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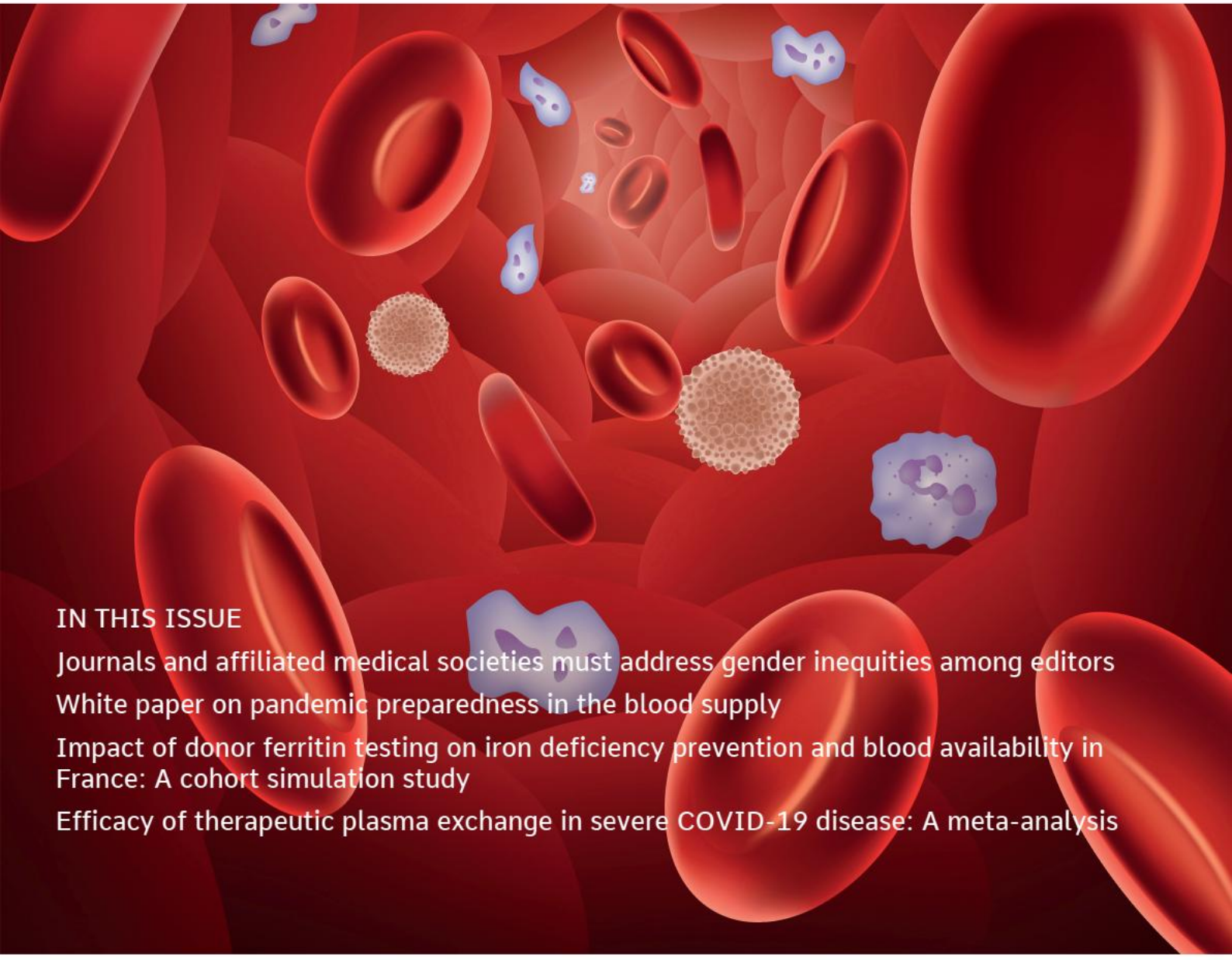

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International Journal of Blood Transfusion

Aims and Scope

Vox Sanguinis reports on all issues related to transfusion medicine, from donor vein to recipient vein, including cellular therapies. Comments, reviews, original articles, short reports and international fora are published, grouped into eight main sections:

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3. Transfusion-transmitted Disease and its Prevention: Identification and epidemiology of infectious pathogens transmissible by blood; Donor testing for transfusion-transmissible infectious pathogens; Bacterial contamination of blood components; Pathogen inactivation.
4. Transfusion Medicine and New Therapies: Transfusion practice, thresholds and audits; Transfusion efficacy assessment, clinical trials; Non-infectious transfusion adverse events; Therapeutic apheresis.
5. Haemovigilance: Near misses, adverse events and side effects throughout the transfusion chain; Monitoring, reporting and analysis of those adverse events and side effects; Activities aiming at increasing the safety of the whole transfusion chain; Standardization of the definition of adverse events and side effects.
6. Patient Blood Management: Caring for patients who might need a transfusion; Transfusion indication decision-making process; Search for the optimal patient outcomes; Study of transfusion alternatives; Autologous blood transfusion.
7. Immunohaematology: Red cell, platelet and granulocyte immunohaematology; Blood phenotyping and genotyping; Molecular genetics of blood groups; Alloimmunity of blood; Pre-transfusion testing; Autoimmunity in transfusion medicine; Blood typing reagents and technology; Immunogenetics of blood cells and serum proteins: polymorphisms and function; Complement in immunohaematology; Parentage testing and forensic immunohaematology.
8. Cellular Therapies: Cellular therapy (sources; products; processing and storage; donors); Cell-based therapies; Genetically modified cell therapies; Stem cells (sources, collection, processing, storage, infusion); Cellular immunotherapy (e.g., CAR-T cells, NK cells, MSC); Cell-based regenerative medicine; Molecular therapy; In vitro manufacturing of blood components.

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COMMENTARY

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The role of CD36 antibodies in haematopoietic stem cell transplantation

In 2017, we came across a case of a Philadelphia chromosome positive (Ph+) acute lymphocytic leukaemia in Japan in a patient who was CD36-deficient and developed platelet transfusion refractoriness (PTR) due to CD36 antibody (case 1 in Table 1). Although there was indication for haematopoietic stem cell transplantation (HSCT), because of the lack of evidence in the literature on the feasibility and the risks of CD36-incompatible HSCT, the physician-in-charge decided to disregard HSCT, and indicated chemotherapy, with a good response. The only report available in the literature at the time was a case reported in a Japanese meeting in 2010 [1] where the successful transplant of a CD36-positive HSC to a CD36-negative patient was reported. Through personal communication with the physician, however, we realized that the patient had developed idiopathic pneumonia syndrome after engraftment (day 84) and died (Dr. T. Ito, personal communication).

