C. JACQUOT AND M. DELANEY

CASE STUDY

268 Hemolysis during therapeutic plasma exchange: A rare phenomena
B. KAKKAR, S. MELINKERI, S. NARAWADE, S. KETKAR AND S. KULKARNI

LETTERS TO THE EDITOR

271 Reflections of a re-engaged donor. A response to 'on consciousness of the decision to discontinue blood donation: Intention to return and effective recovery activities'
S. P. HIBBS

272 The lack of anti-PF4 antibodies in convalescent plasma from COVID-19 infected blood donor
A. PICCIN, B. MULLIN, A. BROWN AND G. BENSON

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Received: 4 January 2022 | Revised: 16 April 2022 | Accepted: 26 July 2022
DOI: 10.1111/tme.12904

COMMENTARY

TRANSFUSION MEDICINE WILEY

Critical appraisal of knowledge, attitude and practice studies for blood donation in India

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Abstract

Background and Objectives

A critical appraisal of the literature helps to assess the strength and weakness of the research and suggests ways to improve future research. Our aim was to critically appraise the knowledge, attitude, and practice (KAP) studies conducted in India for blood donation.

Materials and Methods

Of 70 articles identified in our search on PubMed, Scopus and Google Scholar, 32 were assessed for quality using an appraisal tool for cross-sectional studies (AXIS) and questionnaire items.

Results

Quality assessment revealed that only 6 of 32 studies had acceptable reporting (≥80% score on the AXIS tool). The most frequently identified shortcomings were failure to address the non-responders, lack of justification for sample size, assessment of outcome variables and demographic results for the survey. Our evaluation of questionnaires revealed that knowledge for need for blood donation, its benefits and site/place for blood donation were assessed by very few studies.

Conclusion

Most published KAP studies for blood donation in India were not appropriately described, especially the methodology and result section. These deficiencies could have led to suboptimal interpretation of the prevalent issues. Use of an open-ended and validated KAP questionnaire with a problem-based approach and inclusion of participants from various socio-cultural

KEYWORDS

KAP, Blood, Transfusion, Donor, Medecine

1 | INTRODUCTION

The incidence of hip fractures continues to increase, along with the global expansion of aging population observed secondary to improved healthcare and quality of life.[1] Subtrochanteric fractures are defined as fractures encountered between the inferior border of lesser trochanter and 5 cm distal to it.[2] They represent a complex subset of injuries surrounding the hip, which are most commonly managed with intramedullary (IM) nailing.[3,4] However, their moderate blood supply and being subjected to high concentration of stresses[5-8] meant these

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have long been the standard method to assess iron status. However, haemoglobin levels can remain sufficient for some time, even when iron stores are dwindling; this is known as iron deficiency non-anaemia.[1]

In contrast to haemoglobin, serum ferritin levels reflect the amount of stored iron.[1] Therefore, they are increasingly used to assess individuals' iron stores when these are at risk, for instance after traumatic blood loss, during pregnancy, or in blood donors.[3] Sanquin, the national blood service in the Netherlands, started measuring ferritin levels in each new donor, and subsequently after every fifth donation, in October 2017. Donating blood has a substantial impact on ferritin levels. Ferritin levels are lower among blood donors than in the general population: cross-sectional studies report lower ferritin levels in donors with a higher number of whole blood donations and a large randomised trial showed that ferritin levels indeed decline with more frequent blood donations.[4,5] Among new donors, large variation in ferritin levels is observed.[4] It is well established that individual characteristics such as sex and age are relevant: women in general, but pre-menopausal women in particular, have considerably lower ferritin levels than men.[4,6,7] Higher body mass index (BMI) is associated with higher ferritin levels.[8] In recent decades, many other factors that affect iron status have been identified: diet,[9,10] genetics,[11,12] ethnicity,[13] and iron supplementation, which is mostly studied among blood donors.[14,15]

Ferritin is also a known acute-phase protein that is elevated in inflammatory conditions, complicating its diagnostic value in individuals with conditions such as inflammatory bowel disease or chronic heart failure.[16] This could also explain the association between BMI and ferritin levels, as adipose tissue is known to promote systemic inflammation.[17] Additionally, exposure to environmental pollutants has been linked to disordered iron homeostasis,[18,19] and ambient particle matter (PM) concentration is correlated with ferritin levels.[19] The biological mechanism behind this is still unclear, but it is postulated that iron attaches to the PM rather than to cell nuclei, effectively creating a functional deficiency.[18,19] In turn, mechanisms start upregulating iron uptake and recycling in an attempt to meet the iron requirement of the cells, thereby altering iron homeostasis. Another suggested mechanism is that when pollutants enter the lungs, iron is transported away from the surface of the lung tissue and stored in ferritin complexes, in order to avoid chemical reactions between iron and the pollutant.[18] Other potential environmental determinants are neighbourhood characteristics, including population density and socio-economic status, which are consistently shown to be related to body weight[20] and blood parameters.[21]

Previous studies on ferritin levels have focused on studying the association with variables in a limited setting, for example, characteristics such as age and BMI, donation-related variables, or environmental pollutants. In this paper, we propose a novel framework that integrates multiple settings, using a structural equation model. By grouping relevant explanatory variables into constructs, we describe relationships with ferritin on a more general level. This enhances the insight into various mechanisms that influence ferritin levels, which is valuable to those who use these as a diagnostic tool. We explore associations between ferritin levels and individual characteristics, donation behaviour and environmental factors, in a large group of newly registered and active whole blood donors.

2 | METHODS

For this cross-sectional study, data collected by Sanquin and the Geoscience and health cohort consortium (GECCO) were analysed. Sanquin is by law the only blood service in the Netherlands, collecting over 400 000 whole-blood donations each year, with collection sites geographically well-distributed throughout the country. Several eligibility criteria exist to ensure the safety of the donors and recipients and the quality of the blood product. Donors must be aged between 18 and 79 years old, and a pre-donation screening visit takes place before the first 500 ml whole blood donation, which includes blood sampling for blood type and infectious disease testing, as well as initial haemoglobin and ferritin measurements. We will refer to these prospective donors, who have not donated yet, as ‘new donors’.

Before every donation, a donor screening is performed, including a donor health questionnaire and measurements of blood pressure, pulse rate and haemoglobin levels to assess whether the donor is eligible to donate. Haemoglobin levels need to be at least 7.8 mmol/L for women and 8.4 mmol/L for men. This is measured by point-of-care testing with a photometer (HemoCue, Angelholm, Sweden). Ferritin levels, are measured in serum samples, using the Architect i2000 (Abbott Diagnostics, Chicago, IL), after the pre-donation screening visit and after every fifth whole blood donation. As such, ferritin measurements are only available in case of successful whole blood donations, and for new donors whose venous samples are taken as part of the pre-donation screening visit.

2.1 | Data

This study included all new and active whole blood donors who gave consent to the use of their data for scientific research (consent given by >99% of all donors) and for whom ferritin measurements were available between 1 October 2017 and 31 December 2019. If multiple ferritin measurements were available for a donor, only the first measurement was used. Information on donors and donation histories was extracted from the blood bank information system (ePROGESA, MAK-SYSTEM International Group, Paris, France). Variables used were sex, age, height, weight, time since previous successful donation, the number of successful donations in the previous 2 years, donor status (new or active donor), and ferritin levels. BMI was calculated from self-reported donor height and weight. Sanquin does not register donor ethnicity, but Duffy negative phenotype was included to function as a proxy for sub-Saharan African descent.

Environmental exposure variables of various characteristics were obtained from the Geoscience and health cohort consortium (GECCO).[22] The exposure data were operationalised based on publicly

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TABLE 1 Grouping of variables into constructs for each model

Variable	Model A	Model B	Model C	Model D
Age	Individual characteristics	Individual characteristics	Individual characteristics	Individual characteristics
Weight				
Height				
BMI				
Duffy phenotype				
Time since previous donation[a]	Donation history	Donation history		
Number of previous donations[a]				
Population density	Environment	Environment	Environment	Environment
Temperature				
Socio-economic status				
Ozone	Pollution		Pollution	
PM2.5				
PM10				
Soot				
NO_2				

Note: All models contain the same observed variables but differ in how these are grouped into latent constructs.
[a]Only available for active donors.

available data. Data from 30 weather stations in the Netherlands—obtained from the Royal Netherlands Meteorological Institute (KNMI)—were used to estimate temperature at a spatial resolution of 1 km. Three options for the measurement level were considered (minimum, average, and maximum daily temperature), as well as three time spans (day, week or month before donation), resulting in nine options in total. The combination that showed the highest correlation with ferritin was included in the final model.

Daily concentrations for particulate matter (PM) 2.5, PM10, NO_2, ozone and soot levels were obtained via the Dutch National Institute for Public Health and the Environment (RIVM), for the years 2017–2019. These variables were imputed on a spatial resolution of 1 by 1 km. Neighbourhood socio-economic status (SES) scores and population density from 2017–2019 were acquired from Statistics Netherlands (CBS), both available on 6-digit postal code level. SES scores are based on percentiles of income, education level and vocational history of households, with a score of 0 being exactly the national average, and positive scores being above average. All spatio-temporal variables were matched with donor and donation data based on donation date and donor postal code. Lastly, the date and time of each donation were included as potential factors to account for seasonal and diurnal variation, as they are known to affect haemoglobin levels and may also affect ferritin levels.

To check for a possible confounding effect of smoking on environmental variables, we analysed the correlation between the percentage of smokers per municipality (data from Statistics Netherlands) and all environmental variables described in the above paragraph.

There were no missing data for environmental datasets from the RIVM and CBS. Donors with no ferritin measurement were excluded from the analysis. There were no missing data for the other donor or donation level variables.

2.2 | Statistical analysis

Structural equation modelling (SEM) was used to investigate which variables relate to serum ferritin and to what extent. Briefly, observed variables and latent constructs are distinguished in SEM. Latent constructs cannot be measured or observed directly, but are inferred from the observed variables. One or more hypothesized sets of relationships and correlations between variables and constructs are specified a priori and shown in a path diagram. For each relationship, a parameter is estimated that indicates its strength. Estimates are obtained by numeric optimization of a fit criterion, using maximum likelihood estimation. A more detailed overview of this method is provided in Appendix A.

We compared four ways to divide the 15 variables included in the analysis into latent constructs, as shown in Table 1. Date and time of the donation were added to the model separate of the constructs, and as such are not included in Table 1. Model A contains four latent constructs, and in models B, C and D different sets of constructs are combined. Confirmatory factor analysis (CFA) was used to test the validity of the specified measurement models, that is, the hypothesized relationships between the latent constructs and their observed variables. The overall fit of the models was assessed by the Tucker-Lewis Index (TLI) and the root mean square error of approximation (RMSEA). A rule of thumb is to exclude variables for which the absolute value of the standardised factor loading is below 0.4, but at sample sizes larger than 300, if the overall model fit is good, exclusion is not necessary and should be judged separately for each variable based on sensible background knowledge.[23]

Pairwise residual correlations between observed variables were calculated to identify whether any covariances needed to be added to the model. Of the four specified models, we continued our analysis with the best fit according to CFA, based on the TLI and RMSEA.

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TABLE 2 Distribution of explanatory variables by donor status and sex

	New donors		Active donors	
	Women	Men	Women	Men
N	40 172	19 424	39 085	39 233
Age (years)	26 (21–37)	28 (23–37)	47 (31–58)	53 (39–62)
Height (cm)	170 (166–175)	183 (178–188)	170 (166–175)	183 (178–188)
Weight (kg)	68 (62–77)	82 (74–90)	70 (64–80)	85 (78–93)
BMI (kg/m^2)	24 (21–26)	24 (22–27)	24 (22–27)	25 (23–27)
Time since previous donation (days)	NA	NA	154 (132–217)	139 (71–147)
Number of previous donations[a]	NA	NA	3 (2–4)	5 (4–7)
Population density (inhabitants per km^2)	1173 (425–2617)	1246 (477–2936)	827 (322–1855)	814 (320–1824)
Duffy phenotype (proportion)	0.25	0.17	0.28	0.16
Temperature (°C)[b]	11.4 (6.4–16.6)	11.7 (6.6–16.7)	10.4 (6.0–16.0)	10.4 (5.9–16.0)
Socio-economic status	0.04 (−0.21 to 0.22)	0.02 (−0.24 to 0.22)	0.10 (−0.10 to 0.25)	0.12 (−0.07 to 0.26)
Ozone (μg/m^3)	46.9 (45.6–48.8)	46.8 (45.5–48.7)	47.2 (45.9–49.2)	47.2 (45.9–49.1)
PM2.5 (μg/m^3)	10.7 (9.7–11.6)	10.7 (9.8–11.6)	10.5 (9.6–11.5)	10.6 (9.7–11.6)
PM10 (μg/m^3)	18.2 (16.8–19.3)	18.2 (16.9–19.3)	18.0 (16.6–19.0)	18.0 (16.7–19.1)
Soot (μg/m^3)	0.66 (0.54–0.78)	0.66 (0.55–0.78)	0.63 (0.52–0.75)	0.65 (0.54–0.76)
NO_2 (μg/m^3)	17.6 (14.9–21.6)	17.8 (15.1–21.8)	16.8 (14.2–19.7)	16.9 (14.3–19.6)
Ferritin (ng/ml)	47 (28–75)	118 (79–170)	30 (17–47)	34 (20–56)

Note: Data are presented as medians (interquartile range) due to non-normal distributions of the variables.
[a]Within 2 years before the ferritin measurement.
[b]The maximum temperature recorded on the day of donation.

To the model with the best fit, we added the structural component, which contains the relationships between the latent variables and ferritin, the outcome variable. A multiple group SEM was carried out with parameters estimated separately for male and female donors, and for new and active donors. Because the assumption of normality of the explanatory variables does not hold in our data, a different estimator than the default maximum likelihood estimator was used: the 'mean and covariance adjusted weighted least squares estimator', which is robust against violations of the normality assumptions in a multivariate setting.[24]

The same model was fitted in all four groups, although the variables belonging to the *donation history* construct (see Table 1) are not available for new donors, as they do not (yet) have a donation history. The overall fit of the SEM model was assessed using the TLI and RMSEA, as well as the R^2 measure.

All analyses were conducted using *R* programming language and environment for statistical computing version 4.0.3,[25] with package *zoo*[26] for pre-processing environmental data, and *lavaan*[27] for CFA and SEM analyses. Path diagrams were created with yEd Live Graph Editor.[28]

3 | RESULTS

3.1 | Sample composition

Table 2 shows descriptive statistics of the study population by sex and donor status. The size of each of the groups was comparable, except for the group of new male donors, which was only half the size of the other groups. Between new and active donors, age differed considerably, new donors being younger than active donors by 17 years on average ($p < 0.001$ using a two-sample *t*-test). In both new and active donors, men were older (by 6 years on average, $p < 0.001$) and heavier (by 13 kg on average, $p < 0.001$) than women. *p*-values were obtained using two-sample *t*-tests. The time since last donation is higher in women than in men, and the number of prior donations is higher in men than in women. These differences are due to differences in the minimum required donation interval: for women, there must be 122 days between two donations with a maximum of 3 donations per year, while for men, the minimum is 57 days between two donations with a maximum of 5 donations per year. Differences in ferritin levels between the groups are as expected from previous studies: men have higher ferritin levels than women, and repeat donors have lower ferritin levels than new donors.

For pollution and environmental variables, there was little difference between the groups, any differences between new and active donors were most likely due to the different age and geographical distribution of the groups. None of these differences were statistically significant.

We found a weak correlation between the percentage of smokers and SES score (Pearson's $r = -0.4$) and a moderate correlation between the percentage of smokers and population density (Pearson's $r = 0.5$). No correlation was found for any of the other environmental variables.

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3.2 | Model selection

CFA did not provide support for the *environment* construct as defined by the three variables *temperature*, *population density* and *socio-economic status*. These variables did not share a high proportion of their variance and consequently there was no convergent validity, effectively ruling out models A and C. In models B and D, variables *Duffy phenotype*, *temperature*, *SES* and *height* were omitted due to very low factor loadings (<0.05). The factor loading for variable *age* was also low (0.35) but this variable was not excluded, as it is expected that this factor loading would be small, considering the other variables in the construct (*weight* and *BMI*) are much more closely related. All other factor loadings were above the suggested threshold of 0.6. All latent constructs (individual characteristics, donation history and environment) showed convergent and discriminant validity in models B and D. Variables *time* and *day of year*, which were added to the model outside the constructs, were also dropped due to very low factor loadings (<0.05).

The presence of a *donation history* construct was the only difference between models B and D, and since new donors do not yet have a donation history, the models only differed for active donors. Model B had a TLI of 0.961 and RMSEA of 0.063, while model D had a TLI of 0.932 and RMSEA of 0.083. Based on these fit measures, model B fit the data best, and was therefore used in the remainder of the analyses.

Based on inspection of the pairwise residual correlations between all observed variables, two covariance terms were added to the model: one for *PM2.5* and *PM10* (residual correlation 0.092–0.102, depending on sex/donor status), and one for *age* and *population density* (residual correlation −0.151 to −0.149, depending on sex/donor status). We also added one covariance term for *weight* and *BMI*, as BMI was calculated using weight and was therefore inherently dependent.

3.3 | Parameter estimates

Figure 1 shows the structure of the final model and the parameter estimates for new donors. Parameter estimates were similar for both sexes, but factor loadings for variables belonging to the *individual characteristics* construct were higher for women than for men, indicating more shared variance. Factor loadings in the *environment* construct did not differ between sexes, showing that the covariance structure of those variables was not dependent on sex. The parameter estimates for the regression coefficients show the relative importance of each latent construct for the outcome variable. Table 3 shows the percentage of variance in ferritin levels that is explained by each construct for each model, adding up to the total percentage of variance explained.

Figure 2 shows the final model for active donors. As in new donors, factor loadings in the *individual characteristics* construct were higher for women than for men, and they were also higher for new donors than for active donors. The relative importance

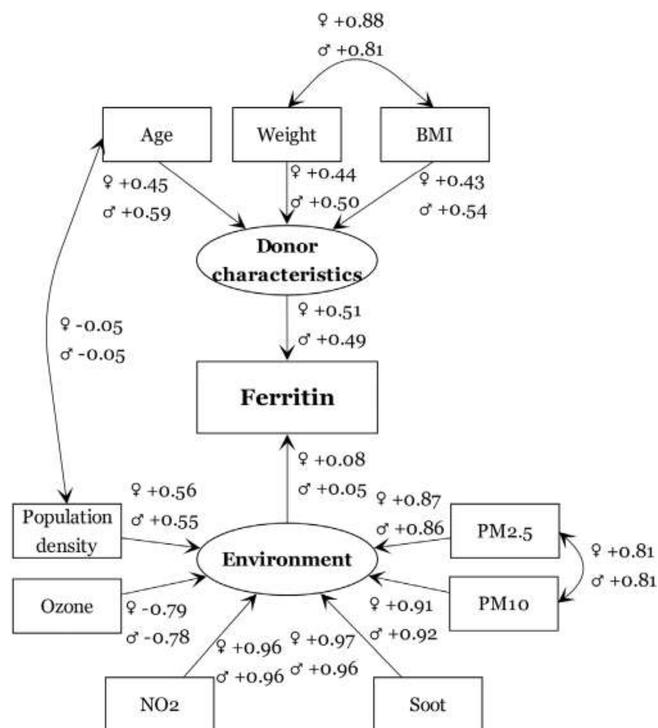

FIGURE 1 Final structural equation model for ferritin determinants in new donors, with parameters estimated separately for men and women. All parameter estimates are standardised so that the variance of each observed variable and latent construct equals 1

TABLE 3 Relative contribution to explanation of variance of ferritin levels per model

	New donors		Active donors	
Construct	Women	Men	Women	Men
Individual characteristics	23%	23%	20%	17%
Donation history	NA	NA	14%	25%
Environment	2%	2%	5%	4%
Total % of variance explained	25%	25%	39%	46%

of individual characteristics and donation history was opposite for both sexes: for men, donation history was correlated with ferritin levels more strongly than individual characteristics (0.66 vs. 0.45), while this was reversed for women (0.43 vs. 0.61). The regression coefficient of the *environment* construct is 0.15 for women and 0.10 for men. The *environment* construct explains twice as much variation in ferritin levels in active donors as in new donors.

As for overall model fit, with a TLI of 0.981 and 0.979 and RMSEA of 0.052 and 0.042, for new and active donors respectively, both models fit very well when compared to commonly used thresholds (TLI > 0.95, RMSEA < 0.06).[29] R^2 was calculated separately by sex: for new donors, R^2 was 0.251 for men and 0.252 for women, and for active donors, 0.458 for men and 0.393 for women.

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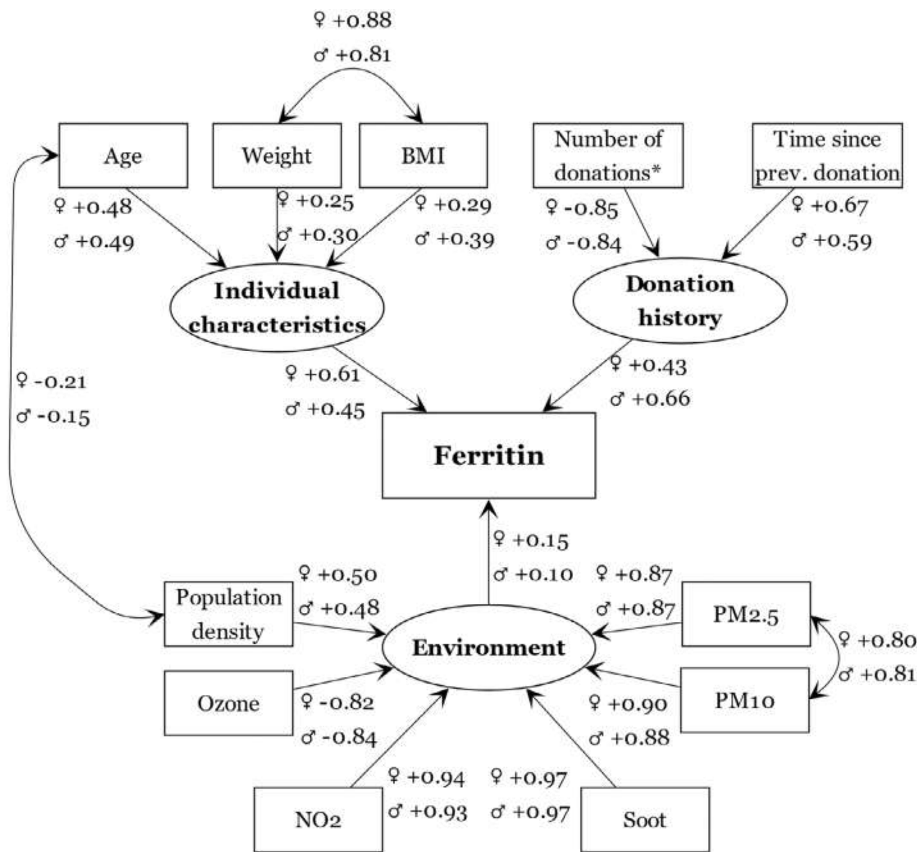

FIGURE 2 Final structural equation model for ferritin determinants in active donors, with parameters estimated separately for men and women. All parameter estimates were standardised so that the variance of each observed variable and latent construct equals 1

4 | DISCUSSION

This study investigated the impact of individual and environmental determinants on ferritin levels in Dutch individuals, using SEM. The model was able to explain 25% of ferritin level variance in new donors for both sexes, and 46% and 39% in active donors for male and female donors, respectively.

We found the construct composed of individual characteristics (age, weight, and BMI) to be the most important determinant of ferritin in female active donors, followed by donation history (time since previous donation, number of donations in the past 2 years). For male active donors, this was the opposite: donation history was a more important determinant than individual characteristics. In both sexes, environmental factors are associated with ferritin levels, albeit to a lesser degree than individual characteristics and donation history.

The relationship between ferritin levels and anthropometric characteristics is well-documented, and the positive correlations we found for ferritin with age, weight and BMI are consistent with those found in other studies.[4,15,30] Men have much higher ferritin levels than women in general and show a larger decrease in ferritin levels after repeated donations. As a result, ferritin levels in active donors are similarly low for women and men.[4] The *donation history* construct explained more variance in ferritin levels in men than in women. Although often not explicitly mentioned, this discrepancy is also found in previous studies, with stronger relationships between variables regarding donation history and ferritin for men than for women.[15] A reasonable explanation for this is that men commonly display more variation in donation history variables due to the possibility of more frequent donations: in many blood services, men are allowed to donate more often than women and are usually less frequently deferred for low haemoglobin levels.[31]

From previous epidemiological studies, we know that environmental factors may play a role in iron metabolism, and that certain pollutants can disrupt iron homeostasis.[32] Our study shows that although environmental factors are less strongly associated with ferritin levels than individual characteristics and donation history, their effects are far from negligible. Because of the wide reach of environmental exposures over geographic areas, even a relatively small influence on individuals can result in a large effect on the population level. As this study includes only data from the Netherlands, which is a relatively small country, associations between environmental variables and ferritin levels were not very strong, as was expected. Repeating this study on a larger, or even global, scale may result in finding a more substantial effect.

Higher values for all but one environmental factor (ozone) were positively correlated with higher ferritin levels. These findings support the hypothesis that air pollution causes higher ferritin levels. The underlying mechanism may be that when certain pollutants enter the lungs, iron is transported away from the lung tissue surface and stored in ferritin complexes to avoid chemical reactions between iron and the pollutant.[18,33] This would imply that using serum ferritin as a proxy for total body iron is less reliable when there is significant air pollution.

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The *environment* construct was more strongly associated with ferritin level in active donors than in new donors. In new donors, environmental factors explain 2% of variance in ferritin levels, while in active donors this increases to 4%–5% depending on sex. This indicates that environmental factors are more important for ferritin recovery after blood loss than for naive ferritin level. A plausible explanation for this difference is that since both exposure to air pollution and donating blood causes significant disruptions to iron homeostasis, these disruptions may interact and together have a larger effect than simply additive.

SEM is a technique well-suited to test hypotheses on how different factors interact and correlate with a specific outcome like ferritin levels, especially when there are many factors to consider. Compared to multiple (linear) regression, more complex models can be tested, and for each variable measurement error is taken into account.[34] Moreover, the percentage of variance explained by groups of related variables can be calculated and compared. The stratified approach in this study also adds to the model validity: parameter estimates can be compared across groups, allowing discovery of implausible results. Our analyses show that the convergent validity of the *individual characteristics* construct is lower for active donors than for new donors. This may indicate that new donors are a more homogenous group than active donors, which is likely due to the more narrow age range of new donors. Other strengths of this study are its large sample size and collection of data throughout the country.

Two main limitations of this study should be noted: its generalizability and its restricted scope. One might be tempted to generalise the results of new donors to the general Dutch population, as these donors have never donated blood before. However, even new donors form a very specific, generally healthier subgroup of the general population, which means that selection bias has likely been introduced. We can speculate that less healthy individuals would show a higher rate of inflammation, which may cause higher serum ferritin levels. On the other hand, iron deficient or anaemic individuals are likely underrepresented in our sample. As this selection bias most likely reduced variance in ferritin levels, this may have attenuated our results.

Regarding the scope, data on some other potentially important determinants of ferritin levels were not available in this study, the two most important being genetics and diet.[9,10] Several genetic polymorphisms that have an effect on iron pathways have been identified, and these are likely to play a role in the recovery speed of ferritin levels after blood donation.[12,35–37] Dietary behaviour, and in particular heme iron intake, is also a determinant of iron status in donors.[9,15] Information on iron supplementation was also not available for this study. Sanquin does not prescribe oral supplementation of iron to donors, and only a small minority (8.7%) uses iron supplements.[9] Information on donors' smoking status is also expected to add value to the model. Had these determinants been available for our analysis, the proportion of variance explained in donor ferritin levels would likely have increased.

This study presents a model to explain variance in ferritin levels in individuals with or without donation history, based on three types of determinants. The model explained a relatively large part of the variance, especially in active donors. Individual characteristics and donation history form the most important determinants of ferritin levels. Although environmental factors accounted for less variance than the individual and donation history constructs, their contribution is meaningful and statistically significant. When clinicians or researchers use serum ferritin as a proxy for total body iron, they should be aware of this potentially confounding effect.

For blood services that are considering implementing ferritin testing for their donors, these results are of particular value. The results can be of use while the blood service is deciding on a sensible threshold for donation: rather than implementing a one-size-fits-all threshold, environmental conditions in the country can be taken into account. If there is a high level of air pollution, ferritin levels are likely to be overestimated, and thus a higher threshold for donation may be desired. It could even be taken further to make ferritin thresholds more tailored to a specific donor, by taking into account a donor's individual characteristics.

AUTHOR CONTRIBUTIONS

Rosa de Groot, Katja van den Hurk, and Jeroen Lakerveld conceptualised the study; Mart Janssen and Marieke Vinkenoog designed the methodology; Marieke Vinkenoog, Rosa de Groot, and Jeroen Lakerveld curated data; Marieke Vinkenoog did the formal analysis and wrote the original draft; all authors reviewed and edited the manuscript; Jeroen Lakerveld, Katja van den Hurk, and Mart Janssen supervised the study.

ACKNOWLEDGEMENTS

Geo-data were collected as part of the Geoscience and Health Cohort Consortium (GECCO), which was financially supported by the Netherlands Organisation for Scientific Research (NWO), the Netherlands Organisation for Health Research and Development (ZonMw), and Amsterdam UMC. More information: www.gecco.nl. This study was funded by Sanquin Blood Supply Foundation, PPO-C grants 14-028 and 18-14/L2337, and the VU University Medical Center.

CONFLICT OF INTEREST

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

Data collected on prospective and active donors by Sanquin Blood Supply Foundation will not be shared due to privacy reasons. The authors are open to research questions from other researchers; proposals for joint research projects may be made to the corresponding author via e-mail. The environmental exposure data provided by the GECCO institute is based on publicly available data, and can be requested via a data access request form available on the website: www.gecco.nl.

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How to cite this article: Vinkenoog M, de Groot R, Lakerveld J, Janssen M, van den Hurk K. Individual and environmental determinants of serum ferritin levels: A structural equation model. *Transfusion Medicine*. 2023;33(2):113-122. doi:10.1111/tme.12902

APPENDIX A

A.1 | STRUCTURAL EQUATION MODELLING OVERVIEW

Structural equation modelling (SEM) comprises a set of statistical methods that enables researchers to assess the support for hypothesized relationships between variables of interest. Its purpose is to account for variation and covariation of the variables in the model. Many different techniques are included in SEM, this appendix explains the approach taken in this particular study. In SEM, observed variables and latent constructs are distinguished. Observed variables are variables in the traditional sense, which are observations in the data set that have been

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collected by the researcher. Latent constructs are theoretical concepts that cannot be measured, but must be inferred from the observed variables; a well-known example is the latent construct *intelligence* that cannot be measured directly, but can be inferred from observed variables such as scores for an IQ test. Intuitively, observed variables that belong to a latent construct represent the same underlying concept, and latent constructs form in a way a dimensionality reduction of the observed variables. Mathematically, latent constructs represent shared variance of the observed variables related to the construct they belong to.

SEM is composed of two main model components: the measurement model, which shows how observed variables are divided among latent constructs, and the structural model, which shows the relationships between latent constructs and outcome variable(s). First, the measurement model is specified, and test its validity using confirmatory factor analysis (CFA). Often, several measurement models are tested and compared to see which division into latent constructs best fits the data. When the measurement model is considered to have a good fit, the structural part of the model is added, and the model fit is assessed for the full SEM model.

A.1.1. | Measurement model

The validity of the latent constructs must be measured in two ways: each construct must have convergent and discriminant validity. Convergent validity occurs when the observed variables belonging to the latent construct share a high proportion of their variance. This is assessed by the factor loadings of the observed variables onto the latent construct: the higher the (absolute value of the) factor loading, the stronger the indication that this variable belongs to this construct. Very generally speaking, factor loadings greater than 0.4 are acceptable for including a variable within a construct, but this threshold depends greatly on the hypothesized interpretation of the latent variable. Variables with low factor loadings are excluded from the construct.

The discriminant validity of a latent construct is a measure for how well the construct can be distinguished from the other constructs in the model. It is measured by the covariances between latent constructs. A high covariance between two constructs can indicate that these constructs are (partly) overlapping, and thus have no discriminant validity.

If convergent and discriminant validity are satisfactory, model fit indices can be calculated for the measurement model. Commonly used indices are the chi-square test, comparative fit index (CFI), Tucker-Lewis index (TLI) and root mean square error of approximation (RMSEA). The CFI and TLI are both relative measures of fit, and compare the fit of the tested model against a null model, which in CFA means that the means and variances of each variable are freely estimated, but no correlations are included. CFI and TLI are on a scale from 0 to 1, with higher values indicating a better fit of the hypothesized model relative to the null model. The TLI is always more conservative (lower value) than the CFI, because the TLI includes a harsher penalty for the number of parameters estimated. Because the two fit indices are highly correlated, only one should be reported. We chose the TLI because of its more elegant penalty for complexity. Values higher than 0.95 indicate good fit.

The RMSEA is an absolute measure of fit that is not sensitive to large sample sizes, unlike the chi-square test. It uses the covariance matrix of the entire data set and of the fitted hypothesized model, and calculates the differences between these two. This results in a measure between 0 and 1, with lower values indicating smaller differences and better model fit. Cut-offs of 0.08, 0.05, and 0.01 indicate mediocre, good, and excellent fits, respectively.

If multiple measurement models are compared, as in this study, the best fitting model is selected, based on the fit indices described above. If these indicate sufficient model fit, the analysis can be continued with inspection of residual correlation between observed variables. If the pairwise residual correlation between two variables is high (absolute value of 0.1 or higher is a common cut-off), this indicates that these two variables share more variance than is currently captured in the model. If this occurs, the researcher needs to decide whether a covariance term for these two variables should be included in the model. However, this should only be done if there is sufficient theoretical support for an interpretable correlation between these variables. Otherwise there is a risk of overfitting the model to the data; after all, in confirmatory factor analysis we build upon a set of relationships that are hypothesized by the researcher. It is not a data-driven method of finding the best set of relationships. If such an approach is desired, exploratory factor analysis (EFA) can be applied instead of CFA.

A.1.2. | Structural model

The structural component is added to the model once the latent constructs are defined, variables with low factor loadings are removed, and necessary covariance terms are added. The structural component consists of the relationships between latent constructs, or between latent constructs and outcome variable(s). With this, we now have three types of parameters for which an estimate must be calculated:

1. Factor loadings (observed variable $\rightarrow$ latent construct).
2. Covariances (observed variable $\leftrightarrow$ observed variable).
3. Regression coefficients (latent construct $\rightarrow$ latent construct or outcome variable).

Each parameter adds one degree of freedom to the model, and the number of parameters determines the identifiability of the model. Parameter estimates can only be obtained when the number of free parameters (the number of 'unknowns') is equal to or smaller than the number of independent elements in the covariance matrix of the data (the number of 'knowns'), which is equal to $k(k+1)/2$, where k is the number of observed variables in the model. If there are more unknowns than knowns, the model is under-identified and no solution can be found. If the numbers are the same, the model is just identified, and a unique solution can be obtained. If there are fewer unknowns than knowns, we have an over-identified model, which means that

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there is no unique solution but multiple, and we can select the best solution based on fit measures. An over-identified model is desired.

In most software packages parameter estimates are obtained by a maximum likelihood estimator by default, but alternative estimators can be chosen as well. In this study most observed variables did not follow a normal distribution, which violates maximum likelihood estimator assumptions. Therefore, the diagonally weighted least squares (DWLS) method was used instead, which is more robust and provides more accurate parameter estimates in case the normality assumption is violated.

If the model is over-identified, fit measures can be reported along with the parameter estimates. Again, TLI and RMSEA are used to assess model fit, with the same thresholds as seen in the CFA (TLI > 0.9, RMSEA < 0.08). If the model fit is acceptable the parameter estimates can be interpreted. The interpretation of the parameter estimates depends on the specification of the model. By default, one factor loading in each latent construct is set to 1, to fix the scale of the latent construct. However, in order to compare factor loadings across constructs it is useful to consider standardized parameter estimates. The variance of the latent construct is then set to 1 and factor loadings are interpreted in terms of a change in variance. In this study, we look only at the standardized parameter estimates, as we are interested in the relative importance of each observed variable and latent construct.

Factor loadings indicate how much variance of an observed variable is shared with the variance of its latent construct. Higher absolute values indicate more shared variance, and the sign of the factor loading specifies the direction of the association. Covariance terms provide the same information for two observed variables, which can belong to the same construct or to different constructs. If they belong to the same construct, a high covariance term indicates that these two variables share more variance with each other than can be explained by the latent construct. Regression coefficients indicate how much variance of the outcome variable is explained by the variance of the latent construct. To find the relative effect of a single observed variable on the outcome variable, its factor loading must be multiplied by the regression coefficient that connects the latent construct to the outcome.

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Received: 11 November 2022 | Revised: 7 February 2023 | Accepted: 8 March 2023

DOI: 10.1111/tme.12969

REVIEW

TRANSFUSION MEDICINE WILEY

The use of prothrombin complex concentrate in chronic liver disease: A review of the literature

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Abstract

Patients with chronic liver disease (CLD) and cirrhosis present a rebalanced hemostatic system in the three phases of haemostasis. This balance is however unstable and can easily tip towards bleeding or thrombosis. Management of both spontaneous bleeding and bleeding during invasive procedures remains a challenge in this patient population. Transfusion of blood products can result in circulatory overload and thereby worsen portal hypertension. As an alternative to fresh frozen plasma (FFP), prothrombin complex concentrates (PCC) may have merit in patients with liver disease because of their low volume. The impact of PCC in in-vitro spiking experiments of cirrhotic plasma is promising, but also warrants cautious use in light of thromboembolic risk. The majority of existing studies carried-out in CLD patients are retrospective or do not have an adequate control arm. A prospective study (the PROTON trial) was set up in 2013 to investigate the utility of PCC in patients undergoing liver transplantation. However, the study has never recruited the planned number of patients. Robust data on PCC safety in CLD is also required. The limited existing evidence does not seem to indicate an excessive thromboembolic risk. Currently, the utilisation of PCC in CLD cannot be routinely recommended but can provide an option for carefully selected cases in which other measures were not sufficient to control bleeding and after delicately weighing risks and benefits.

KEYWORDS

bleeding, chronic liver disease, cirrhosis, prothrombin complex concentrate, thromboembolic risk

1 | INTRODUCTION

1.1 | Concept of rebalanced haemostasis

Currently, the concept of rebalanced haemostasis is widely accepted for patients with chronic liver disease (CLD) and cirrhosis. Multiple procoagulant and anticoagulant perturbations give rise to a fragile balance, elevating both hemorrhagic and thrombotic risk. The three phases of haemostasis are affected: primary haemostasis, coagulation cascade and fibrinolysis (Figure 1).[1] Thrombocytopenia is common in CLD patients but seems to be counterbalanced by an increase in von Willebrand factor.[2,3] The study of platelet function is much more complex and conflicting reports exist.[4,5] Some describe decreased platelet function[6,7] while others report enhanced platelet activation.[3,8] Fibrinogen plays a role in both primary haemostasis (platelet aggregation) and coagulation (fibrin formation). Levels of fibrinogen can be elevated in early liver disease but hypofibrinogenemia is often seen in more advanced stages, leading to reduced clot formation potential.[1] Qualitative defects (hypersialated fibrinogen, high carbonyl content) have been described.[9,10] The majority of coagulation factors is synthetized by hepatocytes except for factor VIII which is predominantly produced by liver sinusoidal endothelial cells.[11] A decrease in factor

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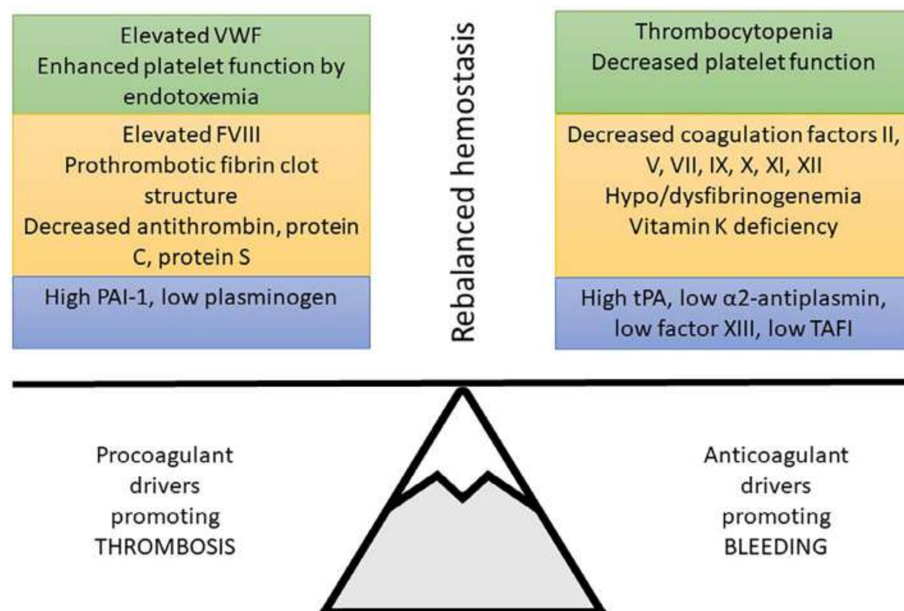

FIGURE 1 Rebalanced hemostatic system in liver disease patients. The three phases of haemostasis are concerned: primary haemostasis (green), coagulation cascade (orange), fibrinolysis (blue). tPA, tissue plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor; PAI-1, plasminogen activator inhibitor-1.

II, V, VII, IX, X, XI and XII but an increase in factor VIII is observed in patients with liver disease. The natural anticoagulants antithrombin, protein S and C decrease progressively with disease severity.[12] Hyperfibrinolysis is often described in CLD.[13] However, changes in both pro-and anti-fibrinolytic drivers are recognised: increased tissue plasminogen activator, decreased alpha 2-antiplasmin, FXIII and thrombin activatable fibrinolysis inhibitor increase fibrinolytic potential whereas high plasminogen activator inhibitor type 1 and decreased plasminogen levels decrease fibrinolytic capacity. This can lead to a rebalanced system.[1,14]

Routine haemostasis tests, for example prothrombin time (PT) and activated partial thromboplastin time, are only sensitive for procoagulant proteins and only reflect 5% of the total amount of thrombin generated during coagulation.[15] Important information on the anticoagulant pathway is therefore not provided. Viscoelastic tests, like ROTEM and TEG, better reflect this rebalanced hemostatic system than conventional testing but also have their drawbacks. A normal viscoelastic test may be useful to select patients that do not need preprocedural prohemostatic treatment. However, an abnormal viscoelastic test may still not require correction, since existing viscoelastic tests underestimate coagulation capacity (no activation of protein C, insensitivity to von Willebrand factor).[16] The European Association for the Study of the Liver (EASL) recommends that routine use of viscoelastic tests presently cannot be recommended for predicting post-procedural bleeding but that it needs further exploration. Because of rarity of bleeding events documented, studies are often not able to show an association between viscoelastic tests and bleeding events.[17] Plasma-based thrombomodulin-modified thrombin generation, in which exogenous thrombomodulin activates protein C, provide a good appreciation of rebalanced haemostasis.[18] They are however not readily available in clinical laboratories. The predictive value for procedural or spontaneous bleeding of thrombin generation assays (TGA) is unknown.

Whereas hemostatic changes may contribute to bleeding or thrombosis in patients with cirrhosis, other important factors may also play a role in hemorrhagic and thrombotic risk in CLD patients. Variceal haemorrhage is primarily due to portal hypertension.[19] Reduced portal blood flow can lead to portal thrombosis. Recently, Turon et al. demonstrated that hypercoagulability likely plays a minor role in development of portal vein thrombosis.[20] Renal disease, bacterial infection and inflammatory changes in endothelial cells can further impair haemostasis, and thereby contribute to bleeding risk.

1.2 | Prothrombin complex concentrates

Non-activated prothrombin complex concentrates or PCCs are virally inactivated, low-volume prohemostatic products containing non-activated vitamin K-dependent coagulation factors: factor II, (factor VII), factor IX and factor X. Some concentrates also contain anticoagulants protein S, protein C, protein Z, antithrombin or heparin. They were originally designed and intended for use in haemophilia B patients. Nowadays, PCC are primarily used for the rapid reversal of oral vitamin K antagonist-related bleeding.[21] Other indications are congenital or acquired factor deficiencies where purified factor concentrates are not available. The concentration of coagulation factors is on average 25 times higher than in FFP.[22] Multiple 3 (FII, FIX, FX) or 4 factor (FII, FVII, FIX, FX) PCC formulations are available globally: Cofact® (Sanquin, Amsterdam, The Netherlands), Confidex® (CSL Behring, Marburg, Germany) and Octaplex® (Octapharma, Brussels, Belgium), Beriplex® (CSL Behring), Uman Complex® (Kedrion, Barga, Italy), Kcentra® (CSL Behring), Prothrombinex™-VF® (CSL Behring),

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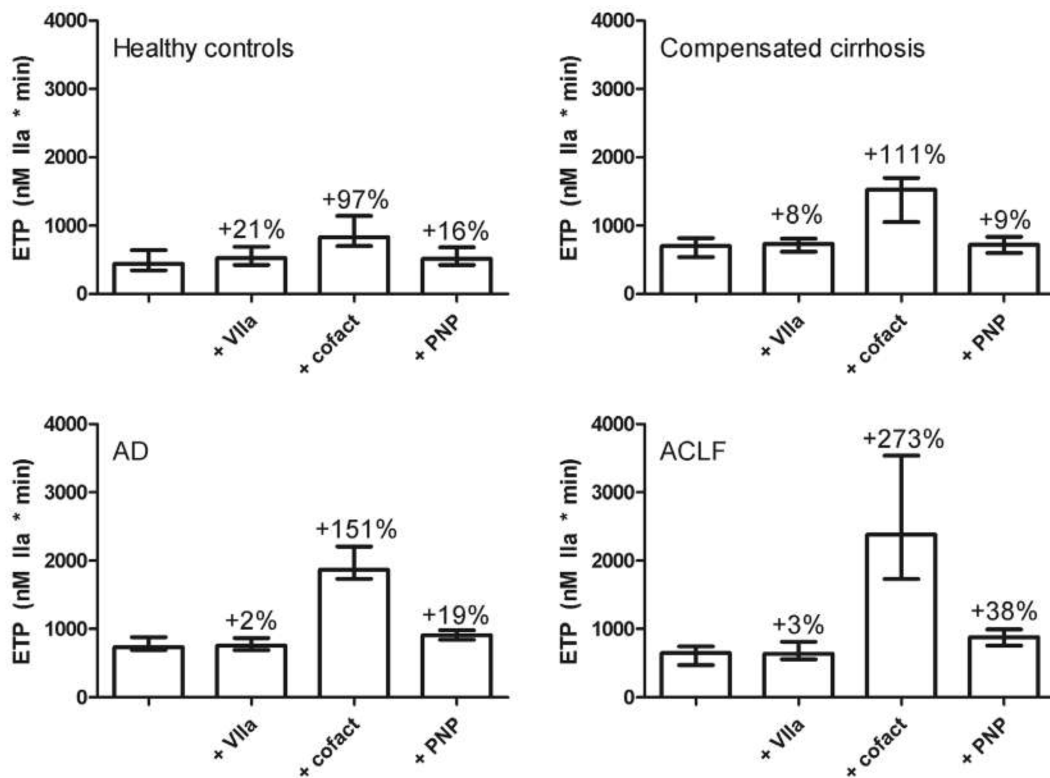

FIGURE 2 Absolute ETP values from thrombomodulin-modified thrombin generation testing in controls and patients with compensated, or acutely decompensated cirrhosis (AD) or acute-on- chronic liver failure (ACLF). VIIa = factor VIIa, Cofact = 4-factor PCC; PNP: pooled normal plasma. Reproduced from Lisman et al. Liver Int. 2018;38(11):1988–1996.

and Profilnine SD® (Grifols Biologicals, Barcelona, Spain). The only activated PCC available is FEIBA (Baxter Healthcare Corporation, Westlake Village, CA). This procoagulant agent has a recognised use as a bypassing agent in haemophilia patients with inhibitors. In this review only non-activated PCCs will be discussed.