CD36 antibody has been reported to be involved in the pathophysiology of transfusion-related acute lung injury (TRALI), a severe complication of blood transfusion. The patho-mechanism of TRALI caused by CD36 antibodies has been explained by the expression of CD36 on human monocytes as well as on endothelial cells [2]. Once platelets and monocytes are activated by CD36 antibodies, they release inflammatory mediators, which increase alveolar vascular permeability, but these antibodies can also directly damage endothelial cells. Thus, the possibility of a TRALI or a TRALI-like reaction could not be ruled out in this transplanted case. In addition, CD36 expression has also been confirmed on erythroblasts [3], suggestive of a role of CD36 antibody as a donor-specific antibody (DSA) in HSCT rejection reaction. Considering these issues, HSCT was avoided, and the patient continued on maintenance regimen consisting of ponatinib + vincristine + prednisolone for 2 years [4]. Since then, a successful case of CD36-incompatible HSCT, after receiving immunosuppression with rituximab and intravenous immunoglobulins for the management of PTR, has been reported in the United States [5], and another case from China [6] who was transplanted with a CD36-positive HSC and developed PTR after the transplant. Unfortunately, we could not get details or know the fate of the latter case.

Taking these facts into account, concerns were raised related to the feasibility of transplanting CD36-negative patients with CD36-positive HSC. Japan is one of the few countries where CD36-compatible platelets can be supplied in a timely manner for the management of PTR due to CD36 antibodies. This is dependent on the highest frequency of CD36-negative individuals, especially type II, who are individuals lacking CD36 expression only on platelets, and thus can donate CD36-negative platelets. Type I deficiency, in which CD36 is absent in all cell types, accounts for less than 1% of the Japanese population, but together with type II deficiency they account for 7%–10%. For this reason, the registry of CD36-negative platelet donors is feasible in Japan. CD36 deficiency varies widely between different ethnic groups, with a reported frequency of 2.6% in Arabians, 3%–11% in Asians, 8% in sub-Saharan Africans and less than 0.4% in Caucasians [7]. Thus, CD36 isoimmunization may be an issue also in other countries, especially among Asians and Africans. CD36 expression, however, is not tested in HSC donors, including cord blood donors [8]. So, we considered it essential to alert haematologists on the potential risks of a CD36-incompatible HSCT [8, 9].

There are two alternatives for the safe conduct of HSCT to these patients. One is the selection of a CD36-compatible donor, who should be type I-deficient individual, which is almost unfeasible even in Japan. The other is to appropriately manage isoimmunization to CD36 and reduce antibody titres before transplant. Meanwhile, we followed two CD36-negative patients (cases 2 and 3 in Table 1) who received HSCT from unrelated donors in Japan. Both received chemotherapy as the initial treatment when they required platelet transfusions. Because initially the CD36 status of the patients was not known, random platelets were transfused, and after PTR due to CD36 antibodies was diagnosed, the patients received CD36-negative platelet transfusions. In Japan, the use of antigen-compatible platelets (called PC-HLA, which includes HPA and CD36) is allowed only in case the causative antibody is identified, confirming immune-mediated PTR, and the cross-match between the donor and patient is negative. The first patient (case 2 in Table 1) was a 38-year-old male, diagnosed as Ph+ acute lymphoblastic leukaemia (Ph+ ALL), who achieved complete molecular response after two cycles of chemotherapy. Then, HSCT (HLA 8/8, CD36-positive donor) was performed, after a conditioning regimen and rituximab administration for the reduction of CD36 antibody titres. CD36 antibody titre was <4 prior to rituximab but was found to be negative thereafter. Neutrophil engraftment was confirmed on day +21 and platelet engraftment on day +38. The patient had an uneventful course and was discharged on day +67. This patient unfortunately relapsed on day +92 and was started on intensification chemotherapy [10].

The second case [11] was a 64-year-old Japanese man (case 3 in Table 1) diagnosed with acute myeloid leukaemia (AML), who achieved morphological remission after one cycle of induction

TABLE 1 Treatment characteristics of the three cases of CD36-negative patients who developed platelet transfusion refractoriness due to CD36 antibody and in whom indication of haematopoietic stem cell transplantation (HSCT) was considered.

	Case 1 (no HSCT)	Case 2 (successful HSCT)	Case 3 (successful HSCT)
Diagnosis	Ph+ ALL	Ph+ ALL	AML with mutated *RUNX1*
Frontline treatment	Dasatinib/ponatinib + hyper-CVAD (four cycles)/ponatinib + vincristine + PSL (maintenance regimen, 2 years)	Dasatinib with hyper-CVAD—two cycles	Daunorubicin and cytarabine (induction)/high-dose cytarabine (consolidation)/azacytidine + venetoclax combination chemotherapy (maintenance) (bridging therapy until HSCT)
HSCT	Not transplanted due to lack of information related to safety of a CD36-incompatible HSCT	Allogeneic, CD36-incompatible HSCT; HLA 8/8 match	Allogeneic, CD36-incompatible peripheral blood stem cell transplantation; HLA 8/8-antigen-level matched but HLA-B 1-allele-level mismatched
Conditioning regimen	-	Flu/CY + TBI12Gy and rituximab on day-8 and day-1	Fludarabine + busulfan + melphalan
GVHD prevention	-	FK + sMTX + MMF 1500 mg/day	FK + MTX
Neutrophil engraftment	-	Day +21	Day +18
Platelet engraftment	-	Day +38	Day +14
Prognosis	Molecular remission confirmed	Recurrence of disease on day +92; chemotherapy restarted	Complete remission confirmed on day +37 by bone marrow examination, without acute or chronic GVHD. Uneventful clinical course after HSCT and discharged in good condition