In contrast to PCC, FFP has several drawbacks. Large volumes of plasma are needed to significantly improve the PT.[23] This can lead to volume overload and elevation in portal pressure in liver disease patients. Thrombin generation, that is often normal in CLD patients, does not improve considerably after infusion of FFP.[24] Beside volume overload, there are allergic, immunological and infectious transfusion risks associated with plasma transfusions. Thawing FFP takes time, which can be an issue in urgent situations. PCC could be a low volume alternative to FFP as it contains both procoagulant and anticoagulant factors.

In this review we aim to provide an concise overview on existing evidence of PCC use in CLD patients.

2 | MATERIALS AND METHODS

We used the PUBMED database searching for the following key elements: “liver diseases” [MESH] and “PCC” [MESH]. References in articles were also screened. All types of articles including case reports and conference proceedings were considered.

3 | PCC IN CLD

3.1 | Impact of PCC on “in vitro” haemostasis

A thrombomodulin-modified TGA enables us to study the effect of different procoagulant agents on the ability to generate thrombin. Lisman et al. performed TGA on normal patient plasma and plasma from cirrhotic patients with differences in severity: compensated, acutely decompensated and acute-on-chronic liver failure.[25] Plasma was spiked with procoagulant agents: normal pool plasma (to mimic FFP), recombinant factor VIIa (rFVIIa) and PCC (Figure 2).

The effect of rFVIIa was minimal, especially in acutely decompensated and ACLF patients. FFP only marginally increased endogenous thrombin potential (ETP) in compensated and acutely decompensated patients, further corroborating the hesitant approach towards FFP use in liver disease. Tripodi et al already underlined the small effect of FFP on thrombin generation in compensated liver disease patients.[26] The American Gastroenterological Association (AGA) practice guidelines on coagulation in cirrhosis state that the utility of FFP is very limited in cirrhotic patients.[27] In concordance to this, the American Association for the Study of Liver Disease (AASLD) and EASL guidelines advice against preprocedural use of FFP.[16,17]

Interestingly, PCC enhanced thrombin generation to a larger extent in cirrhotic plasma compared to plasma from healthy individuals and this enhanced effect was proportional to disease severity.

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TABLE 1 Overview of studies published on PCC use in liver disease.

Author	Study type	Year	# patients	Indication	PCC	TE events	INR outcome	clinical outcome
Huang et al.[31]	Retrospective study	2016	85 (LD = 31/non-LD = 54)	Bleeding	Kcentra	3.2% (LD); 14.8% (non-LD)	Suboptimal correction in LD compared to non-LD	No data
Drebes et al.[35]	Retrospective study	2019	105	Prophylactic/bleeding	Beriplex/Octaplex	3%	Significant reduction	No data
Lesmana et al.[36]	Prospective study, non-randomised	2016	30	Prophylactic	Cofact	No data	Significant reduction	No bleeding events
Kwon et al.[37]	Retrospective study	2016	45 (15 PCC, 15 rFVIIa, 15 FFP)	Prophylactic	Kcentra	7%	Greater reduction in PCC group than in FFP group	Less blood product use in PCC group than FFP group, idem bleeding
Leal-Villalpando et al.[38]	Retrospective study	2016	30 (PCC = 25)	Prophylactic	Confidex/Kcentra	No data	No data	Lower FFP administration
Srivastava et al.[39]	Retrospective study	2018	262 (PCC = 60), propensity score-matched	Prophylactic	No data	0%	No data	Lower RBC/FFP administration in PCC group
Richard-Carpentier and Rioux-Masse[46]	Retrospective study	2013	51	Prophylactic/bleeding	Beriplex/Octaplex	6%	Better INR reduction in CTP A/B compared to CTP C	Poor bleeding control
Small et al.[47]	Retrospective study	2021	58 (PCC = 21)	Bleeding	Kcentra	No data	Significant reduction	No difference
Hartmann et al.[48]	Retrospective study	2019	372 (PCC = 70)	Bleeding	PCC (CSL Behringer)	No data	No data	PCC not independently correlated with mortality
Zamper et al.[51]	Interventional before-after comparative study	2018	237 (54 intervention/183 control; PCC = 6)	Bleeding	No data	Idem TE rate intervention versus control	No data	Reduction allogeneic blood product use
Colavecchia et al.[52]	Retrospective study	2017	212 (PCC: $n = 39$)	Prophylactic	Kcentra	No data	No data	No lower blood product administration
Tischendorf et al.[58]	Retrospective study	2019	347	Prophylactic/bleeding	No data	5.50%	No data	No data
Kirchner et al.[59]	Retrospective study	2014	266 (156CFC group/110 non-CFC group)	Prophylactic (ROTEM guided)	Beriplex	No difference CFC (7.1%)/on-CFC group (4.5%)	No data	No data
Shakowski and MacLaren[60]	Retrospective study	2016	70 (PCC = 27/rFVIIa = 43)	Prophylactic	No data	11.1% (PCC); 14% (rFVIIa)	No data	No difference PCC/rFVIIa in major bleeding
Scott et al.[61]	Retrospective study	2017	41 (LD = 44%)	Prophylactic/bleeding	No data	0%	No difference LD versus non-LD	No data

Abbreviations: CFC, coagulation factor concentrates; CTP, child turcotte pough; FFP, fresh frozen plasma, INR, international normalised ratio; LD, liver disease; PCC, prothrombin complex concentrates; TE, thromboembolic events.

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Cautious use may therefore be warranted in light of thrombotic risk in these acutely ill patients. Conservative dosage may have to be applied. This is an important finding: high dosing could be tempting when attempting to correct PT values.

A similar spiking experiment has been done by Werner et al.[28] in plasma from 20 paediatric patients with end stage liver disease before and during LT. Age-matched controls were included. They also demonstrated that FFP had little effect on TGA in controls and patients before LT. Adding FFP to plasma samples during LT significantly increased ETP, although not to physiological levels. PCC remarkably enhanced thrombin generation in both controls and pre-LT patients. During LT an intensified response was seen which inversely correlated with pre-LT factor II.

Taking into account the high in vitro impact of PCC seen in the aforementioned studies, dose adjustments could be necessary when administering PCC in CLD patients to lower thromboembolic risk. However, these in vitro studies do not reflect the patient's characteristics possibly affecting pharmacokinetics.

3.2 | Data on the current PCC use

Table 1 summarises the studies published on PCC use in CLD.

Older papers already demonstrated the ability of PCC to improve the PT, although its effect was suboptimal compared to non-CLD patients.[29–31] However, clear data on improvement of bleeding or blood loss are not available. Because of improved surgical and anesthesiologic techniques, transfusion requirements in LT and other major surgery have greatly diminished over the past two decades. In a study on 500 liver transplantations, 79.6% did not require transfusion of blood products.[32] Several authors demonstrated reduced survival after blood product transfusion.[33,34]

In the following paragraphs, we will review the literature concerning the use of PCC in a prophylactic context (before interventions, surgery) and in an active bleeding context. We will also discuss thromboembolic risk associated with PCC.

3.2.1 | Preoperative/procedural management

In a retrospective, single-center study 194 episodes of PCC administration (105 patients with chronic and acute liver disease) were analysed for improvement of PT.[35] Indications were treatment of bleeding and preprocedural prophylaxis with a variety of different interventions (e.g. TIPS, paracentesis, thoracocentesis, surgery and LT). Improved PT was demonstrated. The design of this study did not allow for information on reduction of administration of blood products. Fibrinogen concentrate or cryoprecipitate were concomitantly used. INR, not representative of rebalanced haemostasis, was used as an outcome measure.

Lesmana et al.[36] evaluated PCC use in various invasive gastrointestinal and hepatobiliary procedures in a small prospective study ($n = 30$) including 14 patients with cirrhosis (46.7%). They concluded improvement of INR and clinical efficacy (control and prevention of bleeding complications). However, no control arm was included. Similarly, Kwon et al.[37] reported improvement of INR and reduction of blood product use in patients receiving PCC ($n = 15$) compared to patients receiving FFP ($n = 15$). Patients underwent an invasive intervention or a minor surgical procedure. Again, no placebo group was included in this study, which makes conclusions regarding clinical efficacy difficult.

In a small cohort of LT recipients ($n = 39$), a retrospective comparison between a group receiving prophylactic PCC ($n = 25$) and a group without prophylactic PCC ($n = 14$) was made. The authors concluded that prophylactic PCC could reduce bleeding and transfusion requirements in LT.[38] Similarly, Srivastava et al.,[39] compared 60 LT patients who received PCC at induction with 60 propensity score-matched LT patients receiving FFP at induction. The administration of PCC and FFP was guided by TEG ($R > 10$). They reported significantly less RBC and FFP administration in the PCC group. Once more, no placebo control arm was included, making it difficult to draw conclusions.

A prospective study (the PROTON trial) was set up in 2013 to investigate the utility of PCC in patients undergoing LT.[40] Adult patients with an INR >1.5, listed for transplantation, and without a history of hemorrhagic or thrombotic disease were included. The study comprised both a PCC and a placebo arm. However, the study never included the planned number of patients.

Different guidelines exist for periprocedural management of CLD patients. The AASLD guidelines state that efficacy and safety data are lacking for the use of PCC in liver disease.[16] PCC use risks overcorrection to a hypercoagulable state. In accordance, the AGA 2019 guidance document says that 4-factor PCC are an attractive low-volume therapeutic to rebalance a disturbed hemostatic system. However, dosage guidance is based on INR, which is problematic, and published experience in liver disease is limited.[27] They also state that for minor interventions (dental extraction, paracentesis, diagnostic endoscopy, central line placement and cardiac catheterization) no prophylactic prohemostatic treatment is generally needed. In the recent AGA 2021 update,[41] no recommendation is made for PCC use. They recommend against blood product use (e.g. FFP and platelets) for stable cirrhosis patients undergoing common gastrointestinal procedures. In patients with severe coagulatopathy decisions about prophylactic blood product use should include discussions about potential benefits and risks in consultation with a haematologist. The International Society on Thrombosis and Hemostasis Scientific and Standardization Committee (ISTH SSC) guidance document[42] state that PCC should be avoided in the periprocedural setting. The updated EASL guidelines,[17] discourage the use of PCC to lower clinically relevant procedural bleeding. Studies are needed to address safety and efficacy of PCC and to assess their optimal dosage in this indication.

3.2.2 | Spontaneous/procedural bleeding

Variceal bleeding in cirrhotic patients is mainly due to high portal pressure and not directly correlated with hemostatic abnormalities.[43]

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Standard approaches to manage portal hypertension, endoscopic treatment and a restrictive transfusion policy are recommended. In decompensated patients non-variceal bleeding also remains a frequent clinical problem.[44] Only a minority of bleeding events are solely due to hemostatic failure.[45] LT still poses a major hemostatic challenge possibly requiring transfusion of blood products.

In the study by Drebes et al,[35] treatment of varied categories of active and recent bleeding was studied. PCC did have an impact on INR but this study did not allow for information on clinical improvement of bleeding.

A small retrospective study included 51 bleeding patients of which 80% had cirrhosis. No data were provided on the nature of bleeding in these patients. PCC dosing was based on the NAC (Canadian national advisory committee) recommendations. The authors concluded a low control of bleeding with PCC and an absence of correlation between INR correction and hemorrhagic control.[46]

The use of a single dose of PCC was also studied in a retrospective study including patients with cirrhosis and intracranial haemorrhage.[47] A stable head CT at 24 h was the outcome measure. Of 59 patients 21 received PCC. No difference was seen in the rate of stable head CT. Mortality was higher in the PCC group. PCC group patients had more severe cirrhosis and higher INR. Results could have been impacted by a treatment bias in this study.

Hartmann et al.[48] did a retrospective data analysis on 372 liver transplantations. The administration of coagulation factor concentrates was guided by a ROTEM based algorithm, published previously.[49] PCC were only used in patients with massive diffuse bleeding and pathologic ROTEM parameters. No FFP was administered. Of the 372 patients undergoing liver transplantation, 50.2% received fibrinogen concentrate, 18.8% PCC, 21.2% platelet concentrates, 4.5% tranexamic acid and 59.4% red blood cell concentrates. The administration of fibrinogen and PCC was not independently correlated with mortality. Infused volume, MELD score and platelet concentrates were independent predictors of mortality. A more recently published ROTEM-based algorithm[50] suggests a dose of 10–15 IU/kg body weight PCC in diffusely bleeding patients. They also consider concomitant administration of antithrombin in patients with a high thromboembolic risk.

Zamper et al.[51] designed a LT before-after study comparing an interventional group with VET based administration of fibrinogen and PCC with a retrospective control group (based on conventional coagulation tests, no fibrinogen/PCC). The intervention group comprised of 54 patients and the control group of 183 patients (46 and 89 after propensity score-matching). Of 54 patients only 6 received PCC based on diffuse bleeding and an EXTEM clotting time > 80s. Overall the authors saw a decrease in allogeneic blood product (RBC and FFP) use in the interventional group. Interestingly, they also saw a decline in upper digestive haemorrhage, possibly reflecting a decrease in volume overload.

Colavecchia et al. studied the impact of factor concentrates on blood product use in patients undergoing LT ($n = 212$; PCC $= 39$). In the presence of major and/or microvascular bleeding during LT and based on other elements (routine haemostasis, VET) the clinician decided if blood products (RBC, FFP or platelets) and/or coagulation factors (fibrinogen, PCC) were warranted. No standard protocol was followed. They did not demonstrate lower blood product administration in patients treated with PCC and fibrinogen concentrate compared to patients not receiving PCC/fibrinogen concentrate.[52] However, a treatment bias could have been present: PCC/fibrinogen concentrate use in patients with more refractory bleeding.

The Liver Intensive Care Group Europe (LICAGE) guidelines on perioperative coagulation management in LT recommends to consider the use of PCC in bleeding patients once fibrinogen and platelets have been replaced and fibrinolysis is excluded. They also state that more data are needed on safety and efficacy of PCC in LT.[53] As already mentioned, Gorlinger et al designed a ROTEM-guided algorithm for the management of bleeding in patients undergoing LT.[50] In this algorithm, PCC are only administered in diffusely bleeding patients with pathological ROTEM parameters. VET can have a role in guiding PCC administration but currently lack validated target levels.[27]

3.3 | Thromboembolic risk of PCC

A review of the literature evaluating the overall safety of four-factor PCC concludes a fairly low thrombotic risk in patients without underlying risk factors.[54] Thrombogenicity of PCC has greatly diminished in today's PCC formulations compared to historic preparations. The main determinant of thrombogenicity in PCC is thought to be prothrombin which has the longest half-life.[55]

Case reports have been published describing the development of disseminated intravascular coagulation after PCC use in decompensated cirrhotic patients.[56,57] A PCC-product not containing anticoagulant factors, was used in one of those two reports.

Tischendorf et al. retrospectively evaluated 347 patients with cirrhosis that received PCC for the prevention or treatment of bleeding. In a 4-week long period after PCC administration they found 19 patients or 5.5% with a thromboembolic event.[58] Richard-Carpentier and Rioux-Masse[46] showed a similar thromboembolic event rate (6%). Drebes et al did not report an excess of thromboembolic events (3% of patients) for a median dose of 22 IU/kg.[35] A retrospective study including 266 patients who underwent orthotopic LT did not see a significant difference in thromboembolic events between groups receiving PCC or not (ROTEM-guided).[59] In three small retrospective CLD cohorts ($n = 70$; $n = 31$ and $n = 41$), no significant difference between factor VIIa and PCC was demonstrated in thrombotic and bleeding events[60] and no excess thromboembolic event rate (3.2%; 0%) was seen.[31,61] Srivastava et al.[39] did not report any thromboembolic events in a retrospective study including 262 LT patients of which 60 received PCC.

Thromboembolic events could be related to high or repeated doses of PCC. In the previous studies the dose of PCC was predominantly based on vitamin K antagonist dosing taking into account patient weight and INR (information provided with the product used; based on FIX content). No guidelines exist on PCC dosing in CLD patients.

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4 | CONCLUSION

Only limited evidence suggests that PCC diminish allogeneic blood product administration in a variety of procedures, LT or other major surgery and are beneficial in bleeding management in CLD patients. The existing evidence relies on retrospective studies lacking power and a control group to prove clinical efficacy. These studies are also very heterogeneous regarding population studied and prophylactic/bleeding protocols used. Despite guidance documents arguing against FFP prophylaxis, there is an on-going urge to correct PT/INR. In carefully selected cases and after weighing risks and benefits, PCC could be an attractive alternative due to the lack of volume overload. In a therapeutic context, where other measures to stop the bleeding were not sufficient, PCC could also be considered. PCC administration is associated with a risk of thromboembolic events. This risk does not seem to be excessive in CLD patients compared to other patient groups (3%–6%). Disseminated intravascular coagulation has been described in decompensated cirrhotic patients.

Randomised controlled trials assessing both prophylactic and bleeding context are needed to evaluate the safety and efficacy of PCC administration in CLD patients. However, patient inclusion will be challenging in these studies.

AUTHOR CONTRIBUTIONS

Véronique Deneys and Marie-Astrid van Dievoet developed the initial idea for this review, which was further refined with Xavier Stephenne, Madeleine Rousseaux, Ton Lisman and Cedric Hermans. Marie-Astrid van Dievoet did the writing of this review with support of all other authors. All authors approved the final version.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest

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How to cite this article: van Dievoet M-A, Stephenne X, Rousseaux M, Lisman T, Hermans C, Deneys V. The use of prothrombin complex concentrate in chronic liver disease: A review of the literature. *Transfusion Medicine*. 2023;33(3):205-212. doi:10.1111/tme.12969

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Received: 4 January 2022 | Revised: 16 April 2022 | Accepted: 26 July 2022
DOI: 10.1111/tme.12904

ORIGINAL ARTICLE

TRANSFUSION MEDICINE WILEY

Iron deficiency in whole blood donors in a resource-poor setting: A cross-sectional study in Uganda

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Abstract

Background and Objective

Blood donation is known to result in iron deficiency (ID), with a higher prevalence in females. There is little published data on the frequency of ID among blood donors in resource-poor settings. We determined the prevalence of ID in blood donors in Uganda.

Methods

We conducted a descriptive cross-sectional study at the Uganda Blood Transfusion Service, Kampala from December 2021 to February 2022. A sample of 500 whole blood donors was enrolled, complete blood count, and serum ferritin. The primary outcome was the proportion of donors with serum ferritin <15 µg/L.

Results

The median (IQR) serum ferritin was 25 (12-47) µg/L and 89 (52-133) µg/L among female and male donors respectively. The prevalence of iron deficiency (serum ferritin <15 µg/L) among donating individuals was 11.5% (8.7-14.9), , but even higher among females younger than 24 years [35.4% (29.2-42.1)]. female donors (15.81, 5.17, 48.28, $p < 0.001$) and a high RDW (6.89, 2.99, 15.90, $p < 0.001$). We found a moderate correlation between serum ferritin and RDW ($r = -0.419$ and -0.487 for males and females respectively).

Conclusion

Iron deficiency is common among blood donors in Uganda, affecting mostly young female donors. Considerations to adopt evidence-based strategies to prevent and manage ID among blood donors-such as serum ferritin monitoring and iron supplementation are highly recommended.

KEYWORDS
KAP, Blood, Transfusion, Donor, Medecine

1 | INTRODUCTION

The incidence of hip fractures continues to increase, along with the global expansion of aging population observed secondary to improved healthcare and quality of life.[1] Subtrochanteric fractures are defined as fractures encountered between the inferior border of lesser trochanter and 5 cm distal to it.[2] They represent a complex subset of injuries surrounding the hip, which are most commonly managed with

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have long been the standard method to assess iron status. However, haemoglobin levels can remain sufficient for some time, even when iron stores are dwindling; this is known as iron deficiency non-anaemia.[1]

In contrast to haemoglobin, serum ferritin levels reflect the amount of stored iron.[1] Therefore, they are increasingly used to assess individuals' iron stores when these are at risk, for instance after traumatic blood loss, during pregnancy, or in blood donors.[3] Sanquin, the national blood service in the Netherlands, started measuring ferritin levels in each new donor, and subsequently after every fifth donation, in October 2017. Donating blood has a substantial impact on ferritin levels. Ferritin levels are lower among blood donors than in the general population: cross-sectional studies report lower ferritin levels in donors with a higher number of whole blood donations and a large randomised trial showed that ferritin levels indeed decline with more frequent blood donations.[4,5] Among new donors, large variation in ferritin levels is observed.[4] It is well established that individual characteristics such as sex and age are relevant: women in general, but pre-menopausal women in particular, have considerably lower ferritin levels than men.[4,6,7] Higher body mass index (BMI) is associated with higher ferritin levels.[8] In recent decades, many other factors that affect iron status have been identified: diet,[9,10] genetics,[11,12] ethnicity,[13] and iron supplementation, which is mostly studied among blood donors.[14,15]

Ferritin is also a known acute-phase protein that is elevated in inflammatory conditions, complicating its diagnostic value in individuals with conditions such as inflammatory bowel disease or chronic heart failure.[16] This could also explain the association between BMI and ferritin levels, as adipose tissue is known to promote systemic inflammation.[17] Additionally, exposure to environmental pollutants has been linked to disordered iron homeostasis,[18,19] and ambient particle matter (PM) concentration is correlated with ferritin levels.[19] The biological mechanism behind this is still unclear, but it is postulated that iron attaches to the PM rather than to cell nuclei, effectively creating a functional deficiency.[18,19] In turn, mechanisms start upregulating iron uptake and recycling in an attempt to meet the iron requirement of the cells, thereby altering iron homeostasis. Another suggested mechanism is that when pollutants enter the lungs, iron is transported away from the surface of the lung tissue and stored in ferritin complexes, in order to avoid chemical reactions between iron and the pollutant.[18] Other potential environmental determinants are neighbourhood characteristics, including population density and socio-economic status, which are consistently shown to be related to body weight[20] and blood parameters.[21]

Previous studies on ferritin levels have focused on studying the association with variables in a limited setting, for example, characteristics such as age and BMI, donation-related variables, or environmental pollutants. In this paper, we propose a novel framework that integrates multiple settings, using a structural equation model. By grouping relevant explanatory variables into constructs, we describe relationships with ferritin on a more general level. This enhances the insight into various mechanisms that influence ferritin levels, which is valuable to those who use these as a diagnostic tool. We explore associations between ferritin levels and individual characteristics, donation behaviour and environmental factors, in a large group of newly registered and active whole blood donors.