Abbreviations: AML, acute myeloblastic leukaemia; CY, cyclophosphamide; FK, tacrolimus; Flu, fludarabine; GVHD, graft versus host disease; HSCT, haematopoietic stem cell transplantation; hyper-CVAD, cyclophosphamide, vincristine, doxorubicin, methotrexate, cytarabine and dexamethasone; MMF, mycophenolate mofetil; MTX, methotrexate; Ph+ ALL, Philadelphia chromosome-positive acute lymphoblastic leukaemia; PSL, prednisolone; TBI, total body irradiation.

chemotherapy and received several platelet transfusions before the end of the consolidation therapy. He developed PTR during the course of chemotherapy and anti-CD36 antibodies (titre: 16), but no anti-HLA antibodies were identified in his plasma. PTR was successfully managed with the transfusion of CD36-compatible platelets. Despite maintenance therapy, AML relapsed. This led to consideration for HSCT from an unrelated donor. The anti-CD36 antibody titre in his serum just before the HSCT had decreased to 4. After the conditioning regimen, the patient received peripheral blood stem cell transplantation from an HLA 8/8-antigen-level matched but HLA-B 1-allele-level mismatched CD36-positive unrelated donor. Platelet engraftment was achieved on day +14, followed by neutrophil engraftment on day +18, and on day +37 complete remission was confirmed by bone marrow examination. The clinical course after HSCT was generally uneventful, and the patient was discharged in good condition. Post-HSCT re-examination on day +169 revealed no CD36 antibodies in his plasma, and CD36 expression was detected on both platelets and monocytes, confirming the successful engraftment.

This was the first case of a CD36-incompatible unrelated HSCT that we could follow in detail and confirm that CD36-negative patients can be successfully transplanted with CD36-positive HSC, provided CD36 antibody titres are appropriately managed.

Since CD36 is expressed on endothelial cells, CD36 antibodies may also cause rejection reaction in different types of organ transplantation, similar to ABO group antigen/antibodies [12]. CD36 is also found on cardiac muscle cells [13], renal tubular epithelial cells [14] and liver cells [15], suggesting their role in organ transplantation; but interestingly, we do not see reports in the literature, possibly due to under-recognition and the co-existing HLA or ABO antibodies.

It is evident that CD36 antibody titres can be reduced by preventing immunostimuli to CD36; therefore, the appropriate management of PTR with CD36-compatible platelet transfusion is an important strategy, although not necessarily available in all countries. However, when CD36-negative platelets are not readily available or HSCT is urgently needed, desensitization protocols similar to DSA, including plasma exchange, intravenous immunoglobulin and/or rituximab, may be acceptable strategies [16].

In summary, we think that CD36 antibodies play a significant role in HSCT, more often as a cause of PTR, that can be circumvented by providing platelet transfusion support from CD36-negative donors or, if they are not available, by applying desensitization protocols before HSCT. But, we need to continue monitoring such cases to establish the best procedures for the management of HSCT.

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N.H.T. designed the paper and wrote the manuscript and M.L. reviewed and edited it.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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REVIEW

Vox Sanguinis ISBT International Society of Blood Transfusion

Frequency of red blood cell transfusions in preterm neonates in Brazil: A systematic review and meta-analysis

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Abstract

Background and Objectives: Red blood cell transfusions are frequent in preterm neonates. The proportion of preterm neonates transfused in Brazil remains unknown. We systematically reviewed the literature to estimate the frequency of red blood cell transfusions in preterm neonates in Brazil.

Materials and Methods: The LILACS, EMBASE, Cochrane, SciELO, MEDLINE (PubMed), Web of Science, Scopus, BDTD and 27 national university institutional databases were searched for studies that analysed red blood cell transfusion in preterm neonates in Brazil without period restriction. The Preferred Reporting Items in Systematic Reviews and Meta-Analyses guidelines were followed, and the GRADE methodology was applied. A random-effects model along with the restricted maximum likelihood method was used, and the Freeman–Tukey transformed proportion was used to estimate effect size.

Results: Nine studies, representing 6548 preterm neonates, were included in the qualitative and quantitative analyses. The mean gestational age ranged from 26.0 to 31.6 weeks. Most of the studies were from the Southeast region. The pooled estimated frequency of red blood cell transfusions was 58.0% (95% confidence interval = 52.0%–64.0%, $p < 0.001$) with low certainty. There was statistically significant heterogeneity among studies ($I^2 = 92.5\%$, $p < 0.001$).

Conclusion: In this current meta-analysis of the evidence available, which included moderate and extremely preterm neonates, the observed frequency of red blood cell transfusions in preterm neonates in Brazil was 58.0% and this estimate can help health programming. Some Brazilian regions were not included in this study, and further research is needed to provide a more representative overview of Brazil.

Keywords

blood transfusion, Brazil, GRADE assessment, meta-analysis, premature infant

Highlights

- There is high heterogeneity among studies reporting red blood cell (RBC) transfusions in preterm neonates.
- We report here a meta-analysis of the existing literature on the frequency of RBC transfusion in preterm neonates in Brazil.

- The estimated frequency of RBC transfusions was 58.0%, but the North and Midwest regions were not represented, and further research is needed to include these regions.

INTRODUCTION

According to the Brazilian Live Births System, in 2013, prematurity occurred in 11.9% of pregnancies, and it was estimated that 1.35% of all live births were <1500 g birth weight [1]. These infants present comorbidities, such as anaemia, which is multifactorial and happens because of erythropoietin immaturity, excessive blood analyses, diseases, restricted iron stores, early umbilical cord clamping, the rapid growth rate of preterm neonates, and may result in severe anaemia that requires red blood cell (RBC) transfusions [2, 3]. A retrospective multicentre cohort observed an 80% transfusion rate in infants <27 weeks' gestation with different pre-transfusion haemoglobin levels [4].

In Brazil, a study that included 16 university hospitals in 2015 estimated that 51.5% of preterm infants were transfused, and this rate varied from 34.1% to 71.8%, reflecting differences in proportions between institutions [5]. There is no consensus regarding the haemoglobin cut-off for RBC transfusions, and clinical protocols are heterogeneous [6]. Although the results of restricted and liberal protocols are conflicting, a multicentre study that included 1824 preterm neonates <1000 g did not observe differences in mortality between these protocols, suggesting that restricted protocols are safe [7].