2 | METHODS

For this cross-sectional study, data collected by Sanquin and the Geoscience and health cohort consortium (GECCO) were analysed. Sanquin is by law the only blood service in the Netherlands, collecting over 400 000 whole-blood donations each year, with collection sites geographically well-distributed throughout the country. Several eligibility criteria exist to ensure the safety of the donors and recipients and the quality of the blood product. Donors must be aged between 18 and 79 years old, and a pre-donation screening visit takes place before the first 500 ml whole blood donation, which includes blood sampling for blood type and infectious disease testing, as well as initial haemoglobin and ferritin measurements. We will refer to these prospective donors, who have not donated yet, as 'new donors'.

Before every donation, a donor screening is performed, including a donor health questionnaire and measurements of blood pressure, pulse rate and haemoglobin levels to assess whether the donor is eligible to donate. Haemoglobin levels need to be at least 7.8 mmol/L for women and 8.4 mmol/L for men. This is measured by point-of-care testing with a photometer (HemoCue, Angelholm, Sweden). Ferritin levels, are measured in serum samples, using the Architect i2000 (Abbott Diagnostics, Chicago, IL), after the pre-donation screening visit and after every fifth whole blood donation. As such, ferritin measurements are only available in case of successful whole blood donations, and for new donors whose venous samples are taken as part of the pre-donation screening visit.

2.1 | Data

This study included all new and active whole blood donors who gave consent to the use of their data for scientific research (consent given by >99% of all donors) and for whom ferritin measurements were available between 1 October 2017 and 31 December 2019. If multiple ferritin measurements were available for a donor, only the first measurement was used. Information on donors and donation histories was extracted from the blood bank information system (ePROGESA, MAK-SYSTEM International Group, Paris, France). Variables used were sex, age, height, weight, time since previous successful donation, the number of successful donations in the previous 2 years, donor status (new or active donor), and ferritin levels. BMI was calculated from self-reported donor height and weight. Sanquin does not register donor ethnicity, but Duffy negative phenotype was included to function as a proxy for sub-Saharan African descent.

Environmental exposure variables of various characteristics were obtained from the Geoscience and health cohort consortium (GECCO).[22] The exposure data were operationalised based on publicly

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TABLE 1 Grouping of variables into constructs for each model

Variable	Model A	Model B	Model C	Model D
Age	Individual characteristics	Individual characteristics	Individual characteristics	Individual characteristics
Weight				
Height				
BMI				
Duffy phenotype				
Time since previous donation[a]	Donation history	Donation history		
Number of previous donations[a]				
Population density	Environment	Environment	Environment	Environment
Temperature				
Socio-economic status				
Ozone	Pollution		Pollution	
PM2.5				
PM10				
Soot				
NO_2				

Note: All models contain the same observed variables but differ in how these are grouped into latent constructs.
[a]Only available for active donors.

available data. Data from 30 weather stations in the Netherlands—obtained from the Royal Netherlands Meteorological Institute (KNMI)—were used to estimate temperature at a spatial resolution of 1 km. Three options for the measurement level were considered (minimum, average, and maximum daily temperature), as well as three time spans (day, week or month before donation), resulting in nine options in total. The combination that showed the highest correlation with ferritin was included in the final model.

Daily concentrations for particulate matter (PM) 2.5, PM10, NO_2, ozone and soot levels were obtained via the Dutch National Institute for Public Health and the Environment (RIVM), for the years 2017–2019. These variables were imputed on a spatial resolution of 1 by 1 km. Neighbourhood socio-economic status (SES) scores and population density from 2017–2019 were acquired from Statistics Netherlands (CBS), both available on 6-digit postal code level. SES scores are based on percentiles of income, education level and vocational history of households, with a score of 0 being exactly the national average, and positive scores being above average. All spatio-temporal variables were matched with donor and donation data based on donation date and donor postal code. Lastly, the date and time of each donation were included as potential factors to account for seasonal and diurnal variation, as they are known to affect haemoglobin levels and may also affect ferritin levels.

To check for a possible confounding effect of smoking on environmental variables, we analysed the correlation between the percentage of smokers per municipality (data from Statistics Netherlands) and all environmental variables described in the above paragraph.

There were no missing data for environmental datasets from the RIVM and CBS. Donors with no ferritin measurement were excluded from the analysis. There were no missing data for the other donor or donation level variables.

2.2 | Statistical analysis

Structural equation modelling (SEM) was used to investigate which variables relate to serum ferritin and to what extent. Briefly, observed variables and latent constructs are distinguished in SEM. Latent constructs cannot be measured or observed directly, but are inferred from the observed variables. One or more hypothesized sets of relationships and correlations between variables and constructs are specified a priori and shown in a path diagram. For each relationship, a parameter is estimated that indicates its strength. Estimates are obtained by numeric optimization of a fit criterion, using maximum likelihood estimation. A more detailed overview of this method is provided in Appendix A.

We compared four ways to divide the 15 variables included in the analysis into latent constructs, as shown in Table 1. Date and time of the donation were added to the model separate of the constructs, and as such are not included in Table 1. Model A contains four latent constructs, and in models B, C and D different sets of constructs are combined. Confirmatory factor analysis (CFA) was used to test the validity of the specified measurement models, that is, the hypothesized relationships between the latent constructs and their observed variables. The overall fit of the models was assessed by the Tucker-Lewis Index (TLI) and the root mean square error of approximation (RMSEA). A rule of thumb is to exclude variables for which the absolute value of the standardised factor loading is below 0.4, but at sample sizes larger than 300, if the overall model fit is good, exclusion is not necessary and should be judged separately for each variable based on sensible background knowledge.[23]

Pairwise residual correlations between observed variables were calculated to identify whether any covariances needed to be added to the model. Of the four specified models, we continued our analysis with the best fit according to CFA, based on the TLI and RMSEA.

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TABLE 2 Distribution of explanatory variables by donor status and sex

	New donors		Active donors	
	Women	Men	Women	Men
N	40 172	19 424	39 085	39 233
Age (years)	26 (21–37)	28 (23–37)	47 (31–58)	53 (39–62)
Height (cm)	170 (166–175)	183 (178–188)	170 (166–175)	183 (178–188)
Weight (kg)	68 (62–77)	82 (74–90)	70 (64–80)	85 (78–93)
BMI (kg/m^2)	24 (21–26)	24 (22–27)	24 (22–27)	25 (23–27)
Time since previous donation (days)	NA	NA	154 (132–217)	139 (71–147)
Number of previous donations[a]	NA	NA	3 (2–4)	5 (4–7)
Population density (inhabitants per km^2)	1173 (425–2617)	1246 (477–2936)	827 (322–1855)	814 (320–1824)
Duffy phenotype (proportion)	0.25	0.17	0.28	0.16
Temperature (°C)[b]	11.4 (6.4–16.6)	11.7 (6.6–16.7)	10.4 (6.0–16.0)	10.4 (5.9–16.0)
Socio-economic status	0.04 (−0.21 to 0.22)	0.02 (−0.24 to 0.22)	0.10 (−0.10 to 0.25)	0.12 (−0.07 to 0.26)
Ozone (μg/m^3)	46.9 (45.6–48.8)	46.8 (45.5–48.7)	47.2 (45.9–49.2)	47.2 (45.9–49.1)
PM2.5 (μg/m^3)	10.7 (9.7–11.6)	10.7 (9.8–11.6)	10.5 (9.6–11.5)	10.6 (9.7–11.6)
PM10 (μg/m^3)	18.2 (16.8–19.3)	18.2 (16.9–19.3)	18.0 (16.6–19.0)	18.0 (16.7–19.1)
Soot (μg/m^3)	0.66 (0.54–0.78)	0.66 (0.55–0.78)	0.63 (0.52–0.75)	0.65 (0.54–0.76)
NO_2 (μg/m^3)	17.6 (14.9–21.6)	17.8 (15.1–21.8)	16.8 (14.2–19.7)	16.9 (14.3–19.6)
Ferritin (ng/ml)	47 (28–75)	118 (79–170)	30 (17–47)	34 (20–56)

Note: Data are presented as medians (interquartile range) due to non-normal distributions of the variables.
[a]Within 2 years before the ferritin measurement.
[b]The maximum temperature recorded on the day of donation.

To the model with the best fit, we added the structural component, which contains the relationships between the latent variables and ferritin, the outcome variable. A multiple group SEM was carried out with parameters estimated separately for male and female donors, and for new and active donors. Because the assumption of normality of the explanatory variables does not hold in our data, a different estimator than the default maximum likelihood estimator was used: the 'mean and covariance adjusted weighted least squares estimator', which is robust against violations of the normality assumptions in a multivariate setting.[24]

The same model was fitted in all four groups, although the variables belonging to the *donation history* construct (see Table 1) are not available for new donors, as they do not (yet) have a donation history. The overall fit of the SEM model was assessed using the TLI and RMSEA, as well as the R^2 measure.

All analyses were conducted using *R* programming language and environment for statistical computing version 4.0.3,[25] with package *zoo*[26] for pre-processing environmental data, and *lavaan*[27] for CFA and SEM analyses. Path diagrams were created with yEd Live Graph Editor.[28]

3 | RESULTS

3.1 | Sample composition

Table 2 shows descriptive statistics of the study population by sex and donor status. The size of each of the groups was comparable, except for the group of new male donors, which was only half the size of the other groups. Between new and active donors, age differed considerably, new donors being younger than active donors by 17 years on average ($p < 0.001$ using a two-sample *t*-test). In both new and active donors, men were older (by 6 years on average, $p < 0.001$) and heavier (by 13 kg on average, $p < 0.001$) than women. *p*-values were obtained using two-sample *t*-tests. The time since last donation is higher in women than in men, and the number of prior donations is higher in men than in women. These differences are due to differences in the minimum required donation interval: for women, there must be 122 days between two donations with a maximum of 3 donations per year, while for men, the minimum is 57 days between two donations with a maximum of 5 donations per year. Differences in ferritin levels between the groups are as expected from previous studies: men have higher ferritin levels than women, and repeat donors have lower ferritin levels than new donors.

For pollution and environmental variables, there was little difference between the groups, any differences between new and active donors were most likely due to the different age and geographical distribution of the groups. None of these differences were statistically significant.

We found a weak correlation between the percentage of smokers and SES score (Pearson's $r = -0.4$) and a moderate correlation between the percentage of smokers and population density (Pearson's $r = 0.5$). No correlation was found for any of the other environmental variables.

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3.2 | Model selection

CFA did not provide support for the *environment* construct as defined by the three variables *temperature*, *population density* and *socio-economic status*. These variables did not share a high proportion of their variance and consequently there was no convergent validity, effectively ruling out models A and C. In models B and D, variables *Duffy phenotype*, *temperature*, *SES* and *height* were omitted due to very low factor loadings (<0.05). The factor loading for variable *age* was also low (0.35) but this variable was not excluded, as it is expected that this factor loading would be small, considering the other variables in the construct (*weight* and *BMI*) are much more closely related. All other factor loadings were above the suggested threshold of 0.6. All latent constructs (individual characteristics, donation history and environment) showed convergent and discriminant validity in models B and D. Variables *time* and *day of year*, which were added to the model outside the constructs, were also dropped due to very low factor loadings (<0.05).

The presence of a *donation history* construct was the only difference between models B and D, and since new donors do not yet have a donation history, the models only differed for active donors. Model B had a TLI of 0.961 and RMSEA of 0.063, while model D had a TLI of 0.932 and RMSEA of 0.083. Based on these fit measures, model B fit the data best, and was therefore used in the remainder of the analyses.

Based on inspection of the pairwise residual correlations between all observed variables, two covariance terms were added to the model: one for *PM2.5* and *PM10* (residual correlation 0.092–0.102, depending on sex/donor status), and one for *age* and *population density* (residual correlation −0.151 to −0.149, depending on sex/donor status). We also added one covariance term for *weight* and *BMI*, as BMI was calculated using weight and was therefore inherently dependent.

3.3 | Parameter estimates

Figure 1 shows the structure of the final model and the parameter estimates for new donors. Parameter estimates were similar for both sexes, but factor loadings for variables belonging to the *individual characteristics* construct were higher for women than for men, indicating more shared variance. Factor loadings in the *environment* construct did not differ between sexes, showing that the covariance structure of those variables was not dependent on sex. The parameter estimates for the regression coefficients show the relative importance of each latent construct for the outcome variable. Table 3 shows the percentage of variance in ferritin levels that is explained by each construct for each model, adding up to the total percentage of variance explained.

Figure 2 shows the final model for active donors. As in new donors, factor loadings in the *individual characteristics* construct were higher for women than for men, and they were also higher for new donors than for active donors. The relative importance

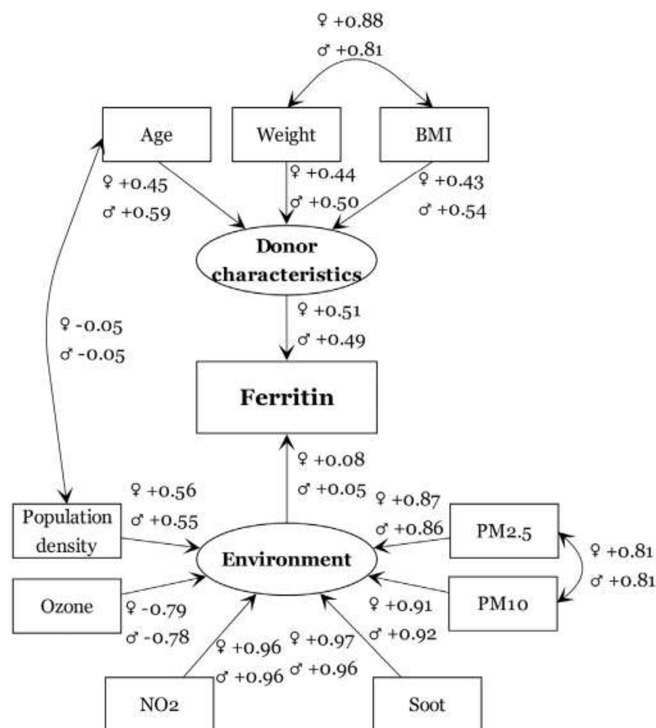

FIGURE 1 Final structural equation model for ferritin determinants in new donors, with parameters estimated separately for men and women. All parameter estimates are standardised so that the variance of each observed variable and latent construct equals 1

TABLE 3 Relative contribution to explanation of variance of ferritin levels per model

	New donors		Active donors	
Construct	Women	Men	Women	Men
Individual characteristics	23%	23%	20%	17%
Donation history	NA	NA	14%	25%
Environment	2%	2%	5%	4%
Total % of variance explained	25%	25%	39%	46%

of individual characteristics and donation history was opposite for both sexes: for men, donation history was correlated with ferritin levels more strongly than individual characteristics (0.66 vs. 0.45), while this was reversed for women (0.43 vs. 0.61). The regression coefficient of the *environment* construct is 0.15 for women and 0.10 for men. The *environment* construct explains twice as much variation in ferritin levels in active donors as in new donors.

As for overall model fit, with a TLI of 0.981 and 0.979 and RMSEA of 0.052 and 0.042, for new and active donors respectively, both models fit very well when compared to commonly used thresholds (TLI > 0.95, RMSEA < 0.06).[29] R^2 was calculated separately by sex: for new donors, R^2 was 0.251 for men and 0.252 for women, and for active donors, 0.458 for men and 0.393 for women.

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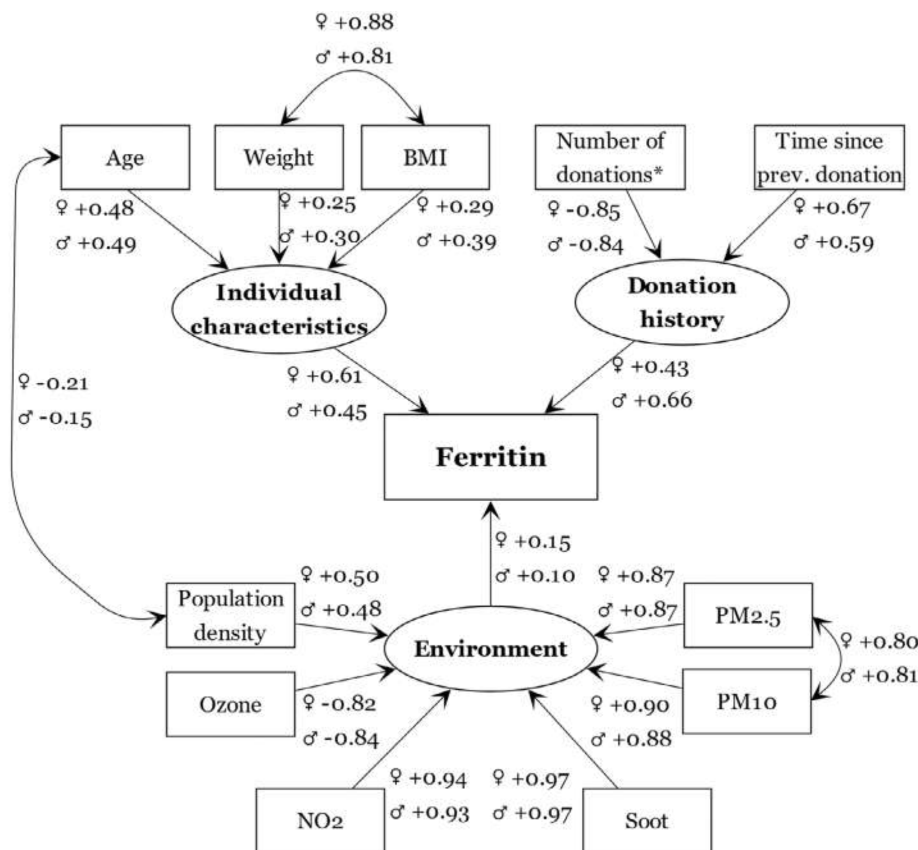

FIGURE 2 Final structural equation model for ferritin determinants in active donors, with parameters estimated separately for men and women. All parameter estimates were standardised so that the variance of each observed variable and latent construct equals 1

4 | DISCUSSION

This study investigated the impact of individual and environmental determinants on ferritin levels in Dutch individuals, using SEM. The model was able to explain 25% of ferritin level variance in new donors for both sexes, and 46% and 39% in active donors for male and female donors, respectively.

We found the construct composed of individual characteristics (age, weight, and BMI) to be the most important determinant of ferritin in female active donors, followed by donation history (time since previous donation, number of donations in the past 2 years). For male active donors, this was the opposite: donation history was a more important determinant than individual characteristics. In both sexes, environmental factors are associated with ferritin levels, albeit to a lesser degree than individual characteristics and donation history.

The relationship between ferritin levels and anthropometric characteristics is well-documented, and the positive correlations we found for ferritin with age, weight and BMI are consistent with those found in other studies.[4,15,30] Men have much higher ferritin levels than women in general and show a larger decrease in ferritin levels after repeated donations. As a result, ferritin levels in active donors are similarly low for women and men.[4] The *donation history* construct explained more variance in ferritin levels in men than in women. Although often not explicitly mentioned, this discrepancy is also found in previous studies, with stronger relationships between variables regarding donation history and ferritin for men than for women.[15] A reasonable explanation for this is that men commonly display more variation in donation history variables due to the possibility of more frequent donations: in many blood services, men are allowed to donate more often than women and are usually less frequently deferred for low haemoglobin levels.[31]

From previous epidemiological studies, we know that environmental factors may play a role in iron metabolism, and that certain pollutants can disrupt iron homeostasis.[32] Our study shows that although environmental factors are less strongly associated with ferritin levels than individual characteristics and donation history, their effects are far from negligible. Because of the wide reach of environmental exposures over geographic areas, even a relatively small influence on individuals can result in a large effect on the population level. As this study includes only data from the Netherlands, which is a relatively small country, associations between environmental variables and ferritin levels were not very strong, as was expected. Repeating this study on a larger, or even global, scale may result in finding a more substantial effect.

Higher values for all but one environmental factor (ozone) were positively correlated with higher ferritin levels. These findings support the hypothesis that air pollution causes higher ferritin levels. The underlying mechanism may be that when certain pollutants enter the lungs, iron is transported away from the lung tissue surface and stored in ferritin complexes to avoid chemical reactions between iron and the pollutant.[18,33] This would imply that using serum ferritin as a proxy for total body iron is less reliable when there is significant air pollution.

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The *environment* construct was more strongly associated with ferritin level in active donors than in new donors. In new donors, environmental factors explain 2% of variance in ferritin levels, while in active donors this increases to 4%–5% depending on sex. This indicates that environmental factors are more important for ferritin recovery after blood loss than for naive ferritin level. A plausible explanation for this difference is that since both exposure to air pollution and donating blood causes significant disruptions to iron homeostasis, these disruptions may interact and together have a larger effect than simply additive.

SEM is a technique well-suited to test hypotheses on how different factors interact and correlate with a specific outcome like ferritin levels, especially when there are many factors to consider. Compared to multiple (linear) regression, more complex models can be tested, and for each variable measurement error is taken into account.[34] Moreover, the percentage of variance explained by groups of related variables can be calculated and compared. The stratified approach in this study also adds to the model validity: parameter estimates can be compared across groups, allowing discovery of implausible results. Our analyses show that the convergent validity of the *individual characteristics* construct is lower for active donors than for new donors. This may indicate that new donors are a more homogenous group than active donors, which is likely due to the more narrow age range of new donors. Other strengths of this study are its large sample size and collection of data throughout the country.

Two main limitations of this study should be noted: its generalizability and its restricted scope. One might be tempted to generalise the results of new donors to the general Dutch population, as these donors have never donated blood before. However, even new donors form a very specific, generally healthier subgroup of the general population, which means that selection bias has likely been introduced. We can speculate that less healthy individuals would show a higher rate of inflammation, which may cause higher serum ferritin levels. On the other hand, iron deficient or anaemic individuals are likely underrepresented in our sample. As this selection bias most likely reduced variance in ferritin levels, this may have attenuated our results.

Regarding the scope, data on some other potentially important determinants of ferritin levels were not available in this study, the two most important being genetics and diet.[9,10] Several genetic polymorphisms that have an effect on iron pathways have been identified, and these are likely to play a role in the recovery speed of ferritin levels after blood donation.[12,35–37] Dietary behaviour, and in particular heme iron intake, is also a determinant of iron status in donors.[9,15] Information on iron supplementation was also not available for this study. Sanquin does not prescribe oral supplementation of iron to donors, and only a small minority (8.7%) uses iron supplements.[9] Information on donors' smoking status is also expected to add value to the model. Had these determinants been available for our analysis, the proportion of variance explained in donor ferritin levels would likely have increased.

This study presents a model to explain variance in ferritin levels in individuals with or without donation history, based on three types of determinants. The model explained a relatively large part of the variance, especially in active donors. Individual characteristics and donation history form the most important determinants of ferritin levels. Although environmental factors accounted for less variance than the individual and donation history constructs, their contribution is meaningful and statistically significant. When clinicians or researchers use serum ferritin as a proxy for total body iron, they should be aware of this potentially confounding effect.

For blood services that are considering implementing ferritin testing for their donors, these results are of particular value. The results can be of use while the blood service is deciding on a sensible threshold for donation: rather than implementing a one-size-fits-all threshold, environmental conditions in the country can be taken into account. If there is a high level of air pollution, ferritin levels are likely to be overestimated, and thus a higher threshold for donation may be desired. It could even be taken further to make ferritin thresholds more tailored to a specific donor, by taking into account a donor's individual characteristics.