The estimation of the frequency of RBC transfusions in preterm neonates may help determine the burden of severe neonatal anaemia. In this study, we present a systematic review of the existing literature on this topic and perform a meta-analysis of studies evaluating RBC transfusions in preterm neonates in Brazil.

MATERIALS AND METHODS

Search methodology

Search methods were conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and the guidelines of the Joanna Briggs Institute for systematic reviews of prevalence [8, 9]. The study was preregistered in PROSPERO on 15 January 2022 (CRD42022298608). This study question was 'What is the frequency of RBC transfusion in preterm infants in Brazil?'. The studied population consisted of preterm neonates (<37 weeks of gestational age [GA]) who received RBC transfusions in Brazil.

Search was applied on 7 March 2022 and included LILACS, EMBASE, Cochrane, SciELO, MEDLINE (PubMed), Web of Science, Scopus, BDTD and 27 national university institutional databases. For some institutional databases, we asked the librarian to search for the studies. One study was received as a photocopy. The search strategy (Supplemental Material 1) was built by a librarian, and there was no period restriction for the studies.

Selection criteria

Studies were imported to Rayyan (https://www.rayyan.ai). First, titles and abstracts were screened by two independent reviewers. Conflicts were decided by a third reviewer. The full text of potentially relevant studies was retrieved to identify studies meeting the inclusion criteria. Inclusion and exclusion criteria were defined a priori. Only full articles evaluating the frequency of RBC transfusions, which could be randomized controlled trials or observational studies (case–control, cohort and cross-sectional studies), were included. We did not make restrictions according to the guidelines for transfusions (restrictive or liberal). We excluded review studies, comments and guidelines. Furthermore, we checked the source of participants and timeframe of data collection to avoid the inclusion of studies with sample overlap. There was no language restriction.

Data extraction, risk of bias and quality assessment

Data extraction, risk of bias and quality assessment were performed using a standardized form by two independent reviewers. A third reviewer checked all the data. Discrepancies were discussed and solved by a third reviewer. The following data were extracted from each study: authors and year of publication, region of the study, study design, setting, timeframe for data collection, inclusion criteria and main results. The outcome measure was the overall frequency of RBC transfusions. If the frequency of RBC transfusions was reported only within groups and not for the overall study population, then an overall frequency was calculated to represent the entire study population. We investigated the mean volume transfused and the number of transfusions per neonate when this was reported.

To investigate publication bias, a funnel plot was created to assess symmetry. No statistical tests were performed to assess for asymmetry, given the small number of included studies. The Newcastle–Ottawa Assessment Scale [10] for critical appraisal was used to assess the risk of bias in observational studies. Studies were scored from 8 to 9. ROB 2 (Cochrane Risk-of-Bias Assessment Tool) [12] was used for randomized controlled trials (one study), and it scored a low risk of bias. Risk of bias was also assessed by the Joanna Briggs Checklist for Prevalence studies, including all studies [11] and a summary table was plotted with the Review Manager program version 5.4.

The totality of evidence was evaluated according to the GRADE methodology (Grades of Recommendation, Assessment, Development and Evaluation) as suggested by Iorio et al. [13]. According to these authors, in contrast to questions of treatment, in which randomized controlled trials begin as high-quality evidence and observational

TABLE 1 Summary of findings of included studies.

Authors	Year	State	Study design	Setting/context	Timeframe for data collection	*n*	Inclusion criteria; mean gestational age in weeks	Key findings
Valete [14]	1999	Rio de Janeiro	Randomized controlled trial	NICU/administration of recombinant erythropoietin and effects on blood transfusion rates	March 1998 to August 1999	27	<35 weeks' GA and BW < 1500 g. Intervention group: mean 32.2 weeks' GA and control group: mean 31.3 weeks' GA	Frequency 59.2% Mean 27.6 mL/kg Number of transfusions per neonate varied from 0 to 9 (mean 1.8)
Miyashiro et al. [15]	2005	São Paulo	Observational, multicentre	NICU/effectiveness of a guideline of strict indications for packed red blood cell transfusions	Period 1: July 1 to December 15, 2000; Period 2: January 16 to October 15, 2001	345	<37 weeks' GA and BW < 1500 g. Period 1: 31.6 weeks' GA and period 2: 31.3 weeks' GA	Frequency 63.4% Mean 20.9 mL/kg Mean number of transfusions per neonate: Period 1: 1.9 Period 2: 1.4
Baldin [16]	2006	São Paulo	Observational	NICU and IU/influence of prematurity on growth and outcomes	January 1999 to December 2003	155	<37 weeks' GA and BW < 1750 g. Mean 30 weeks' GA	Frequency 43.2%
Venâncio et al. [17]	2006	São Paulo	Observational	NICU/effectiveness of a guideline of strict indications for packed red blood cell transfusions in very low birth weight neonates	Period 1: January 1995 to April 1996; Period 2: June 1997 to December 1998	151	<37 weeks' GA and BW < 1500 g. Period 1: 31.9 weeks' GA and Period 2: 29.8 weeks' GA	Frequency 58.9% Number of transfusions per neonate varied from 0 to 13 Mean number of transfusions per neonate: Period 1: 4.6 Period 2: 4.0
Mimica et al. [18]	2008	São Paulo	Observational	NICU/verification of a very strict guideline for erythrocyte transfusions and reduction of transfusions	Period 1: 1 May 2002 to 31 December 2003	69	<37 weeks' GA and BW < 1500 g. Period 1: 29.0 weeks' GA	Frequency 78.3% Mean 36.0 mL/kg Number of transfusions per neonate varied from 0 to 20 (mean 3.4)
Santos et al. [19]	2011	São Paulo, Rio de Janeiro, Rio Grande do Sul	Observational, multicentre	NICU/analyse if red blood cell transfusion was associated with intra-hospital mortality	January 2006 to December 2007	1077	<37 weeks' GA and BW < 1500 g. Mean 29.0 weeks' GA	Frequency 53.3% Mean 3.3 transfusions per neonate
Santos et al. [5]	2015	São Paulo, Rio de Janeiro, Rio Grande do Sul, Paraná, Pernambuco, Maranhão, Minas Gerais	Observational, multicentre	NICU/analyse the frequency of red blood cell transfusions in very low birth weight neonates and associated factors	January 2009 to December 2011	4283	<37 weeks' GA and BW < 1500 g. Mean 29.9 weeks' GA	Frequency 51.5%

TABLE 1 (Continued)

Authors	Year	State	Study design	Setting/context	Timeframe for data collection	*n*	Inclusion criteria; mean gestational age in weeks	Key findings
Alves [20]	2018	São Paulo	Observational	NICU/analyse the profile of red blood cell transfusions in neonates with BW < 1500 g	January 2015 to December 2016	170	<37 weeks' GA and BW < 1500 g. Transfused: mean 27.7 weeks' GA and non-transfused: mean 30.3 weeks' GA	Frequency 56.5% Mean 1.2 transfusions per neonate
Lopes [21]	2020	Paraná	Observational	NICU/verify if red blood cell transfusion is a risk factor for ROP	1 January 2011 to 31 December 2015	271	<32 weeks' GA and/or BW < 1500 g. Without ROP: mean 30.1 weeks' GA and with ROP: mean 27.4 weeks' GA	Frequency 62.7% Number of transfusions per neonate varied from 1 to 17 Mean number of transfusions per neonate: Without ROP: 2 With ROP: 7

Abbreviations: BW, birth weight; GA, gestational age; IU, intermediate unit; NICU, neonatal intensive care unit; ROP, retinopathy of prematurity.

studies as low-quality, in the field of prognosis and prevalence meta-analysis, a body of longitudinal cohort studies initially provides high confidence. The certainty of evidence was evaluated with GRADEpro software (McMaster University and Evidence Prime, 2022. Available from gradepro.org).

Five domains were considered: risk of bias, inconsistency, imprecision, indirectness and publication bias. Each one was classified as not serious, serious or very serious. The resultant certainty could be very low, low, moderate or high.

Statistical analysis

A meta-analysis was performed to evaluate the frequency of transfusion in preterm neonates. Statistical analysis was performed using Stata software (version 18.0, Stata Corp, L.C.). High heterogeneity was found ($I^2 = 92.5\%$). We performed subgroup analysis considering studies with less than 100 participants and the others, and a meta-regression of the number of participants, and observed the effect with the restricted maximum likelihood (REML) method.

A random-effects model with the REML method was used to estimate between-study variance, and the Freeman–Tukey transformed proportion was used to estimate effect size. Forest plots including 95% confidence intervals (95% CIs) were used to describe the estimates for each study included in the meta-analysis and the pooled frequency. The measure of effect was the frequency of transfusions.

RESULTS

Study characteristics

A total of 1744 studies were identified. Two hundred and seventeen duplicates were removed. Based on title and abstract, 1508 studies were excluded because they did not meet the inclusion criteria. Seventeen studies were fully reviewed, and from these, eight were excluded due to not reporting the frequency of transfusions, because this could not be calculated with the data reported or because samples were overlapping. We included nine studies in our analysis, published between 1999 and 2020, representing 6548 preterm neonates [5, 14–21]. The mean GA ranged from 26 to 31.6 weeks, reflecting extremely and moderately preterm neonates. The frequency of transfusions ranged from 38.5% to 78.3%, and the volume transfused ranged from 20.9 to 36.0 mL/kg. The mean number of transfusions per neonate varied from 1.2 to 4.6. Eight studies were observational, and one was a randomized controlled trial (Table 1).

One study had two periods of observation [18] and overlapped the sample included in another study [19], so only period 1 was considered. Figure 1 presents the PRISMA flow diagram of this systematic review.