AUTHOR CONTRIBUTIONS

Rosa de Groot, Katja van den Hurk, and Jeroen Lakerveld conceptualised the study; Mart Janssen and Marieke Vinkenoog designed the methodology; Marieke Vinkenoog, Rosa de Groot, and Jeroen Lakerveld curated data; Marieke Vinkenoog did the formal analysis and wrote the original draft; all authors reviewed and edited the manuscript; Jeroen Lakerveld, Katja van den Hurk, and Mart Janssen supervised the study.

ACKNOWLEDGEMENTS

Geo-data were collected as part of the Geoscience and Health Cohort Consortium (GECCO), which was financially supported by the Netherlands Organisation for Scientific Research (NWO), the Netherlands Organisation for Health Research and Development (ZonMw), and Amsterdam UMC. More information: www.gecco.nl. This study was funded by Sanquin Blood Supply Foundation, PPO-C grants 14-028 and 18-14/L2337, and the VU University Medical Center.

CONFLICT OF INTEREST

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

Data collected on prospective and active donors by Sanquin Blood Supply Foundation will not be shared due to privacy reasons. The authors are open to research questions from other researchers; proposals for joint research projects may be made to the corresponding author via e-mail. The environmental exposure data provided by the GECCO institute is based on publicly available data, and can be requested via a data access request form available on the website: www.gecco.nl.

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How to cite this article: Vinkenoog M, de Groot R, Lakerveld J, Janssen M, van den Hurk K. Individual and environmental determinants of serum ferritin levels: A structural equation model. *Transfusion Medicine*. 2023;33(2):113-122. doi:10.1111/tme.12902

APPENDIX A

A.1 | STRUCTURAL EQUATION MODELLING OVERVIEW

Structural equation modelling (SEM) comprises a set of statistical methods that enables researchers to assess the support for hypothesized relationships between variables of interest. Its purpose is to account for variation and covariation of the variables in the model. Many different techniques are included in SEM, this appendix explains the approach taken in this particular study. In SEM, observed variables and latent constructs are distinguished. Observed variables are variables in the traditional sense, which are observations in the data set that have been

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collected by the researcher. Latent constructs are theoretical concepts that cannot be measured, but must be inferred from the observed variables; a well-known example is the latent construct *intelligence* that cannot be measured directly, but can be inferred from observed variables such as scores for an IQ test. Intuitively, observed variables that belong to a latent construct represent the same underlying concept, and latent constructs form in a way a dimensionality reduction of the observed variables. Mathematically, latent constructs represent shared variance of the observed variables related to the construct they belong to.

SEM is composed of two main model components: the measurement model, which shows how observed variables are divided among latent constructs, and the structural model, which shows the relationships between latent constructs and outcome variable(s). First, the measurement model is specified, and test its validity using confirmatory factor analysis (CFA). Often, several measurement models are tested and compared to see which division into latent constructs best fits the data. When the measurement model is considered to have a good fit, the structural part of the model is added, and the model fit is assessed for the full SEM model.

A.1.1. | Measurement model

The validity of the latent constructs must be measured in two ways: each construct must have convergent and discriminant validity. Convergent validity occurs when the observed variables belonging to the latent construct share a high proportion of their variance. This is assessed by the factor loadings of the observed variables onto the latent construct: the higher the (absolute value of the) factor loading, the stronger the indication that this variable belongs to this construct. Very generally speaking, factor loadings greater than 0.4 are acceptable for including a variable within a construct, but this threshold depends greatly on the hypothesized interpretation of the latent variable. Variables with low factor loadings are excluded from the construct.

The discriminant validity of a latent construct is a measure for how well the construct can be distinguished from the other constructs in the model. It is measured by the covariances between latent constructs. A high covariance between two constructs can indicate that these constructs are (partly) overlapping, and thus have no discriminant validity.

If convergent and discriminant validity are satisfactory, model fit indices can be calculated for the measurement model. Commonly used indices are the chi-square test, comparative fit index (CFI), Tucker-Lewis index (TLI) and root mean square error of approximation (RMSEA). The CFI and TLI are both relative measures of fit, and compare the fit of the tested model against a null model, which in CFA means that the means and variances of each variable are freely estimated, but no correlations are included. CFI and TLI are on a scale from 0 to 1, with higher values indicating a better fit of the hypothesized model relative to the null model. The TLI is always more conservative (lower value) than the CFI, because the TLI includes a harsher penalty for the number of parameters estimated. Because the two fit indices are highly correlated, only one should be reported. We chose the TLI because of its more elegant penalty for complexity. Values higher than 0.95 indicate good fit.

The RMSEA is an absolute measure of fit that is not sensitive to large sample sizes, unlike the chi-square test. It uses the covariance matrix of the entire data set and of the fitted hypothesized model, and calculates the differences between these two. This results in a measure between 0 and 1, with lower values indicating smaller differences and better model fit. Cut-offs of 0.08, 0.05, and 0.01 indicate mediocre, good, and excellent fits, respectively.

If multiple measurement models are compared, as in this study, the best fitting model is selected, based on the fit indices described above. If these indicate sufficient model fit, the analysis can be continued with inspection of residual correlation between observed variables. If the pairwise residual correlation between two variables is high (absolute value of 0.1 or higher is a common cut-off), this indicates that these two variables share more variance than is currently captured in the model. If this occurs, the researcher needs to decide whether a covariance term for these two variables should be included in the model. However, this should only be done if there is sufficient theoretical support for an interpretable correlation between these variables. Otherwise there is a risk of overfitting the model to the data; after all, in confirmatory factor analysis we build upon a set of relationships that are hypothesized by the researcher. It is not a data-driven method of finding the best set of relationships. If such an approach is desired, exploratory factor analysis (EFA) can be applied instead of CFA.

A.1.2. | Structural model

The structural component is added to the model once the latent constructs are defined, variables with low factor loadings are removed, and necessary covariance terms are added. The structural component consists of the relationships between latent constructs, or between latent constructs and outcome variable(s). With this, we now have three types of parameters for which an estimate must be calculated:

1. Factor loadings (observed variable $\rightarrow$ latent construct).
2. Covariances (observed variable $\leftrightarrow$ observed variable).
3. Regression coefficients (latent construct $\rightarrow$ latent construct or outcome variable).

Each parameter adds one degree of freedom to the model, and the number of parameters determines the identifiability of the model. Parameter estimates can only be obtained when the number of free parameters (the number of 'unknowns') is equal to or smaller than the number of independent elements in the covariance matrix of the data (the number of 'knowns'), which is equal to $k(k+1)/2$, where k is the number of observed variables in the model. If there are more unknowns than knowns, the model is under-identified and no solution can be found. If the numbers are the same, the model is just identified, and a unique solution can be obtained. If there are fewer unknowns than knowns, we have an over-identified model, which means that

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there is no unique solution but multiple, and we can select the best solution based on fit measures. An over-identified model is desired.

In most software packages parameter estimates are obtained by a maximum likelihood estimator by default, but alternative estimators can be chosen as well. In this study most observed variables did not follow a normal distribution, which violates maximum likelihood estimator assumptions. Therefore, the diagonally weighted least squares (DWLS) method was used instead, which is more robust and provides more accurate parameter estimates in case the normality assumption is violated.

If the model is over-identified, fit measures can be reported along with the parameter estimates. Again, TLI and RMSEA are used to assess model fit, with the same thresholds as seen in the CFA (TLI > 0.9, RMSEA < 0.08). If the model fit is acceptable the parameter estimates can be interpreted. The interpretation of the parameter estimates depends on the specification of the model. By default, one factor loading in each latent construct is set to 1, to fix the scale of the latent construct. However, in order to compare factor loadings across constructs it is useful to consider standardized parameter estimates. The variance of the latent construct is then set to 1 and factor loadings are interpreted in terms of a change in variance. In this study, we look only at the standardized parameter estimates, as we are interested in the relative importance of each observed variable and latent construct.

Factor loadings indicate how much variance of an observed variable is shared with the variance of its latent construct. Higher absolute values indicate more shared variance, and the sign of the factor loading specifies the direction of the association. Covariance terms provide the same information for two observed variables, which can belong to the same construct or to different constructs. If they belong to the same construct, a high covariance term indicates that these two variables share more variance with each other than can be explained by the latent construct. Regression coefficients indicate how much variance of the outcome variable is explained by the variance of the latent construct. To find the relative effect of a single observed variable on the outcome variable, its factor loading must be multiplied by the regression coefficient that connects the latent construct to the outcome.

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Check for updates

Received: 8 December 2022 | Revised: 30 January 2023 | Accepted: 16 February 2023

DOI: 10.1111/tme.12962

ORIGINAL ARTICLE

TRANSFUSION MEDICINE WILEY

Evaluation of two temperature storage conditions for full blood count samples from Lifeblood's donors

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Abstract

Background and Objectives: Lifeblood completes full blood count samples for selected donors to assess their suitability for future donations. Removing the current practice for refrigerated (2–8°C) storage and aligning with room temperature (20–24°C) storage of other donor blood samples would produce significant efficiencies in blood donor centres. This study aimed to compare full blood count results under two temperature conditions.

Materials and Methods: Paired full blood count samples were collected from 250 whole blood or plasma donors. These were stored either refrigerated or room temperature for testing on arrival at the processing centre and the following day. The primary outcomes of interest included differences between mean cell volume, haematocrit, platelet count, white cell and differential counts, and the need to produce blood films, based on existing Lifeblood criteria.

Results: A statistically significant ($p < 0.05$) difference for most full blood count parameters results was found between the two temperature conditions. The number of blood films required was similar under each temperature condition.

Conclusion: The clinical significance of the small numerical differences in results is considered minimal. Furthermore, the number of blood films required remained similar under either temperature condition. Given the significant reductions in time, processing and costs associated with room temperature over refrigerated processing, we recommend a further pilot study to monitor the broader impacts, with the intent to implement national storage of full blood count samples at room temperature within Lifeblood.

KEYWORDS
full blood count, room temperature, testing protocols

1 | INTRODUCTION

Full blood count (FBC) samples are routinely taken from Australian Red Cross Lifeblood (Lifeblood) donors prior to their first plasmapheresis or all plateletpheresis donations as recommended by the European Committee on Blood Transfusion.[1] The results of FBCs are important for guiding suitability of donors, including ensuring minimum platelet counts for plateletpheresis donors.[2] Additionally, FBCs may be completed along with a serum ferritin in the investigation of anaemia in blood donors. The FBC samples are routinely collected at

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donor centres, via venepuncture into ethylene diamine tetra acetate (EDTA) tubes, and transported to Lifeblood laboratories for processing using automated analysers. Current standard procedures for FBC testing at Lifeblood involves storage at a refrigerated temperature between 2 and 8°C. This study was conducted to assess differences in outcomes between samples stored at room temperature and the current standard refrigerated process. The results will be assessed for statistical significance as well as suitability for clinical practice. Removing the current requirement for storage of these FBC samples at refrigerated temperature and aligning with other donor blood samples room temperature processing would produce significant efficiencies in blood donor centres. However, the accuracy of FBC results is important to ensure suitable donor follow up and to reduce laboratory processes associated with erroneous results.

Duration since collection and ambient temperature of sample are two main variables that impact FBC results.[3] Manufacturers of automated analysers advise blood specimens yield reliable results for up to 24 h at either room or refrigerated temperature.[4–6] The International Council for Standardisation in Haematology (ICSH) recommend processing of blood samples within 4 to 8 h of collection, though state that room temperature sample results are within the limits of error for up to 24 h.[3,7] The time between collection and testing is dependent on the distance between Lifeblood donation centres and laboratories, with some samples requiring overnight transportation. Inaccuracy in results due to storage conditions carries a risk of an increased need for blood film reviews, a manual process by which blood samples are reviewed, and donor follow up. However, refrigeration of samples for this duration can have significant impacts on workloads and costs, and may have further impact on time to testing due to availability of transport options.

Following collection, changes in blood samples occur due to cellular elements having limited stability when stored in EDTA, whilst higher ambient temperatures also amplify these changes.[6,8] Red cells are known to swell over time, causing an increase in the haematocrit (HCT) and mean cell volume (MCV), however, haemoglobin (Hb) concentration is usually unaffected.[8] Higher temperatures also risk cell lysis, causing a decrease in red and white blood cell counts.[8] Platelets are known to swell, aggregate or disintegrate over time, which may lead to their decline.[7,9] Previous studies of FBC parameters have shown varying results when samples have been stored at different temperatures over different periods of time.[4–7,9,10] However, these studies have included low sample numbers and comparison of results from immediately following venepuncture.[4–6,9–13] Studies assessing room temperature FBC samples have identified red cell count (RCC), white cell count (WCC) and Hb to be stable anywhere from 24 to 48 h.[4–6,11] Whilst, MCV has been found to increase, yet, statistical significance of these difference in results vary from 6 to 48 h after collection.[6,11,13] Platelet counts have been reported to be stable at room temperature.[4,11] However, Gunawardena et al found a decrease in platelet numbers from 6 h when stored at room temperature, which is consistent with the expected physiological change.[6] White cell differential counts have been reported to be most dependent on the type of analyser used. Variations have been proposed to be due to an analyser's difficulty to assess the loss of individual cell characteristics that occurs as the cells age.[4,10] These differential results are also further complicated by very low numbers present in samples, often being reported in nanolitres or percentage of white cell count.

This study aimed to assess the differences in FBC results between samples stored at refrigerated (2–8°C) and room temperature (20–24°C). These outcomes will be used to guide future practices at Lifeblood. The primary outcomes of interest included differences between mean cell volume (MCV), haematocrit (HCT), platelet count (PLT), white cell count (WCC) and white blood cell differential counts. A secondary outcome for this study included the need to progress to blood film review based on initial results.

2 | MATERIALS AND METHODS

Donors were selected for this prospective comparison study through convenience sampling at one Lifeblood collection centre and consent obtained using the standard Donor Declaration. Paired FBC samples were collected from 250 adults (≥ 18 years) donating plasma or whole blood from a Lifeblood donor centre in Melbourne during March 2022. To be included in the study, individuals were required to be eligible for blood donation as per the current 'Guidelines for the Selection of Blood Donors' and complete the Donor Declaration. Donation occurred via a closed system, therefore, due to a minimum of 10 mls required for this study, donors with additional testing requirements were excluded to prevent risk of insufficient collection volumes. This included new donors, individuals donating platelets, or donors who required a 'sample only' collection related to other donation purposes.

The FBC samples were collected in two EDTA tubes, with blue labels representing the refrigeration sample and red labels identifying the room temperature sample. The control samples were refrigerated at the collection centre and transported maintaining temperatures between 2–8°C. The test samples were stored at room temperature, between 20–24°C in collection centres. Transport for room temperature samples was aligned with other Lifeblood pathology samples by using National Association of Testing Authorities (NATA) accredited and validated transport containers. These containers are controlled to maintain ambient temperatures between 4–25°C with the use of cold packs. Following less than an hour of transport, samples were returned to room temperature (20–24°C) whilst in the processing centre. All samples were tested using the DxH 800 Haematology automated analyser on arrival at the processing centre and again the following day, aiming within 24 h of the initial collection. This created 4 distinct groups of results: day 1 refrigerated, day 1 room temperature, day 2 refrigerated, day 2 room temperature.

The FBC results consisted of the following parameters; haemoglobin (Hb), haematocrit (HCT), red cell count (RCC), mean cell volume (MVC), platelet (PLT) count, white cell count (WCC), and white cell differentials of neutrophil, lymphocyte, monocyte, eosinophil and basophil counts. The primary outcomes of interest included the difference in results of mean cell volume (MCV), haematocrit (HCT), white cell count (WCC) and white blood cell differential counts. FBC results may also require the preparation of a blood film if analyser results occur

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TABLE 1 Results of FBC samples under each condition

Parameter	Day 1 Refrigerated	Day 1 Room temperature	Day 2 Refrigerated	Day 2 Room temperature
Mean (SD)				
Haemoglobin (g/L)	139.3 (10.9)	139.3 *(10.8)*	139.4 *(11.0)*	139.2 *(11.0)*
Haematocrit (L/L)	0.413 *(0.029)*	0.415 *(0.030)*	0.414 *(0.030)*	0.422 *(0.031)*
Red Cell Count (x10^{12}/L)	4.61 *(0.42)*	4.61 *(0.42)*	4.63 *(0.43)*	4.62 *(0.43)*
Mean Cell Volume (fL)	89.8 *(4.72)*	90.3 *(4.71)*	89.7 *(4.68)*	91.6 *(4.84)*
White Cell Count (x10^9/L)	5.72 *(1.37)*	5.61 *(1.34)*	5.75 *(1.38)*	5.63 *(1.36)*
Neutrophils (x10^9/L)	3.35 *(1.07)*	3.26 *(1.04)*	3.37 *(1.09)*	3.33 *(1.07)*
Platelet Count (x10^9/L)	225.8 *(61.6)*	228.1 *(62.3)*	229.7 *(63.1)*	230.5 *(63.8)*
Median (IQR)				
Lymphocyte Count (x10^9/L)	1.57 *(1.38–1.97)*	1.60 *(1.39–1.96)*	1.59 *(1.39–1.95)*	1.59 *(1.41–1.94)*
Monocyte Count (x10^9/L)	0.431 *(0.364–0.533)*	0.429 *(0.350–0.531)*	0.429 *(0.368–0.539)*	0.376 *(0.309–477)*
Eosinophil Count (x10^9/L)	0.135 *(0.084–0.208)*	0.131 *(0.079–0.194)*	0.140 *(0.087–0.219)*	0.119 *(0.071–0.200)*
Basophil Count (x10^9/L)	0.035 *(0.026–0.048)*	0.034 *(0.021–0.046)*	0.041 *(0.032–0.054)*	0.038 *(0.026–0.053)*

Abbreviation: IQR, Interquartile range 25%–75%; SD, standard deviation.

significantly outside of Lifeblood's reference ranges or if abnormal cellular morphology is identified, such as blasts or excessive immature granulocytes. Blood film reviews are uncommon in the healthy blood donor population and are time consuming which results in increased laboratory workloads. The secondary outcome measure included the difference in need to produce blood films, based on existing Lifeblood criteria, between sample groups.

Initial descriptive statistics for results will be provided using mean and standard deviation or median and interquartile range for those with a normal and non-normal distribution, respectively. Paired t-testing and Bland–Altman plots were used to assess the mean difference and agreement in results between the sample groups. A McNemar's test was used to determine if differences in the number of blood films required between the sample groups occurred. Statistical significance was defined by a p value <0.05.

The study protocol was designed and implemented and reported in accordance with the NHMRC National Statement on Ethical Conduct in Human Research and approved by the Australian Red Cross Lifeblood Ethics Committee. All samples were also labelled with donor unique identifiers to be used in the event that medical officer contact was required for abnormal results obtained from the day one refrigerated samples; as per the normal Lifeblood practice for following up test results.

3 | RESULTS

A total of 500 FBC samples were tested twice from the 250 donors. Donors were relatively evenly split between male and female, with 135 male donors (54%). The average time (hh:mm) from collection to initial test was 8:41 (SD 2:11) and 7:58 (SD 1:58) for refrigerated and room temperature samples (p = <0.01), respectively. The day two tests occurred an average of 22:18 (SD 1:55) after initial venepuncture. Twenty percent of these samples were tested more than 24 h after initial collection. As expected, majority of results fell within Lifeblood's predefined parameter reference ranges. All parameters except lymphocyte, monocyte, eosinophil and basophil counts had a fairly normal distribution of results (Table 1). There was a strong positive correlation ($r > 0.9$) between temperature condition or time of testing for all parameters, except basophils which has a moderate positive correlation ($r = 0.5$–0.6). There was strong agreement between temperature condition and/or time of testing for most parameters (>95% limit of agreement), and minimal deviation from the mean occurred when test results were compared to control results. All results also fell within acceptable limits for clinical use.

Hb was the most stable parameter, with only a statistically significant difference in mean results ($p = 0.02$) occurring between refrigerated and room temperature samples on day two of testing. The mean difference in Hb results under all testing variables was less than 0.2% (Figures 1 & 2). Conversely platelet results showed a statistically significant mean difference (p = <0.01) under all conditions except when comparing refrigerated and room temperature samples on day two ($p = 0.07$).

3.1 | Temperature effect

The mean difference in results between refrigerated and room temperature samples were found to be statistically significant for MCV, HCT, WCC (p = <0.01) when testing occurred on the day of and day following collection. White cell differentials showed a statistically significant mean decrease in results between refrigerated and room temperature samples ($p < 0.05$) on both day one and two, except for day one basophil results ($p = 0.29$) and day one lymphocyte results which there was a mean increased of 0.01 x 10^9/L ($p = 0.02$). The percentage mean different in results remained minimal for all but monocytes, eosinophils and basophils, which saw a maximum percentage mean decrease in room temperature from refrigerated samples of 13.2% 5.6% and 6.8%, respectively (Figure 1).

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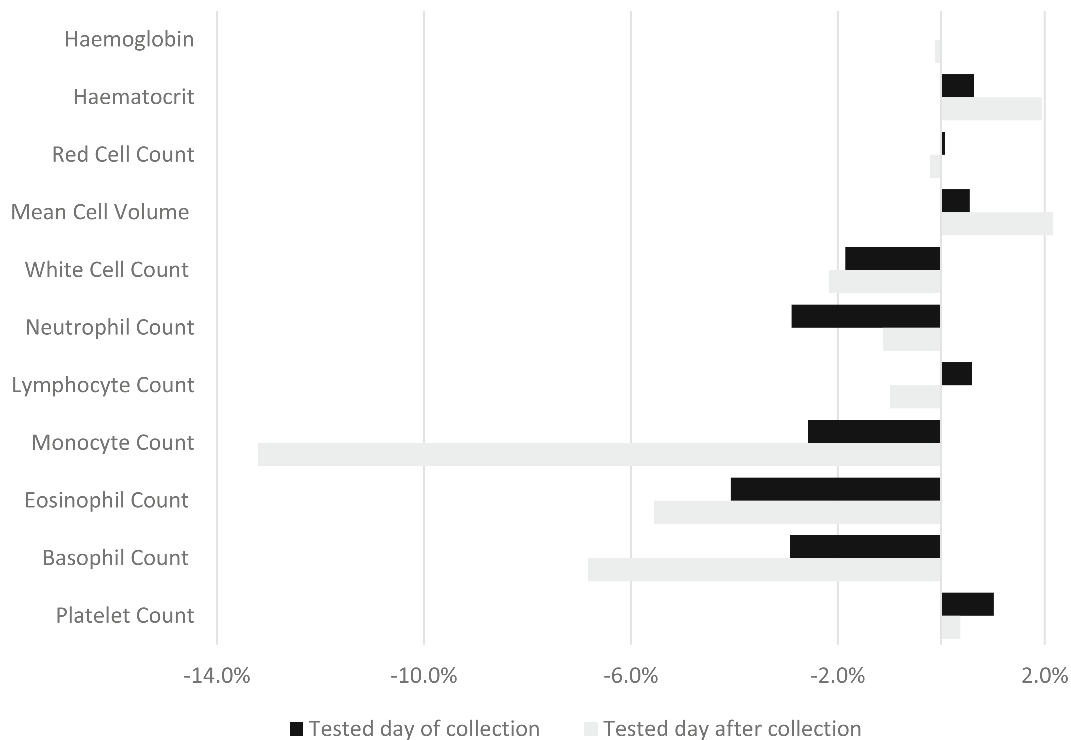

FIGURE 1 Percentage mean difference between room temperature and refrigerated sample results.