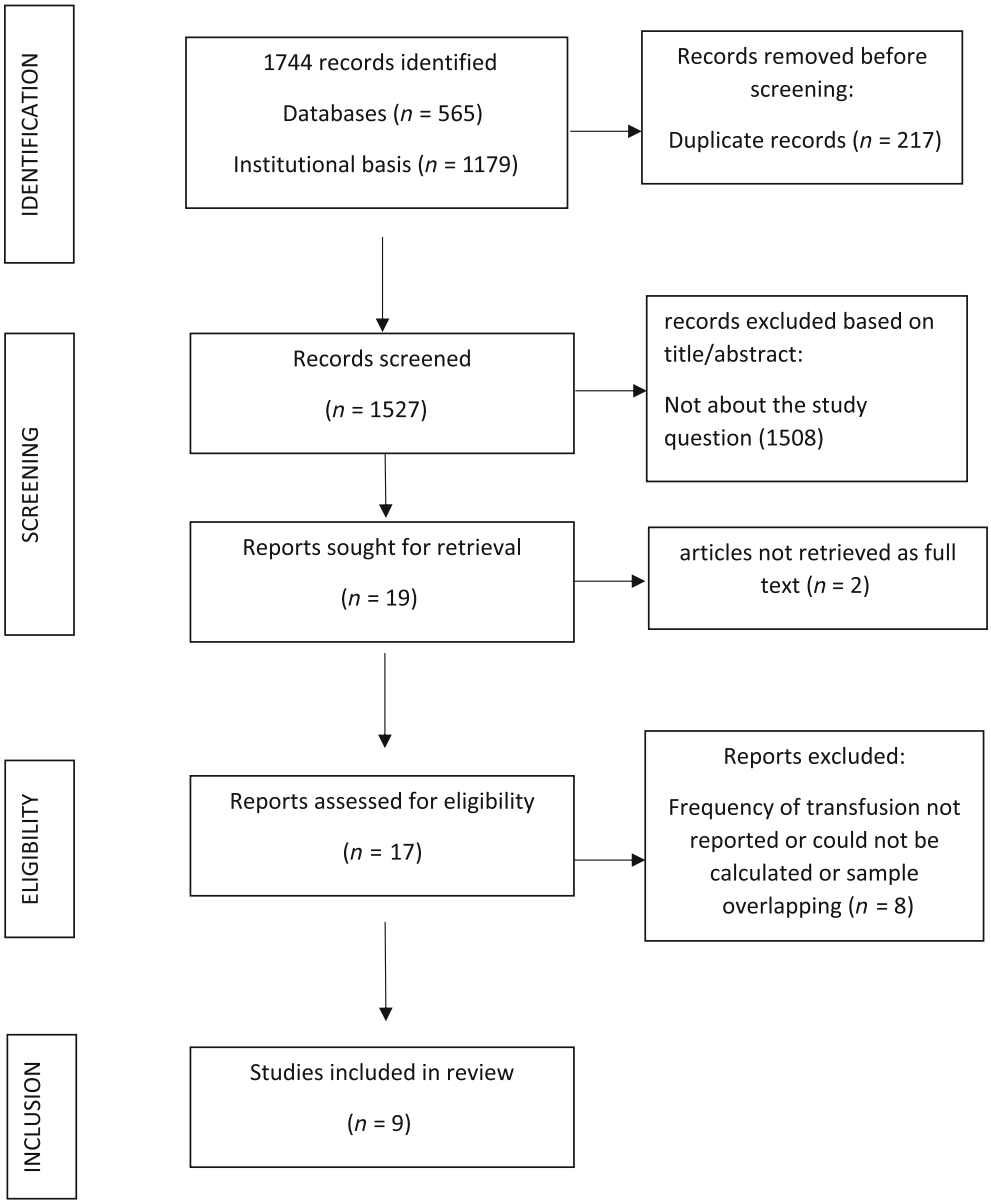

FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) search diagram.

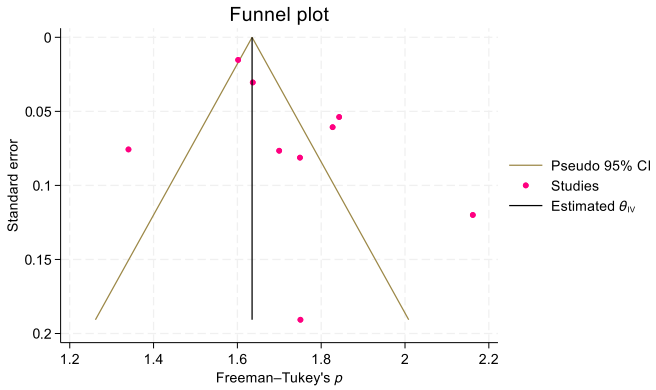

FIGURE 2 Funnel plot of included studies. CI, confidence interval.

Risk of bias and quality assessment

Assessment of methodological issues and risk of bias of individual studies provided low, unclear and high risk of bias ratings. Most of the studies presented a low risk of bias (Supplemental Material 2). The quality assessment resulted in a low quality of evidence. We considered inconsistency to be not serious, and publication bias was considered strongly suspected (Supplemental Material 3). The funnel plot demonstrated some asymmetry (Figure 2).

The estimated pooled frequency was 58.0% with a 95% CI that ranged from 52.0% to 64.0% (Figure 3). The subgroup analysis revealed that the test of group differences was not significant (Figure 4) and the meta-regression analysis ($p = 0.33$) suggested that changes in sample size did not change the estimation.

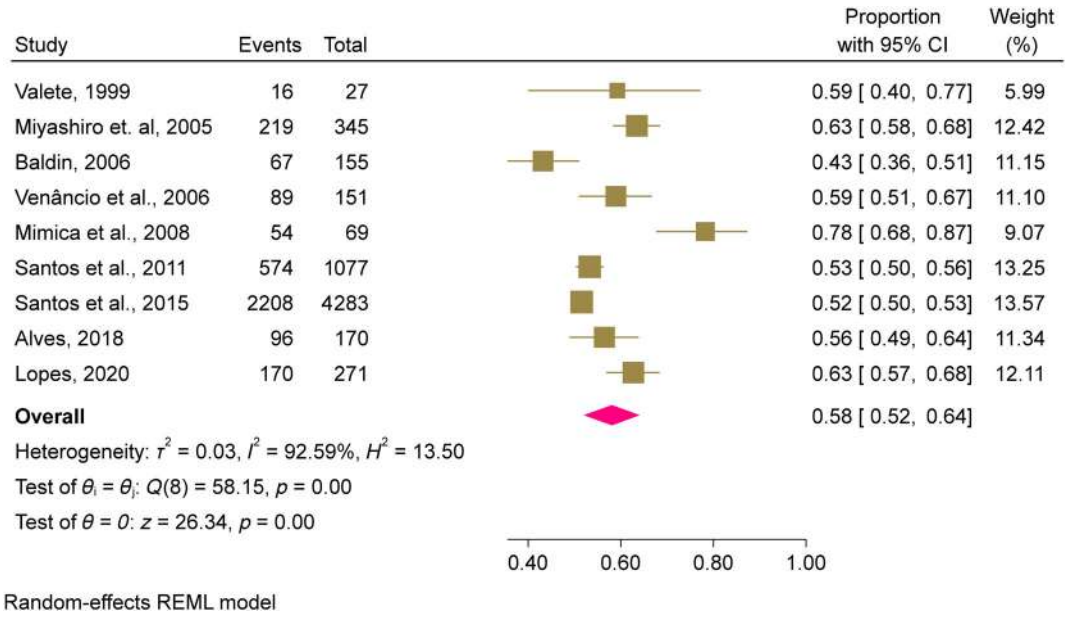

FIGURE 3 Random-effects meta-analysis of studies on the outcome frequency of red blood cell transfusion in preterm neonates. The forest plot contains 95% confidence intervals (CIs), and studies are weighted. REML, restricted maximum likelihood.