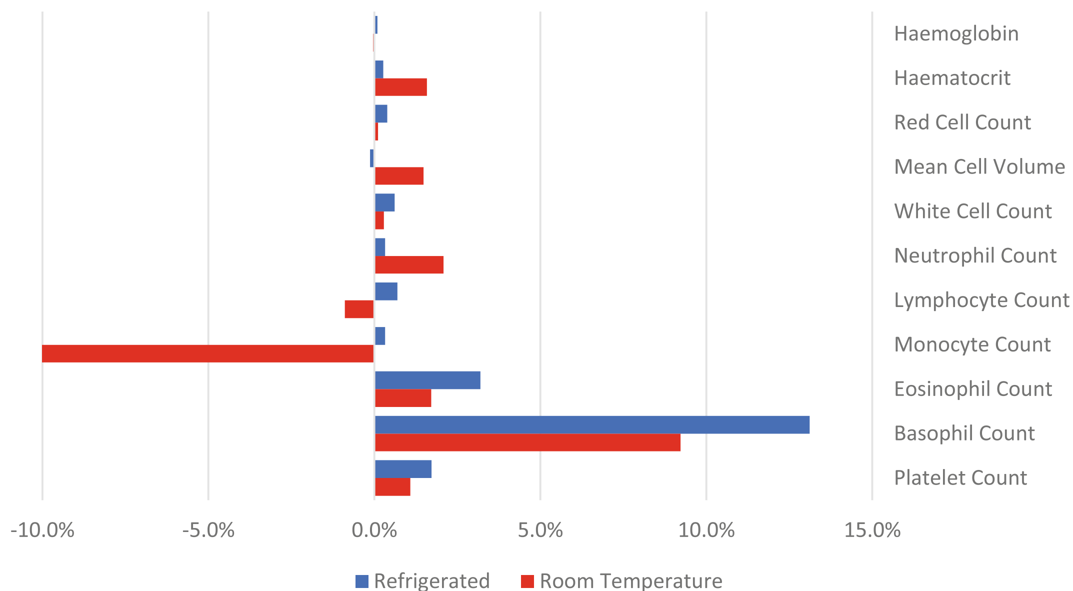

FIGURE 2 Percentage mean difference between day of collection and day after collection sample results.

3.2 | Time delay

The mean difference in results between samples tested on day one and two were found to be statistically significant for MCV, HCT, WCC (day 2 WCC $p = 0.03$, others $p = <0.01$) when tested at either refrigerated or room temperature. White cell differentials showed a mean difference in results between day one and two samples ($p < 0.05$) for both refrigerated and room temperature samples, except for refrigerated sample neutrophils ($p = 0.10$) and monocytes ($p = 0.48$), and room temperature sample eosinophils ($p = 0.33$). Mean results tended to increase over time, except for room temperature sample lymphocytes and monocytes which experienced a mean decrease of 0.02 (0.9%) and 0.04 (10.3%) x 10^9/L, respectively, between day one and two (Figure 2).

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TABLE 2 Blood film production (*n*) under each condition

Blood film Indicator	Day 1 Refrigerated	Day 1 Room temperature	Day 2 Refrigerated	Day 2 Room temperature
Numeric	4	5	2	5
Cell Morphology	5	8	1	4
Total	9	13	4	9

Note: Blood films required on all 4 of the testing occasions regardless of sample storage condition not included. Nineteen remaining donor samples contributed to blood film production on 1 to 3 of the testing occasions with numeric, cell morphology or both as indicators.

3.3 | Secondary outcome

Numeric or cell morphology indicators for blood film review occurred for 32 donors. Results from three of these donors showed borderline results and did not require the production of a blood film. Of the 29 remaining donors, 10 donors would have required the production and review of a blood film based on the indicator (9 numeric, 1 cell morphology) regardless of the sample storage condition. The remaining films required under each condition can be seen in Table 2. There was no statistically significant difference in the number of blood films produced between storage conditions or testing time.

Finally, results for the 3 test conditions were assessed for their sensitivity and specificity to detect individuals with results outside Lifeblood's FBC reference ranges when compared with the gold standard refrigerated day of collection sample results. Sensitivity and specificity for all parameters except HCT and platelet results were >99% under all conditions of storage. The sensitivity of HCT results was lowest for room temperature samples tested on the day after collection at 73%, while specificity remained >96%. Platelet results were 100% specific under all testing conditions when compared with refrigerated day of collection results, but sensitivity was again at its lowest at 77% for room temperature samples tested on the day after collection.

4 | DISCUSSION

The population of this study included blood donors who likely represent a healthy population given the stringent requirements for blood donation in Australia. Hence, as expected most results in our study were within the specified normal parameters. The timing of the initial testing of samples occurred on average at the upper threshold of the ICSH recommendations, with samples at room temperature being tested an average of 35 min prior to samples which were stored and transported at refrigerated temperature.[3] This comparative improvement in time to testing is projected to increase when samples are collected at outer metropolitan or rural donor collection centres, due to the decreased resource requirements that occur when transporting room temperature samples.

Our study, similar to previous,[4–6,11] found Hb to remain stable, both at refrigerated and room temperatures, and over 24 h. In contrast, we identified differences in WCC, with mean results at room temperature being slightly lower than refrigerated samples and a mild mean increase between day of and day after collection results. An increase in the mean HCT results were noted between temperatures and over time, this was most pronounced with the day after collection room temperature sample. Clinically, this may risk low HCT results being elevated above minimum thresholds when stored at room temperature or tested on the day after collection. Interestingly, the mean MCV increased over time when stored at room temperature but mildly decreased when samples were refrigerated. Mean platelet count was higher in the room temperature samples and increased over time in both temperature conditions. This is in contrast to Gunawardena et al[6] and indicates platelet swelling or aggregation over time was not significant in our study. However, sensitivity of the test conditions to produce results below Lifeblood thresholds were suitably appropriate for clinical use when compared to the gold standard refrigerated day of collection results.

While statistically significant differences were demonstrated between the data sets, the small numerical differences are minimal and clinically acceptable for our blood donor cohort. The proportion of medical follow up of donors, as per specific Lifeblood criteria, has not been affected by our results. Furthermore, those donors requiring deferral or medical review due to abnormal results were identified under all testing conditions. Finally, the need to perform blood films was similar under all conditions. Hence, no significant change in laboratory staff work load or donor medical follow up is anticipated from these results.

Implementation of room temperature storage negates the need for temperature monitored refrigerators in collection centres and refrigerated transport conditions. Furthermore, room temperature storage of FBC samples was shown to reduce transport time, as a result of samples being transported with donations and other pathology samples stored at room temperature, rather than end of day collection due to packaging requirements of refrigerated samples. This shorter duration to testing of FBC samples may also allow for a higher proportion of donor samples to be tested on the day of collection. Additionally, this is likely to improve accuracy of results. It is also likely our results are an over representation of what differences would be expected from donor testing if implementation of room temperature storage of samples occurred. This is due to 20% of the donor samples being tested at greater than 24 h since collection on day two. If there is likely to be a prolonged delay from collection to testing, then samples can also be

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refrigerated once at a processing centre rather than being stored at room temperature overnight as in this study.

A limitation to this study includes the wide temperature range for the room temperature samples when being transported (4–25°C). The validated containers used for transport likely had minimal effect on lowering the sample temperatures from the ambient storage temperature. Furthermore, this was only a short portion of the overall duration between collection and testing. This study was also completed in a cooler climate, and hence the effects of a warmer and more humid climate has not been evaluated. Finally, this study addressed FBC results of healthy individuals with a high proportion of results within normal range, the effect of a higher proportion of pathological results remains unevaluated.

5 | CONCLUSION

Despite the differences in results for many of the parameters when stored and tested under the different conditions, mean results remained within normal limits with very minor variations in results. The results remained appropriate for clinical use in the case of screening for blood donation and did not increase laboratory processing requirements due to increased necessity for blood film assessments or medical follow up. There is a moderate cost saving and time benefit to storing FBC samples at room temperature. Given the significant reductions in time, processing, and costs associated with room temperature over refrigerated storage and transport, we recommend a further pilot study to monitor the broader impacts, with the intent to implement national storage of FBC samples at room temperature within Lifeblood. However, this should be implemented with caution and further evaluation of results of samples stored in warmer climates and with various transport times is required to ensure the results remain clinically appropriate for national scaling.

AUTHOR CONTRIBUTIONS

Georgina Jacko prepared the draft manuscript and analysed data; **Anna Green**, **James Peberdy** and **James Daly** designed the research study; **Leo Lycett** prepared and analysed the data; **Sue Ismay** process and data owner, **James Daly** supervised the research. All authors reviewed and edited the manuscript.

Jade Johnstone – donor services project sponsor; Christine Nilsson and her team at Airport West – sample collection; Alana Sarec – collection staff training.

FUNDING INFORMATION

The Australian Government funds Australian Red Cross Lifeblood for the provision of blood, blood products, and services to the Australian community.

CONFLICT OF INTEREST STATEMENT

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

Data available on request.

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How to cite this article: Jacko G, Green A, Lycett L, Ismay S, Peberdy J, Daly J. Evaluation of two temperature storage conditions for full blood count samples from Lifeblood's donors. *Transfusion Medicine*. 2023;33(3):221-226. doi:10.1111/tme.12962

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Check for updates

Received: 29 January 2022 | Revised: 23 December 2022 | Accepted: 2 February 2023

DOI: 10.1111/tme.12961

ORIGINAL ARTICLE

TRANSFUSION MEDICINE WILEY

Rh(D) immune globulin administration in pregnancy: Retrospective audit of patient safety events followed by targeted educational intervention with Bayesian analysis

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Abstract

Objectives: To examine local patient safety events related to the administration of anti-Rh(D) immune globin (RhIG) during pregnancy, and to follow-up with targeted educational intervention to improve knowledge of this process.

Background: Administration RhIG is established treatment for the prevention of haemolytic disease of the foetus and newborn (HDFN). However, patient safety events in relation to its correct use continue to occur.

Methods: A retrospective audit of patient safety events related to RhIG administration during pregnancy was performed. Targeted educational intervention in the form of PowerPoint® presentation were given to nursing staff, laboratory staff and physicians and evaluated with pre- and post-tests using multiple-choice questions given immediately before and after the presentation.

Results: An annual incidence of 0.24% of patient safety events related to the administration of RhIG during pregnancy was found. These events were mostly in the preanalytical phase, for example mislabelled samples or samples for D-rosette/Kleihauer–Betke testing drawn from the baby, not the mother. Using Bayesian analysis, the probability of positive effect for the targeted educational intervention was 100% with a median improved score of 29%. This was compared with a control group using standard curriculum education intervention based on the current curriculum for nursing, laboratory and medical students which showed a median improved score of only 4.4%.

Conclusions: Administration of RhIG during pregnancy is a multistep process involving health care professionals of several disciplines providing opportunities to enhance the curriculum for nursing, laboratory and medical students and to ensure on-going education.

KEYWORDS
education, Rh immunoglobulin patient safety events pregnancy

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1 | INTRODUCTION

The prevention of haemolytic disease of the foetus and newborn (HDFN) due to anti-D by the administration of anti-Rh(D) immune globin (RhIG) as immunoprophylaxis is one of the greatest milestones in obstetrics. HDFN due to anti-D is a potentially fatal condition that occurs when there is an immunologic reaction that results in the destruction of the Rh-D positive red blood cells (RBC) of the foetus by maternal anti-D immunoglobulin G (IgG) alloantibody.[1] Maternal exposure and mixing with foetal RBC can be caused by many events such as ectopic pregnancy, versions, miscarriage, antenatal bleeding, abdominal trauma and delivery. Also, procedures that could increase the incidence of anti-D immunization include amniocentesis, chorionic villus sampling, cordocentesis and uterine curettage.[2,3] All of these events during pregnancy could sensitise the Rh negative woman.

Anti-D alloantibody that targets the D antigen on the RBC is the most common alloantibody known to cause HDFN. Anti-D IgG antibodies can cross the foetal-maternal placental barrier.[1] Therefore, if an Rh negative female is sensitized to D-positive foetal antigen, then the antibodies formed during her first pregnancy would traverse the placenta and bind to the foetal RBC of her second pregnancy. The binding of the anti-D on the foetal RBC causes HDFN of differing severity. In the most extreme cases, the subsequent haemolysis can lead to severe foetal anaemia, brain damage, seizures, jaundice, hydrops foetalis, heart failure and even foetal death.[4] The potential burden of HDFN due to anti-D shows ethnic variation; with for example 15% of the Caucasian population being Rh negative.[5]

Rh immunization which produces anti-D occurs in the event of foetal-maternal haemorrhage (FMH) and/or transfusion. The likelihood of the Rh immunization to occur is proportional to the volume maternal exposure to foetal Rh-positive RBC. Even a volume of FMH of less than 0.1 mL Rh(D) positive foetal RBC has been observed to be associated with 3% maternal Rh(D) immunization.[6] During pregnancy, attention to the Rh blood group system becomes a serious health concern, especially if the woman is Rh negative (D negative) and exposed to the foetal Rh positive (D positive) RBC. By the sixth gestational week, D antigen can be present on the foetal RBC.[7]

RhIG is obtained from human plasma of individuals with a high titre of IgG antibodies to the D antigen. RhIG is administered in the antenatal and postnatal period to Rh(D) negative women to prevent the formation of anti-D antibodies by blocking sensitization from exposure to Rh(D) positive foetal RBC. A dose of 300 micrograms (μg) of RhIG is sufficient to prevent Rh(D) immunization from an exposure of up to 15 mL of Rh(D) positive foetal RBC or 30 mL of foetal whole blood.[8] Guidelines for the administration of RhIG during pregnancy vary from country to country. Current Canadian guidelines are for all Rh negative non-sensitized women to receive a 300 μg dose of RhIG at 28 weeks gestation, and additionally, after delivery of an Rh positive infant, all Rh negative mothers receive another 300 μg dose of RhIG within 72 h.[9] When administered at 28 weeks gestation and within 72 h postpartum, RhIG decreased the incidence of alloimmunisation from 12%–13% to 1%–2% in at risk pregnancies.[10]

The Rh locus has many variants including weak D. Women with serological weak D phenotype have reduced surface expression of Rh(D). Approximately, 0.2%–1.0% of the Caucasian population expresses the serological weak D phenotype, and of these, the majority (80%) were weak D types 1, 2 or 3. Therefore, the Work Group on *RHD* Genotyping suggests that molecular genotyping should be performed on individuals with weak D phenotype to determine whether they are types 1, 2, 3 or other weak D types.[11] Women with weak D types 1, 2 or 3 can be considered as Rh positive because these individuals express sufficient surface D-antigen to avoid anti-D alloimmunisation and therefore, administration of RhIG prophylaxis is unnecessary and should be avoided. For Rh(D) women with Rh variants other than weak D types 1, 2 or 3, a 300 μg dose of RhIG is recommended at 28 weeks gestation and within 72 h postpartum.[12]

Additional testing to assess the volume of FMH may be useful to determine the amount of RhIG that is required to prevent alloimmunisation for Rh(D) negative women who deliver an Rh(D) positive infant. The D-rosette test (qualitative test) is used to screen for FMH in the maternal circulation by revealing the amount of Rh positive foetal RBC among Rh negative maternal RBC. If the D-rosette test is positive (>5 mL of FMH), then Kleihauer–Betke test or flow cytometry (quantitative tests) should follow to estimate the volume of the haemorrhage and guide the dosage of RhIG.[1,13] As mentioned previously, since the 300 μg standard dose only covers 15 mL of Rh positive foetal RBC (or 30 mL of foetal whole blood), additional vials of RhIG are required to prevent alloimmunisation and protect the next pregnancy if the FMH exceeds this volume.

Since its introduction in the 1960s, RhIG has saved countless lives. In at risk gestations, the study by Joseph & Kramer[14] demonstrated Rh sensitisation rates decreased by 7 per 1000 births and perinatal deaths fell from 1.1 per 1000 to 0.06 per 1000 after the administration of RhIG antepartum and postpartum. Despite the widespread adoption of this practice, patient safety events relating to this prophylaxis continue to occur from a variety of causes which may include antenatal Rh(D) sensitisation prior to 28 weeks gestation, inadequate prenatal care and inappropriate dosing of RhIG.[15,16]

An audit cycle and feedback followed up by academic detailing and educational outreach offers a systematic method for a healthcare system to implement behavioural change over time and improve clinical outcomes.[17] To address patient safety issues with RhIG administration, we first performed a retrospective audit of patient safety events related to RhIG administration during pregnancy and followed this with a targeted educational intervention to improve knowledge of this process. Assessment tools to evaluate transfusion medicine knowledge[18] and educational programmes[19,20] have been well received and shown to be helpful to identify areas needing additional support. Applying knowledge to the practice of transfusion medicine is more challenging. Lin et al. showed positive rating for transfusion medicine knowledge as very important in patient care, although objective evidence of change in behaviour was not part of their study.

2 | MATERIALS AND METHODS

2.1 | Audit

Files from the patient safety learning system (PSLS) of the Ottawa Hospital from January 2011 to July 2016 were initially selected based on location (paediatrics, neonatal, obstetrics and special care nursery wards). A key word search (Rh, RhIG, WinRho, rosette, and Kleihauer) was performed on these files to identify PLSL events related to RhIG administration during pregnancy. For the calculation of the incidence of these patient safety events, the average total annual number of RhIG doses issued by the Transfusion Medicine Laboratory at the Ottawa Hospital for this time period was tabulated.

3 | EDUCATIONAL INTERVENTION

3.1 | Development of educational tool

Knowledge items were developed from audited patient safety events described above, necessitating review of D-rosette and Kleihauer–Betke test methods and the RhIG administration process in pregnancy. The threshold of question difficulty was tailored to address theoretical and procedural knowledge gaps needed to correctly identify, quantify and dose RhIG prophylaxis that exist across the multidisciplinary team. This was site specific to the professionals at the Ottawa Hospital and Eastern Ontario Regional Laboratory Association in clinical and laboratory medicine pertaining to their different scopes of practice. Multiple-choice questions were mapped to the topic of Rh administration during pregnancy using the Canadian Blood Services professional educational article[21] and Society of Obstetrics and Gynaecologists of Canada guidelines.[9] Questions were validated using a control group of pathology laboratory personnel. Question item difficulty index was set at 80%, avoiding the 85% threshold which has been suggested to indicate too easy a question.[18] Questions which were correctly answered by less than 80% of the control participants on the pre-test were considered valid and used as the assessment tool. As per classical test theory, the multiple-choice questions were scored for correct answers only.[22] There was no negative marking, and no correction for guessing was applied.

3.2 | Study design

To assess the effect of the educational intervention, the method used was a mixed between- within quasi-experimental design using pre-test and post-test with a control group crossover.[23,24] For the educational intervention group, the quasi-experimental design was the pre-test as the initial observation, the targeted educational PowerPoint® presentation as the intervention and the post-test as the observation to assess the effect. For the control group, there was crossover design. For the control group standard curriculum educational intervention, the pre-test was the initial observation, the standard curriculum PowerPoint® presentation was the intervention and the post-test was the observation to assess the effect. The control group was then crossed over to receive the targeted educational PowerPoint® presentation as the intervention and the post-test was given again as the observation to assess the effect. The pre-test and post-test results were not matched. The sampling strategy for the control group was chosen as a matter of convenience, and used the pathologists, laboratory technologists and trainees regularly attending the departmental pathology grand rounds. The sampling strategy for the targeted educational intervention was to focus on laboratory technologists and nurses involved in the testing and administration of RhIG; a few physicians were also included. A schematic of the study design is shown in Figure 1.

3.3 | Control group: Standard curriculum educational intervention

A PowerPoint® presentation containing information from the current curriculum for RhIG administration during pregnancy for medical students, nursing students and laboratory technology students was prepared and presented to a control group in English. The topics of the current curriculum presentation included description of the Rh blood group, pathogenesis of HDFN, serological weak D testing, and prophylactic use of RhIG in pregnancy. The current curriculum did not include the topics of D-rosette test, Kleihauer–Betke test and genetic weak D types 1, 2 and 3 test. The control group presentation took place as one of the regularly scheduled 1-h pathology department grand rounds, with the audience consisting of the usual attendees of physician pathologists, medical laboratory technologists and trainees. The attendees did not have any recent education on the topic of Rh administration during pregnancy. The first 25 min was taken up by the control group presentation, with pre-test and post-test administration (all five questions). The remaining 35 min was taken up by the crossover of the audience to the targeted educational intervention, see section on control group: Targeted Educational Intervention, followed by the administration of the five post-test questions. The pre-test and post-test were distributed to the attendees as paper copies, and collected immediately upon completion. Pre-test results were compared to the post-test results, which were administered after the curriculum presentation, and used to assess the quality and quantity of current medical, nursing and laboratory technology curriculum.

3.4 | Clinical group: Targeted educational intervention

Targeted educational intervention material was prepared as detailed PowerPoint® presentations in English and French, with the full presentation reviewing the theory, pathophysiology and use of RhIG administration during pregnancy, including the biological principals underlying therapeutic use of RhIG in pregnancy, the guiding principles for routine use of RhIG immunoprophylaxis, the lab testing (including D-rosette and Kleihauer–Betke) required to guide use of RhIG in pregnancy and after delivery in routine situations, in the event of FHM, including RhIG dose calculation and in patients with weak D, including the role of Rh genotyping. This

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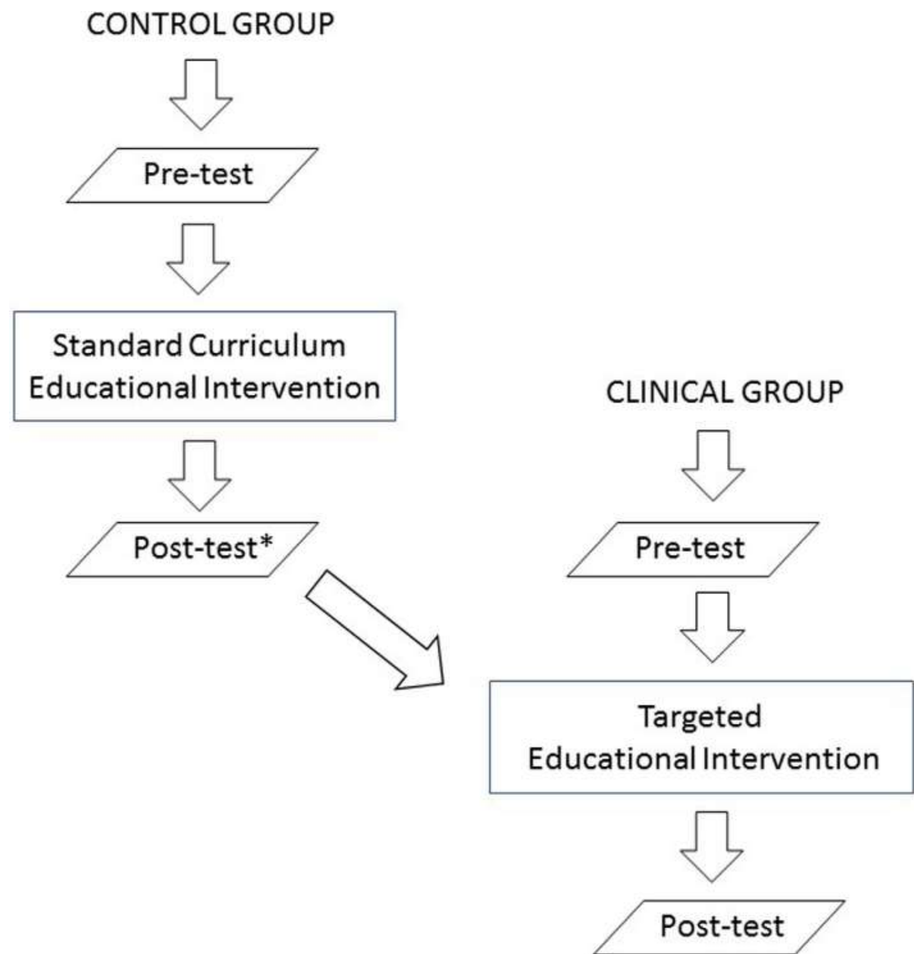

FIGURE 1 Schematic highlighting the study design of the educational intervention. Note the crossover design for the control group, with the first post-test results (indicated by *) used again as the control group pre-test results before the targeted educational intervention.

presentation was given during 2019 in person as part of regular in-service/cercle de qualité meetings to a variety of clinical health-care professionals including labour and delivery nurses and nurse practitioners at The Ottawa Hospital (TOH), physicians and laboratory technologists at the Children's Hospital of Eastern Ontario (CHEO), nurses, medical scientific staff and laboratory technologists at Renfrew Victoria Hospital (RVH) in English and laboratory technologists and emergency room physicians and nurses at the Montfort Hospital (MH) in French. Because of time constraints on some busy clinical services, shortened presentation was given as a 10-min presentation including biological principals, and dosing of RhIG in routine pregnancies and following FMH; attendees of the 10-min presentations were the labour and delivery nurses and nurse practitioners at TOH and the emergency department staff at MH.