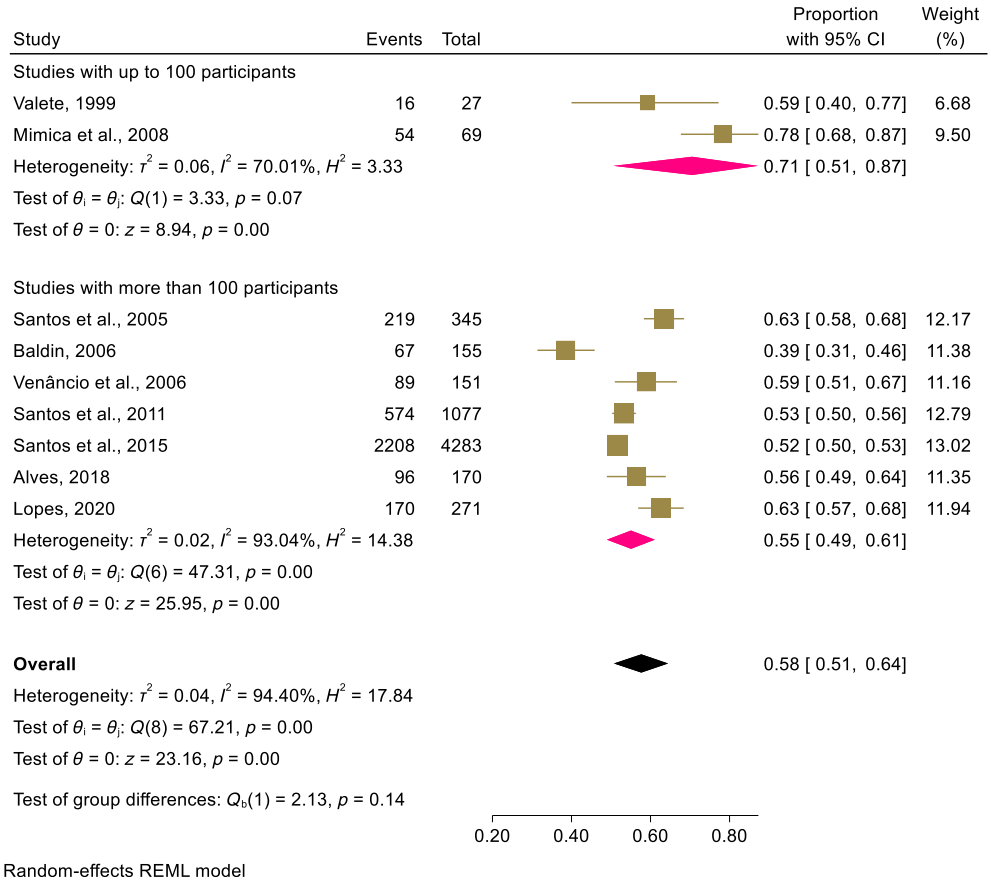

FIGURE 4 Subgroup analysis between studies with up to 100 participants and those with more for the outcome frequency of red blood cell transfusions in preterm neonates. CI, confidence interval; REML, restricted maximum likelihood.

DISCUSSION

The present study aimed to investigate the frequency of RBC transfusions in preterm neonates in Brazil. The current meta-analysis showed an overall pooled frequency of red cell transfusion of 58.0%, considering studies with different guidelines for transfusion, giving an overall burden of this condition in Brazil. GA ranged from 26 to 31.6 weeks, reflecting moderate and extremely preterm neonates. This occurs

probably because most of the RBC transfusions are related to anaemia of prematurity, which affects these neonates preferentially [22].

Of note, three studies included in this meta-analysis with the same mean GA (29.0 weeks) reported transfusion frequencies that ranged from 51.5% to 78.3%, suggesting that differences in severity and clinical practices may influence transfusion frequencies [5, 18, 19]. We do not think that these differences impaired the achievement of an overall point estimate for the frequency of these transfusions, and we know that these transfusion practices vary, so we aimed to include all the frequencies. Noteworthy, a survey that investigated transfusion practices and included 343 neonatal intensive care units revealed that transfusion thresholds and aspects of administration varied widely across Europe [23]. In the present review, only three studies reported the mean volume transfused per infant, which ranged from 20.9 to 36 mL/kg. Also, consideration should be given to the reported number of transfusions per neonate, which varied from 1.2 [20] to 4.6 [17]. The main difference between these studies was the strict protocol implemented by Venâncio et al. [17], who unexpectedly reported a higher mean number of transfusions. The mean GA in these two studies varied from 29.8 to 31.9 weeks [17] and 27.7 to 30.3 weeks [20], and surprisingly, a higher number of transfusions was observed for those who were more mature, suggesting that other factors influenced these transfusions, such as severity of disease or adherence to protocols. We suggest that studies that compare before and after a protocol implementation report their protocol adherence.

The heterogeneity observed was high, and some level of statistical heterogeneity is usually observed in meta-analyses of prevalence. Considering that nine studies were included, a lower degree of freedom would imply a higher numerator in the calculation of I^2. Also, the CIs between studies were most of all narrow, except for the two studies with smaller samples. Migliavaca et al. emphasized that the most important thing is to interpret heterogeneity [24]. Iorio et al. pointed out that it is a challenge to interpret heterogeneity in the context of prognostic studies [13]. In the present study, the two smaller studies had lower heterogeneity compared to the seven larger studies, with narrow CIs. Examining all these aspects, we considered this a not-serious inconsistency at GRADE.

A subgroup analysis was performed with the included studies, considering sample sizes. It was not possible to make subgroups considering GA or birth weight, as studies did not inform the results of transfusion frequencies for different GAs or birth weights. This can be identified as an opportunity to improve the reports of transfusion in preterm neonates in Brazil, as this information was unavailable. Iorio et al. reinforced the need to explore sample size as an explanation for heterogeneity [13]. In this study, it seems that sample size did not influence heterogeneity.

Publication bias was considered. Also, we observed that most studies were from the southeast region and university hospitals that reported protocols for transfusion, and two Brazilian regions were not represented in the analysis. It is possible that those who implemented protocols tended to compare outcomes and publish results differently from others. We followed what was suggested by Iorio et al. [13] and Yusef et al. [25] and considered publication bias to be strongly suspected in GRADE.

Brazil is a country of continental dimensions with socio-economic inequalities that are reflected in population health and in research [26]. As a consequence, a national scope is a challenge. We did not include in this review studies from the North and Midwest regions. This result claims publication for this issue in these regions.

The results of this study have implications for practice, as they can add to the programming of resources needed for the care of preterm neonates. The lower margin of the CI (52.0%) suggests that considering the available Brazilian literature about this issue, one in two preterm neonates with a mean GA ranging from 26.0 to 31.6 weeks will need at least one RBC transfusion.

The potential limitations of this study need to be discussed. First, there were a few studies included in the meta-analysis, and most of them were from the southeast of Brazil. The North and Midwest regions were not represented in this study. It was not possible to make subgroup analyses considering Brazilian regions and GA, as the number of studies was small and, in some cases, the lack of information in the studies did not allow this comparison. Future investigations and the publication of studies performed in the North and Midwest regions are necessary to add epidemiological information from these regions. To the best of our knowledge, this is the first meta-analysis to estimate the frequency of RBC transfusions in preterm neonates in Brazil. This study included a quality assessment evaluation, and we consider this a strength, as few meta-analyses of prevalence analyse the quality of evidence [23].