3.5 | Control group: Targeted educational intervention

The same detailed PowerPoint® targeted educational presentation (full presentation) given to the clinical group, was also given to the control group, immediately following the standard curriculum educational presentation to the control group, and was followed by the repeat administration of the post-test, with all five questions asked.

3.6 | Assessment of targeted educational intervention

Each educational intervention presentation was preceded by pre-test question(s) and effectiveness of the presentation was assessed by asking the same question(s) as a post-test immediately after the presentation. From 1 to 5 questions were used, depending on the length of the in-service presentation, as there were time constraints because of the clinical duties of the audiences. The shortest presentation was 10 min in length to the labour and delivery nurses and nurse practitioners at TOH, and in the emergency department at MH, with only the first question asked. The full presentation given as a 30-min presentation with a lunch & learn format was given to the laboratory staff at MH with only questions 1, 2 and 3 asked. The full presentation was given at a slower pace to the CHEO staff, RVH staff and the standard curriculum control group, with all five questions asked.

The control group took the pre-test before the standard curriculum educational intervention, and took the post-test immediately following. These post-test results were used again as the control group pre-test before the targeted educational intervention, and the post-test was administered again to the control group immediately following the targeted educational intervention.

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TABLE 1 Patient safety learning events.

Phase of patient safety events	Details of patient safety events	Number of events (%)
Preanalytical		15/22 (68%)
	Lab requisitions not signed	4
	Mislabelled	2
	Mother Rh positive	3
	Preformed allo anti-D antibodies	2
	Samples for D-rosette/Kleihauer drawn from baby, not mother	2
	Kleihauer order missed	1
	Foetal/maternal haemorrhage not quantified	1
Analytical		2/22 (9%)
	Misinterpretation of antibody screen as positive but was due to RhIG (corrected)	1
	Erroneous Kleihauer result reported (and then corrected)	1
Postanalytical		5/22 (23%)
	RhIG vial mishandled after issue/lost or returned to TM lab unused	2
	RhIG given to wrong patient	1
	Underdosing of RhIG	1
	RhIG not administered prior to discharge	1

4 | STATISTICAL ANALYSIS

Proportional probabilities were calculated based on the Bayesian analysis, with P(A|B), where $P =$ probability of improved score on post-test (A) following educational intervention (B).[25] Statistical analysis was performed using the statistical programming language R using the 'Bayesian First Aid' package.[26] Combinatorial calculation was performed using the Monte Carlo chain module.

5 | ETHICS STATEMENT

The study was submitted to the Ottawa Health Science Network (OHSN) Research Ethic Board (REB) with the decision letter indicating that this project fell within the context of quality initiative, quality improvement, and therefore review by the OHSN REB was not required. The participants in our study did not provide informed consent and the participants' pre- and post-tests results were anonymized, and not paired to individual participants.

6 | RESULTS

6.1 | Audit

The retrospective audit of RhIG administration at the Ottawa Hospital 2011–2016 identified 22 patient safety events. Over the same time period, an average of approximately 1500 RhIG doses were administered annually, thus the annual incidence of patient safety events related to RhIG administration during pregnancy was very low (0.24%). The patient safety events were categorized into preanalytical, analytical and postanalytical events (Table 1). Similar to other laboratory

TABLE 2 Assessment tool.

Question 1 (correct answer a)

When is RhIG indicated for women after delivery? (Choose all that apply)

a. Rh(D) negative mum with a negative antibody screen and a Rh(D) positive baby
b. Rh(D) positive mum with a positive antibody screen and a Rh(D) negative baby
c. Rh(D) positive mum with a negative antibody screen and a Rh(D) positive baby
d. Rh(D) negative mum with a positive antibody screen showing anti-D antibody and a Rh(D) negative baby

Question 2. (correct answer d)

What is standard dose of RhIG administered at 28 weeks and again within 72 hours of delivery?

a. 30ug
b. 120ug
c. 250ug
d. 300ug
e.1500ug

Question 3. (correct answer c)

A single vial of 300 ug RhIG administered will prevent formation of maternal anti-D antibodies for up to what volume of whole blood exposure in a FMH?

a. 10 mL
b. 15 mL
c. 30 mL
d. 50 mL

Question 4. (correct answer b)

For Kleihauer–Betke and D-rosette tests, which statement(s) is/are correct? (Choose all that apply)

a. Using cord blood sample after delivery, the D-rosette test is performed first to screen for FMH, and if positive is followed by the Kleihauer–Betke test to quantify the FMH
b. Using mum's blood sample after delivery, the D-rosette test is performed first to screen for FMH, and if positive is followed by the Kleihauer–Betke test to quantify the FMH
c. Using either mum's blood sample or cord blood sample after delivery, the D-rosette test is performed first to screen for FMH, and if positive is followed by the Kleihauer–Betke test to quantify the FMH
d. Using mum's blood sample after delivery, the Kleihauer–Betke test is performed first to screen for FMH, and if positive is followed by the D-rosette test to quantify the FMH

Question 5. (correct answer c)

If the pregnant woman has weak D phenotype and a negative antibody screen and the weak D molecular testing confirms that the Rh(D) genotype is not Type 1, 2 or 3:

a. RhIG not needed
b. Administer RhIG between 8–12 weeks of gestation and after delivery
c. Administer RhIG at 28 weeks of gestation and after delivery
d. Administer RhIG at 28 weeks of gestation, but not after delivery

Note: Set of questions that were used as pre- and post-test for all the groups of this study to evaluate the effectiveness of the presentation.

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TABLE 3 Control group pre-test and post-test results after standard curriculum educational intervention.

	N (pre-test)	*N* (post-test)	Pre-test	Post-test	Proportional improvement in score (± confidence interval)	Non-parametric comparison p-value (Chi-square, (degrees of freedom = 1)
Question 1	38	37	66% (25/38)	62% (23/37)	−0.04 (−0.24–0.17)	0.931
Question 2	38	37	66% (25/38)	70% (26/37)	0.03 (−0.16–0.25)	0.866
Question 3	38	37	39% (15/38)	35% (13/37)	−0.04 (−0.24–0.17)	0.881
Question 4	38	37	37% (14/38)	37% (14/37)	0.01 (−0.21–0.22)	1.000
Question 5	38	37	29% (11/38)	54% (20/37)	0.24 (0.03–0.44)	0.048*

TABLE 4 Clinical group pre-test and post-test results after targeted educational intervention.

	N (pre-test)	*N* (post-test)	Pre-test	Post-test	Proportional improvement in score (± confidence interval)	Non-parametric comparison p-value (Chi-square, degrees of freedom = 1)
Question 1	38	38	50% (19/38)	84% (32/38)	0.33 (0.13–0.52)	0.003*
Question 2	23	23	87% (20/23)	100% (23/23)	0.12 (−0.03–0.29)	0.232
Question 3	18	18	67% (12/18)	89% (16/18)	0.20 (−0.05–0.46)	0.229
Question 4	10	10	30% (3/10)	70% (7/10)	0.34 (−0.02–0.68)	0.180
Question 5	10	10	40% (4/10)	100% (10/10)	0.50 (0.20–0.79)	0.015*

deviations, most of the safety events (68%) occurred at the preanalytical phase. These events were related to patient specimen labelling, requisitions, missed orders, selection of incorrect type of patient for testing, and drawing blood sample from baby instead of mother. Analytical errors were the lowest (9%), and were both interpretive errors. One was miscalculation of the Kleihauer–Betke result. The other was misinterpretation of the transfusion medicine antibody panel, with the antibody panel initially reported as 'non-specific' when there was evidence in the panel of weak anti-D due to recent administration of RhIG. Both of these errors were corrected before there was any impact on patient care. Postanalytical safety events (23%) included underdosing of RhIG, RhIG given to the wrong patient, RhIG not administered prior to discharge and RhIG vial lost or returned to blood bank unused.

6.2 | Educational intervention assessment tool

The five questions used as the pre-test and post-test are shown in Table 2. For test validation, all five questions obtained less than 80% correct when given as a pre-test to the control group (see Table 3, pre-test results).

The pre-test and post-test results are compared in Tables 3–6, including the pre-test and post-test per cent scores as a function of group, and with the Bayesian analysis proportional improvement in score ± confidence interval for each question and each group, which are also illustrated in Figure 2. There are no confidence intervals or error estimates for the pre-test and post-test per cent scores as a function of group, as the testing was done only once. With the lack of pre-test post-test matching, the non-parametric comparison using Chi-square analysis was also performed using the statistical programming language R. *P*-values are supplied, defining a value of <0.05 as statistically significant, indicated by * in Tables 3–6.

6.3 | Control group: Standard curriculum educational intervention

As shown in Table 3, 38 participants completed the pre-test and 37 participants completed the post-test before and after the standard curriculum educational intervention. The test results demonstrated low improvement in the post-test scores for questions 1–4, with proportional improvements 0.03 or less and no significance demonstrated with non-parametric comparison. The greater improvement for question 5 still shows a wide confidence interval, and is interpreted as getting the right answer for the wrong reason, as the importance of giving RhIG at 28 weeks gestation and within 72 hours postpartum was emphasized, but information about RhIG administration and weak D genotyping was not provided in this presentation.

6.4 | Clinical group: Targeted educational intervention

Table 4 shows the results of pre- and post-tests before and after the targeted educational intervention consisting of the detailed PowerPoint® presentation given to the clinical group as part of the regular in-service/cercle de qualité meetings. The number of participants answering the questions ranged from 38 for question 1, to 10 for questions 4 and 5. The targeted educational intervention resulted in improved test performance on all 5 questions, with proportional improvement in scores ranging from 0.12 up to 0.50 and reaching statistical significance with non-parametric comparison for questions 1 and 5.

TABLE 5 Control group pre-test and post-test results after targeted educational intervention.

	N (pre-test)	*N* (post-test)	Control group Pre-test	Post-test	Proportional improvement in score (± confidence interval)	Non-parametric comparison p-value (Chi-square, degrees of freedom = 1)
Question 1	37	34	62% (23/37)	79% (27/34)	0.16 (−0.04–0.36)	0.183
Question 2	37	34	70% (26/37)	100% (34/34)	0.28 (0.13–0.43)	0.002*
Question 3	37	34	35% (13/37)	85% (29/34)	0.47 (0.28–0.66)	<0.001*
Question 4	37	34	37% (14/37)	65% (22/34)	0.53 (0.35–0.70)	<0.001*
Question 5	37	34	54% (20/37)	76% (26/34)	0.21 (0.00–0.42)	0.084

TABLE 6 Subgroup analysis. Nurses and medical laboratory technologists.

	N (pre-test)	*N* (post-test)	Pre-test	Post-test	Proportional improvement in score (± confidence interval)	Non-parametric comparison p-value (Chi-square, degrees of freedom = 1)
Nurses	12	12	17% (2/12)	67% (8/12)	0.44 (0.11–0.73)	0.038*
Medical laboratory technologists	12	12	92% (11/12)	100% (12/12)	0.06 (−0.15–0.31)	1.00

Note: Clinical group pre-test and post-test results for Question 1 after targeted educational intervention.

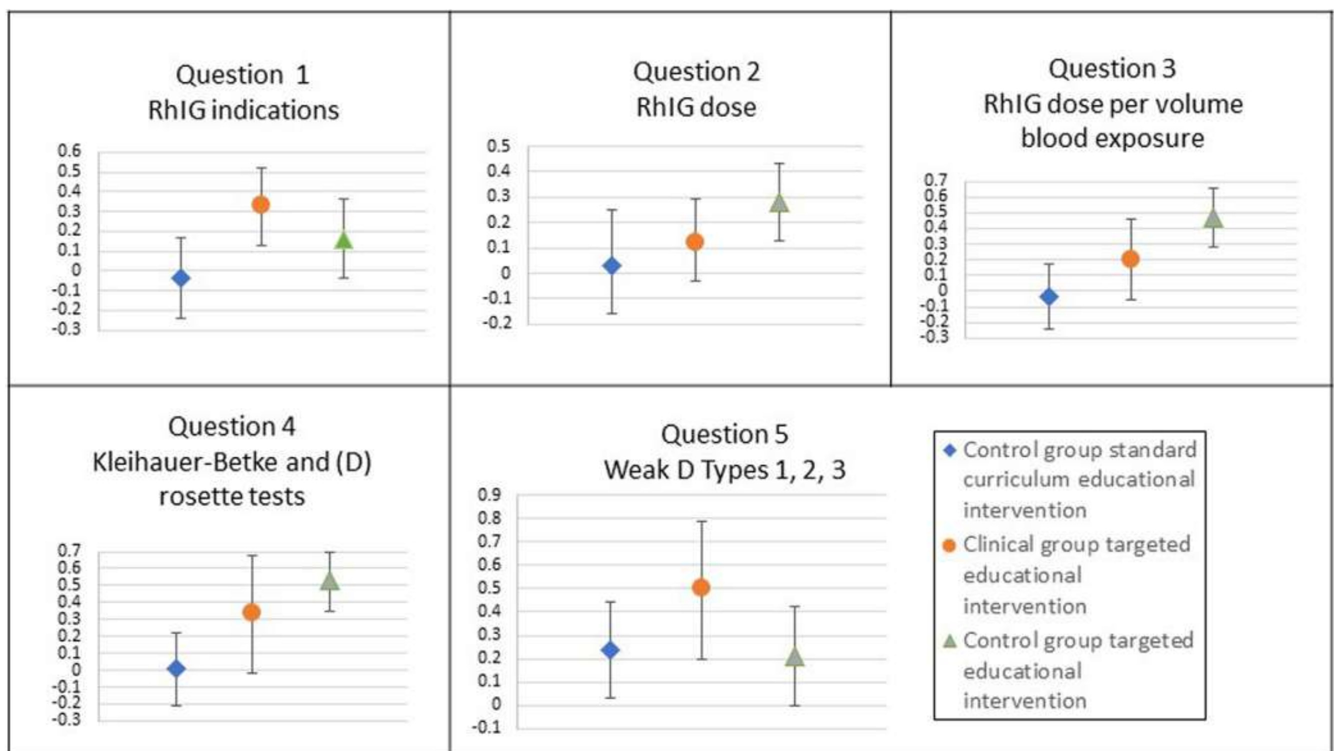

FIGURE 2 Bayesian analysis proportional improvement in score of post-test results ± confidence intervals for each question and each group.

6.5 | Control group: Targeted educational intervention

Table 5 shows the pre- and post-tests results before and after the targeted educational intervention consisting of the detailed PowerPoint® presentation given to the control group of laboratory professionals immediately after the current standard curriculum presentation. A total of 37 participants completed the pre-test and 34 participants completed the post-test. The targeted educational intervention resulted in improved test performance on all 5 questions, with

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proportional improvement in scores ranging from 0.16 up to 0.53 and reaching statistical significance with non-parametric comparison for questions 2, 3 and 4.

6.6 | Subgroup analysis clinical group: Targeted educational intervention

In the clinical group of targeted educational intervention, a total of 12 nurses and 12 medical laboratory technologists completed pre- and post-test question 1. As shown in Table 6, subgroup analysis showing medical laboratory technologists (MLTs) achieved higher scores than nurses for Question 1 both before and after the clinical group: targeted educational intervention. The pre-test score for nurses was 2/12 (17%) correct and for the medical laboratory technologists was 11/12 (92%) correct. The post-test score for nurses was 8/12 (67%) correct and for the medical laboratory technologists was 12/12 (100%) correct. The targeted educational intervention was very effective for the nurses, with a 0.44 proportional improvement (95% CI 0.11–0.73) and reaching statistical significance with non-parametric comparison, $p = 0.038$. The proportional improvement in score for the MLTs was only 0.06 (95% CI—0.15–0.31) due to their high score on the pre-test. The number of participants answering questions 2–5 was insufficient for subgroup analysis.

6.7 | Bayesian analysis of targeted educational intervention

Using the probability density function, the proportional probabilities based on Bayesian analysis showed the targeted educational intervention was more effective than the standard curriculum educational intervention as currently taught to medical students, nursing students and laboratory technology students. Figure 3A shows that although the standard curriculum educational intervention had a probability of a positive effect of 80.9%, the overall improvement in score was only 4.4%. Figure 3B shows the probability of positive effect for the targeted educational intervention for the clinical group was 100%, with a median improved score of 29%. Figure 3C shows the probability of a positive effect for the targeted educational intervention for the control group was 100%, with a median improved score of 30%.

7 | DISCUSSION

As shown by our audit of patient safety events, correct administration of RhIG in pregnancy is a multistep process involving many healthcare professionals, making it prone to errors and deviations.[27] A survey of laboratory practice by the College of American Pathologists demonstrated more than 20% of laboratories may have recommended inaccurate doses of RhIG.[28] Also, gaps have been identified in physician knowledge of certain aspects of RhIG administration, including correct FMH testing in an Rh-negative mother giving birth to an Rh-weak D-positive infant[29] and correct calculation of RhIG in cases of FMH.[30] A recent Canadian survey found that not all centres quantify FMH to ensure that sufficient RhIG is administered to prevent alloimmunisation (Survey Report[31]). Our audit disclosed one misinterpretation of an antibody panel as 'non-specific', but investigation before release of these results determined the observed reactions were due to recent administration of RhIG. It is very important to distinguish between passive and immune anti-D, and accurate documentation of RhIG administration, quantification of antibody levels along with serial monitoring may assist in ambiguous cases.[32] This will avoid poor outcome, as illustrated by the neonatal death which occurred from complications after allo-anti-D antibody was erroneously thought to be passive anti-D from RhIG administration.[33]

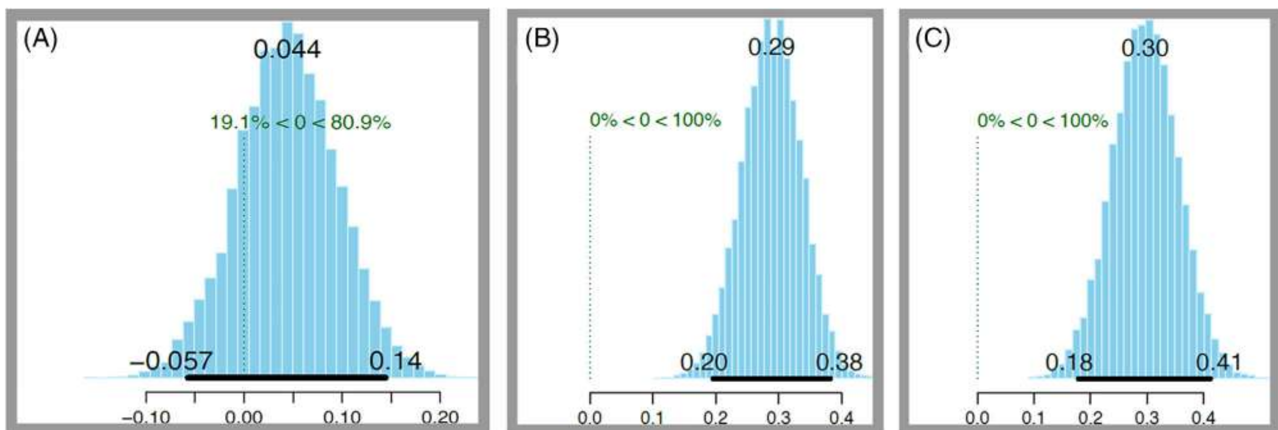

FIGURE 3 Posterior distributions of the education intervention effect. Shown are probabilities based on the Bayesian proportional analysis. The green dotted lines at 0.0 show the point of no effect; green text indicates probability the intervention effect is above or below zero; where the entire distribution does not cross zero, the probability of effect is 100%. **A.** The median control group standard education effect was 4.4% (95% CI − 5.7 to +14%); the probability of a positive effect was 80.9%. B. The median targeted educational effect in the clinical group was 29% (95% CI 20%–38%); the probability of a positive effect was 100%. C. The median targeted educational effect in the control group was 30% (95% CI 18%–41%); the probability of a positive effect was 100%.

Our targeted educational intervention to provide knowledge to maximize patient safety around RhIG administration during pregnancy showed improved knowledge immediately after the presentations. One of the strengths of this study is that the presentations were given to the wide variety of health care professionals involved in the process of correct administration of RhIG during pregnancy, including nurses, laboratory technologists and physicians. Our study showed that with the standard curriculum educational intervention, there was less improvement in knowledge, especially on the topics of details of RhIG administration, FMH and correct administration of RhIG in pregnant women with weak D. Education in transfusion medicine for medical students needs to be enhanced[34] and next steps might be to adapt this targeted educational intervention as an on-line self-learning module for undergraduate medical students. However, knowledge alone is not sufficient by itself to change behaviour. Using theories relevant to transfusion medicine behaviour may aid designing efficacious interventions for implementing behaviour changes.[35] Quality improvement projects may be one strategy to closing the evidence to practise gap in transfusion medicine.[36] Other approaches may include improved dissemination of guidelines and better quality of information provided to Rh(D) negative women.[37]

There are several limitations to this study. The selection of the participants in this study was based on convenience, with small sample sizes and effect sizes, and no matching of pre- and post-test results. No a-priori hypotheses were given and there was no power calculation, impeding null results interpretation. The analysis should be considered exploratory, and replication of these findings would be required before translation into practice. The sampling was not inclusive, as some physician groups (family medicine, obstetrics), as well as midwives, were not included in this study. This may have resulted in bias leading to exaggeration of the positive effects of the targeted educational intervention, as these groups may be more knowledgeable than the control group used in the crossover design. Multiple choice questions are vulnerable to guessing and process of elimination strategies, giving them lower discrimination indices than constructed response questions. In our study, penalty for guessing or leaving a question blank was not applied, which may have decreased the discrimination effect.[22] Limitations to the targeted educational intervention include that the pre- and post-tests were administered on the same day of the presentation, with no follow-up education or testing.

Another limitation is the low number of participants which may be an issue with non-parametric statistical analysis. However, the low participant number provided the opportunity to apply Bayesian analysis. Frequentist statistics are thoroughly embedded in the scientific literature, and have the advantage of high recognition of *p*-values, although not necessarily accompanied by high understanding. Well-known disadvantages of frequentist statistics include undue effect of sample size, with small sample size predisposing to unobtainable significant *p*-value even though significance is really present (false negative) and conversely with large sample size predisposing to easily obtaining significant p value, when actually no significance exists (false positive). Dependent *t*-test was not suitable for our study, as the pre- and post-tests were anonymized, and not paired to individual participants. The small sample size in this study made it particularly suited to Bayesian analysis.

Our study confirms more attention needs to be paid to the complex process of correct administration of RhIG during pregnancy, including more opportunities for education of involved personnel and patients, to decreased morbidity and mortality from this preventable condition.[38]

AUTHOR CONTRIBUTIONS

Kim Pyra, Dima Hage and Ruth Padmore designed and performed the study. Kim Pyra and Ruth Padmore performed the audit. Christopher McCudden performed the statistical analysis. Dima Hage wrote the paper, and Dima Hage, Kim Pyra, Ruth Padmore and Christopher McCudden revised it critically.

ACKNOWLEDGEMENTS

This work was funded in part by an unrestricted educational grant from Octapharma Canada, Inc. The authors thank Dr. Tim Wood, Professor, University of Ottawa, for guidance in the design of this project.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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How to cite this article: Hage D, Pyra K, McCudden C, Padmore R. Rh(D) immune globulin administration in pregnancy: Retrospective audit of patient safety events followed by targeted educational intervention with Bayesian analysis. *Transfusion Medicine*. 2023;33(3):244-253. doi:10.1111/tme.12961

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Check for updates

Received: 10 May 2022 | Revised: 21 December 2022 | Accepted: 21 February 2023

DOI: 10.1111/tme.12964

SHORT COMMUNICATION

WILEY

Cultured CD71$^+$ erythroid cells modulate the host immune response

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Abstract

Objective: The study aimed to determine the impact of Red Blood Cells (RBCs) generated from peripheral blood mononuclear cells (PBMCs) on T cell proliferation and host response following whole blood stimulation.

Background: Culturing RBCs is a potential solution for donor shortage. The impact of immature cultured RBCs which express CD71$^+$ on host immune response is not known.

Methods/Materials: PBMCs were seeded in an erythroid expansion medium. CD71$^+$ cells were isolated at days 14 and 21 of culture and incubated with either purified T cells or with LPS-stimulated whole blood. Controls were incubated with medium.

Results: At day 9, the percentage of cells that expressed CD45 and CD71 reached to the highest level (32.9%, IQR; 26.2–39.05) while the percentage of cells that expressed CD71 and CD235a reached to the highest level on day 17 (70.2%, IQR; 66.1–72.8). Incubation of T cells with days 14 CD71$^+$ cells and day 21 CD71$^+$ cells increased T cell proliferation. In a whole blood stimulation assay, day 21 CD71$^+$ cells, but not day 14 CD71$^+$ cells, inhibited the production of IL-6 and TNFα.

Conclusion: Cultured erythroid cells can modulate the immune response by promoting T cell proliferation and inhibiting cytokine secretions following whole blood stimulation.

KEYWORDS

erythroid cells, immunomodulation, reticulocytes, red blood cells

1 | INTRODUCTION

Nearly 120 million blood donations are collected yearly to fulfil the growing global blood transfusion demand.[1] The current blood donation system is based on a voluntary basis which is often insufficient to cover the need. The number of donors is decreasing steadily in the past years,[2,3] and the ongoing COVID-19 pandemic has further decreased donations.[4] The ex vivo production of red blood cells (RBCs) may be a strategy to cover the shortage and provide consistent blood supply. Several attempts have been made to produce RBCs from stem cells in cord blood.[5] Recently, RBCs have been generated from peripheral blood mononuclear cells (PBMCs) and induced pluripotent stem cells.[6,7]

During RBC maturation, erythroid cells lose CD45 and start gradually express transferrin receptor antigen (CD71), which controls the cellular uptake of iron as part of maturation during the reticulyte stage. Similarly, reticulocytes gradually lose CD71 as they enter the circulation and mature to RBCs.[8] Immature RBCs, which express

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CD45 and/or CD71, have been found to cause immunomodulatory effects in neonates and cancer patients.[9,10] To the best of our knowledge, no previous study has investigated if cultured erythroid cells have an impact on the host immune response. Therefore, this report aimed to generate RBCs from PBMCs, characterise their expression of CD45 and CD71, and explore their effects on the host response using T cell proliferation and whole blood stimulation assays.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Blood studies were approved by the Medical Ethical Committee of Sanquin Research and performed in accordance with the 2013 Declaration of Helsinki. PBMCs were isolated from whole blood of healthy volunteers using Percoll (GE Healthcare) with a density of 1.076 g/ml and seeded at 10 x 10^6 cells/ml in erythroid expansion medium, as described previously.[6] Media was changed every 2 days with the same expansion medium but without IL-3 starting from day 2 and onwards until day 10. On day 11, cells were cultured in differentiation medium containing Cellquin medium supplemented with EPO (2 U/ml), 5% Omniplasma (provided by Sanquin Plasma Products, Amsterdam, The Netherlands), and heparin (5 U/ml; LEO Pharma BV). On day 14 and day 21, cells were purified using CD71 Magnetic Activated Cell Sorting (MACS; Miltenyi Biotec) following the manufacturer's instructions. Expression of CD45, CD71, and CD235a (Anti-CD45, APC; anti-CD71, PE; anti-CD235a, FITC; BD Biosciences) were analysed at days 0, 3, 6, 9, 11, 13, 17, and 20 of culturing.

2.2 | Cytospin

Cells were centrifuged and concentrated on slides using Shandon Cytospin 4 (Thermo Scientific). Cells were fixed in methanol and then stained with May-Grunwald-Giemsa. Slides were dried and examined under the microscope.

2.3 | T cell proliferation

T cells were indirectly isolated from ABO-matched donors using Pan T cell isolation MACS (Miltenyi Biotec) in which all blood cells except T cells (CD3 positive cells) were depleted by magnetic separation. T cells were then labelled with Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies). T cells were then cultured in 96 well flat-bottom plates for 5 days in the presence of anti-CD3 (1:1000; Sanquin, Amsterdam, The Netherlands) and anti-CD28 (5 mg/ml; Sanquin), as described previously.[11] As a negative control for proliferation, polymorphonuclear leukocytes (PMNs) were added (60 000 cells per well) in the presence of tumour necrosis factor-alpha (TNFα; 10 ng/ml; PeproTech EC). Cultured erythroid cells were incubated with T cells to assess the effect of Day 14 CD71 cells and Day 21 CD71 cells on T cell proliferation. The ratio of T cell proliferation was calculated by the number of proliferated cells (cells in G2; Figure SS1B) on the number of original T cells (Cells in G1; Figure SS1A).

2.4 | Whole blood stimulation

Whole blood (0.5 ml) from ABO-matched donors was stimulated with Lipopolysaccharide (LPS; 10 ng/μl; Escherichia coli; Sigma-Aldrich) and incubated for either 4 or 24 h in RPMI1640 (Gibco, ThermoFisher) in the presence or absence of purified CD71 cells. After incubation, whole blood was centrifuged (600 x g, 10 min, 20°C), and supernatants were stored at −80°C until cytokines analyses. Interleukin(IL)-6, IL-10, and TNFα concentration were measured using an enzyme-linked immunosorbent assay (ELISA; R&D systems).

2.5 | Statistical analysis

Statistical analyses were performed using SPSS® version 26.00 software. Graphical representation was generated using GraphPad Prism® version 8.00 and Excel (Microsoft® Office 2016). Flow cytometry data were measured using LSRFortessa flow cytometer (BD Biosciences) and analysed with FlowJo software (FlowJo v10). An Independent Kruskal-Wallis test was used to assess differences between groups. Medians and interquartile ranges (IQR) were reported for all parameters. A p-value of less than 0.05 was considered statistically significant for all analyses.

3 | RESULTS

3.1 | In vitro generation of RBCs from PBMCs

Expansion and differentiation of erythroid cells from PBMCs are shown in Figure 1. The percentage of cells that expressed CD45 and CD71 was 0.3% (IQR; 0.2–0.4) on day 0, which reached to 32.9% on day 9 (IQR; 26.2–39.05). The percentage of cells that expressed CD71 and CD235a reached to the highest point on day 17 (70.2%, IQR; 66.1–72.8). Microscopic examination shows that erythroblast cells start to be seen on day 6 of culturing, and these cells increased more on day 11. At the end of culturing on day 20, reticulocytes were the predominant cell type.

3.2 | Cultured CD71+ cells induce T-cell proliferation

Figure 2 shows the ratio of T cell proliferation co-incubated with Day 14 $CD71^+$ cells and Day 21 $CD71^+$ cells. The ratio of T cell proliferation was 2.2 (IQR; 1.3–2.9), which increased significantly to 4.5 (IQR;

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3.8–7.2) in the presence of Day 14 CD71^{+} cells Similarly, T cell proliferation significantly increased in the presence of Day 21 CD71^{+} to 4.6 (IQR; 3.0–6.1) compared to the condition with T cell only. In addition, Day 14 CD71^{+} cells also enhanced the T cell proliferation in the presence of PMNs and TNFα compared to the condition with T cell only.

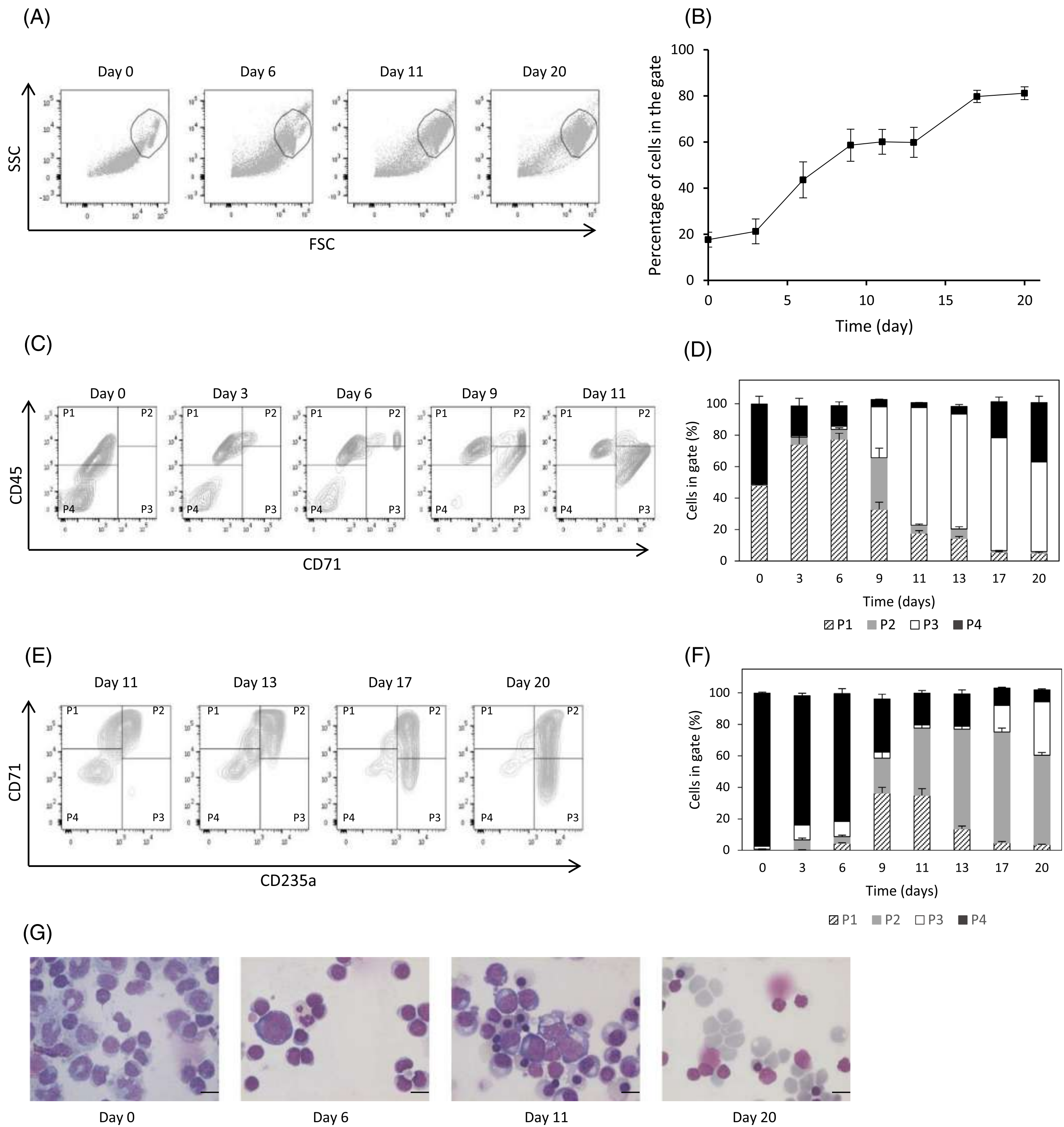

FIGURE 1 Expansion and differentiation of erythroid cells from PBMCs. (A) Representative dot plots showing forward scatter (FSC) and sideward scatter (SCC) of cultured PBMCs at days 0, 6, 11, and 20. (B) Percentage of erythroid cells in the gate indicated in (A) ($n = 4$). (C) Representative density plots indicating the expression levels of CD45 and CD71 in cultures at days 0, 3, 6, 9, and 11. (D) Representative bar graphs of cell number in quadrants' gates (P1-P4) indicated in (C) ($n = 4$). (E) Representative density plots indicating the expression levels of CD71 and CD235a in cultures at days 11, 13, 17, and 20. (F) Representative bar graphs of cell number in quadrants' gates (P1-P4) indicated in (E) ($n = 4$). (G) Morphology of the erythroid cells generated from PBMCs at days 0, 6, 11, and 20 (original magnification x40). Scale bar, 10 μm.

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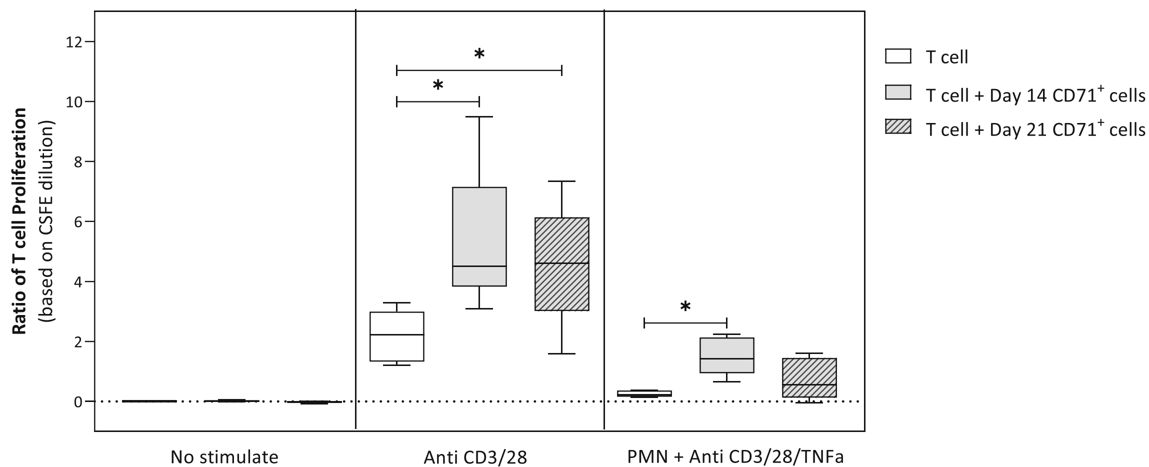

FIGURE 2 The ratio of T cell proliferation. T cells were cultured with purified CD71 cells at day 14 (Day 14 CD71$^+$ cells) and purified CD71 cells at day 21 (Day 21 CD71$^+$ cells) in the presence or absence of the stimulus (anti CD3 and anti CD28). T cell proliferation was suppressed with polymorphonuclear leukocytes (PMNs) in the presence of anti CD3, anti CD28, and TNFa. $n = 8$ per group. *denotes a significant difference (p value <0.05) compared to T cell condition.

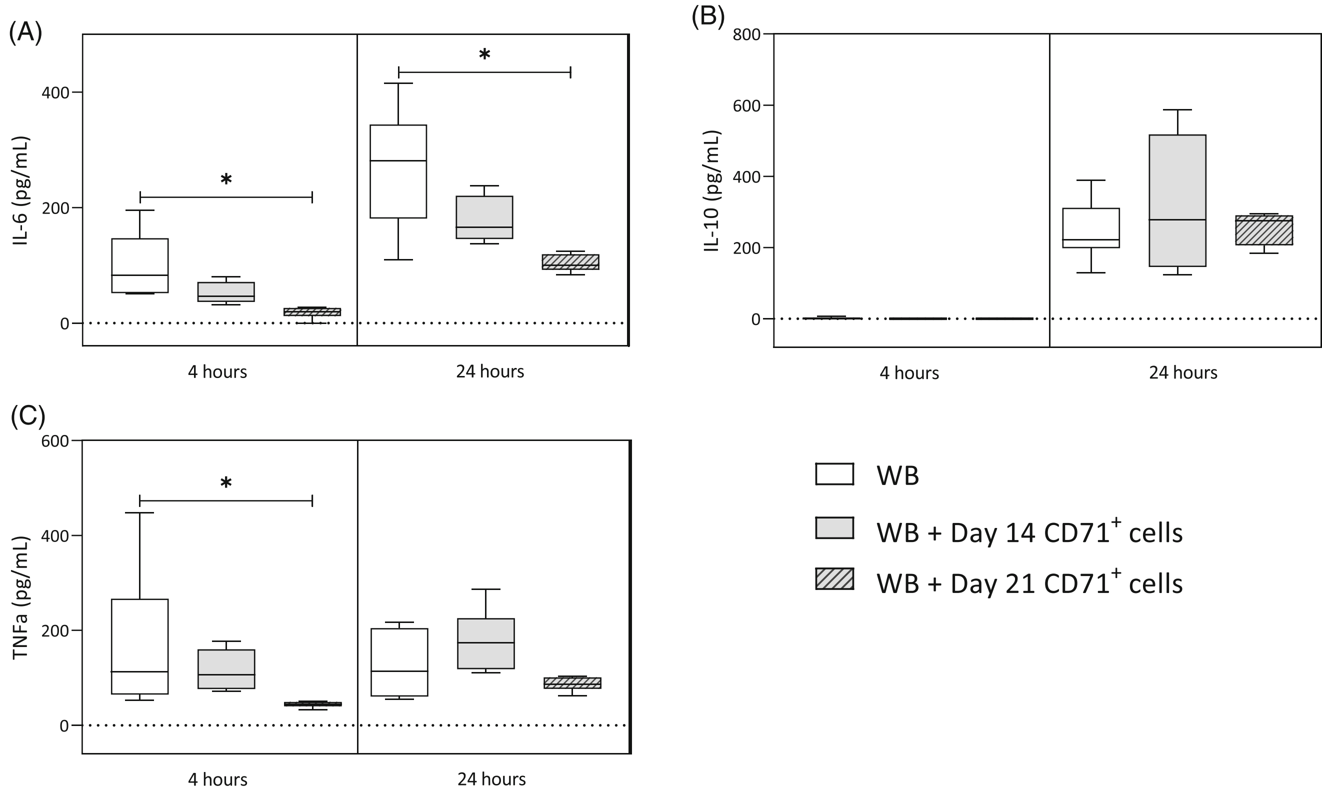

FIGURE 3 Whole blood (WB) stimulation. Concentrations of (A) IL-6, (B) IL-10, and (C) TNFa were evaluated at 4 and 24 h after stimulating whole blood in the presence or absence of Day 14 CD71$^+$ cells and Day 21 CD71$^+$ cells. $n = 8$ per group. *denotes a significant difference (p value <0.05) compared to WB condition.

3.3 | Cultured CD71$^+$ cells alter the release of cytokines

Concentrations of IL-6, IL-10, and TNFa were evaluated after stimulating whole blood with LPS in the presence or absence of Day 14 CD71$^+$ cells and Day 21 CD71$^+$ cells (Figure 3). In a whole blood stimulation assay, the production of IL-6 was significantly decreased in the presence of Day 21 CD71$^+$ cells compared to the condition with whole blood only. There was no significant difference in IL-10 secretion between groups. The presence of Day 21 CD71$^+$ cells also decreased the concentration of TNFα after 4 h compared to the condition with whole blood only .

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4 | DISCUSSION

Previous studies have extensively investigated structural and functional properties of cultured RBCs. Although it is dependent on the culturing methods, cultured erythroid cells display relatively similar characteristics to those in the body, including haemoglobin content, oxygen affinity, blood grouping, morphology and deformability.[5,6] Transfusion of ex vivo generated RBCs to animals showed that they can survive the circulation with low clearance rates, similar to those of standard RBC products.[12] However, much uncertainty still exists about the safety of cultured RBCs. Using a recently developed technique to produce RBCs directly from PBMCs,[6] this study generated two populations of reticulocytes (early reticulocytes which are mostly expressing CD71 and CD45, and late reticulocytes which express CD71 and CD234a) and examine their immunogenicity using T cell proliferation and whole blood stimulation.

Results of this report indicate that both early and late reticulocytes promote T cell proliferation. This finding does not support previous research. A recent study showed that erythroid cells were associated with suppression of T cell proliferation and that the degree of suppression was related to the expression of CD45 in an anaemic mice model with advanced tumour.[13] Hence, erythroid cells expressing CD45 have higher suppression capabilities compared to $CD45^{-}$ erythroid cells. Other studies showed that erythroid cells expressing CD71 isolated from cord blood of human or mice can suppress T cell proliferation.[14,15] A possible explanation for the inconsistency between results of the current study and those in the literature may be related to the origin of erythroid cells. In the majority of previous work, cells were derived from patients suffering from various conditons of either anaemic, neonates, or cancer patients or mice. In line with this explanation, several studies have suggested phenotypic differences between $CD71^{+}$ erythroid cells originated from neonates and adults, and those differences influence their mechanisms of suppressing T cell proliferation.[15,16]

Another finding of the current study was that late reticulocytes suppressed the pro-inflammatory response following whole blood stimulation. In accordance with the present results, a previous study showed that $CD71^{+}$ cells suppress cytokines production in an in vitro bacterial stimulation of PBMCs.[15] Thereby, immature cells appear to have differential immunomodulatory effects, as shown before with $CD71^{+}$ cells from neonates.[14]

Culturing of cells was stopped at later stages of reticulocytes prior to maturing to red blood cells. It is therefore undetermined whether mature cells do not have immunomodulatory effects. Thereby, it is not clear whether the observed immune changes are related to the process of culturing, or to the age of RBCs. Another limitation is that the current study did not evaluate whether the differences in T cell proliferation are related to incubating cultured erythroid cells with T cells from ABO-matched donors. In other words, it is unknown if such differences will be noticed when incubating cultured erythroid cells with T cells from same donors.

Taken together, cultured erythroid cells can modulate immune response as they promote T cell proliferation and inhibit cytokines secretion following whole blood stimulation. Whether these changes translate into increased risk of nosocomial infections, tumour progression or other immunomodulatory effects of transfusion, is not determined from this study. In addition, whether cells cultured for a longer duration have less impact on the host immune response remains to be determined.

AUTHOR CONTRIBUTIONS

Abdulrahman Alshalani, Robin van Bruggen, and Nicole P. Juffermans conceived and planned the study. Abdulrahman Alshalani and Boukje M. Beuger collected data for the study. Abdulrahman Alshalani performed data analyses and drafted the manuscript. Boukje M. Beuger, Robin van Bruggen, Jason P. Acker, and Nicole P. Juffermans provided critical feedback and helped shape the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We would like to acknowledge blood volunteers and staff of Sanquin for their help in collecting blood samples. Abdulrahman Alshalani is supported by the Deanship of Scientific Research, King Saud University through the Vice Deanship of Scientific Research Chairs; Research Chair of Medical and Molecular Genetics.

FUNDING STATEMENT

None.

CONFLICT OF INTEREST STATEMENT

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

Inquiries to access original data included in this study can be directed to the Corresponding author.

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How to cite this article: Alshalani A, Beuger BM, van Bruggen R, Acker JP, Juffermans NP. Cultured CD71^{+} erythroid cells modulate the host immune response. *Transfusion Medicine*. 2023;33(3):257-262. doi:10.1111/tme.12964

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