In conclusion, in this current meta-analysis of the evidence available, which included moderate and extremely preterm neonates, the observed frequency of RBC transfusions in preterm neonates in Brazil was 58.0%, and this estimate can help health programming. The North and Midwest Brazilian regions were not included in this study, and further research is needed to provide a more representative overview of Brazil. We suggest that other countries report their frequencies, and an estimate of overall transfusion rates can be done in the future.

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C.O.S.V. and E.A.L.F. designed the study, C.O.S.V., E.A.L.F., C.P.M., M.C.A.P., M.O.D.R.W. and S.M.W. performed the literature search and data interpretation, C.O.S.V. performed data analysis C.O.S.V., E.A.L.F., M.O.D.R.W. and S.M.W. wrote the manuscript including revisions.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Iron supplementation: A qualitative study on the perception of blood donors, blood collection staff and donor physicians

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Abstract

Background and Objectives: Iron supplementation is an effective strategy to mitigate donation-induced iron deficiency in blood donors. However, evidence on the perception of individuals involved in blood donation on iron supplementation as a blood service policy is lacking. This study aimed to evaluate the knowledge and perception of whole blood donors (donors), blood collection staff (collection staff) and donor physicians (physicians) regarding donation-induced iron loss and iron supplementation.

Materials and Methods: Online focus group discussions had four to six participants and followed a structured questioning approach. All participants had to be fluent in Dutch to participate, and donors had donated at least five times. Sixteen donors, eight collection staff members and four physicians participated in this study. Recordings were transcribed, coded and analysed using a grounded theory approach.

Results: Awareness of donation-induced iron loss was limited in donors. Donors and physicians were predominantly positive towards iron supplementation; the primary motivator for donors was to prevent deferral and reduce iron-deficiency-related symptoms. Improving donor health was the main argument for physicians to advocate iron supplementation. Staff had a critical view on iron supplementation as a policy, as they perceived it as unethical and possibly ineffective. A knowledge gap might underlie their concerns.

Conclusion: Most individuals involved in blood donation are positive towards iron supplementation as a blood service policy. If implemented, guidance and monitoring is desired and adequate education of all stakeholders is required.

Keywords

blood donation, focus group discussion, iron deficiency, iron supplementation

Highlights

- Donors and blood service staff are predominantly positive towards iron supplementation as a blood service policy.
- Sufficient information, guidance, iron status monitoring, and involvement of a donor physician are important conditions for successful implementation of iron supplementation for donors.

- Awareness of donation-related iron loss and knowledge of iron supplementation is limited in donors and blood collection staff.

INTRODUCTION

As a result of the loss of haemoglobin (Hb)-bound iron during donation, frequent whole blood donors (donors) are at increased risk of developing iron deficiency [1]. To safeguard the donors' iron status, blood services are required to measure pre-donation Hb levels to assess donor eligibility for donation [2, 3]. Yet, despite Hb monitoring, many donors develop non-anaemic iron deficiency (ferritin <15 μg/L). This leads to a significantly higher risk of low Hb levels at their next donation [4, 5]. Therefore, extended donation intervals and iron supplementation are increasingly implemented internationally as additional iron management policies [6–8]. Extended donation intervals and iron supplementation help replenish iron stores (i.e., ferritin), which in turn prevents anaemia.

Extended ferritin-guided donation intervals allow for a longer recovery period but often do not lead to full iron store recovery and may not improve iron-deficiency-related symptoms [9, 10]. Furthermore, an extended deferral period lowers donor availability, demoralizes donors and decreases donor return [10–13]. Some blood services provide donors with iron supplements [8]. Iron supplementation enhances iron store recovery, allowing donors to maintain their regular donation frequency and to reduce the risk of developing iron deficiency (anaemia) [14, 15].

Previous research into iron supplementation for donors has mainly focused on the effectiveness of iron supplementation in a research study setting [14–16]. However, the success of iron supplementation as a blood service policy is highly dependent on the level of support by those involved in the donation process. Iron supplements, for instance, may cause gastrointestinal side effects and might be perceived as a more invasive intervention compared with deferral [17]. Therefore, one could speculate that iron supplementation may deter subgroups of donors who are not open towards supplements, while enhancing return in others. However, research regarding the knowledge and perception of donation-induced iron loss and iron supplements of those involved in donation besides donors is limited.

This study aims to obtain an overview of the knowledge and perceptions regarding donation-induced iron deficiency and iron supplementation as a blood bank policy amongst donors, blood collection staff (collection staff) and donor physicians (physicians) by means of focus group discussions.

METHODS

Setting

Sanquin Blood Bank is the only organization authorized to collect, process and distribute blood products in the Netherlands [18]. Blood donation in the Netherlands is voluntary and non-remunerated. A donor's eligibility to donate is evaluated through a donor health questionnaire and on-site interview and physical assessment by the collection staff or a physician [19]. The collection staff evaluates the eligibility of regular donors prior to donation, is responsible for the blood collection process and provides the donor with (medical) support during and after the donation. Physicians perform the eligibility screening in new donors and have the final responsibility for the quality of the regular donor eligibility evaluation and blood collection. At least one donor physician is available at every blood collection location. Donors are allowed to donate when they are in good health, at least 18 years old and not at risk for any transfusion-transmissible infections. The regular donation interval for male and female donors is 56 and 122 days, respectively. In accordance with European legislation, Hb cut-offs for donor eligibility for male and female donors are >135 and >125 g/L (measured with the HemoCue 201, Angelholm, Sweden), respectively. Donors with Hb levels below the cut-off are not eligible to donate for 3 months. Additionally, Sanquin has gradually implemented a ferritin-guided donation interval policy between November 2017 and November 2020. Ferritin levels are measured in newly registered donors and after every fifth whole blood donation. Donors with ferritin levels ≥15 and ≤30 μg/L or <15 μg/L are deferred for 6 or 12 months, respectively [20].

Participants and procedures

Focus group discussions were used as a qualitative study approach to assess the perception and knowledge of donors, collection staff and physicians regarding donation-induced iron deficiency and iron supplementation as a blood service policy. This explorative study approach was chosen in order to allow participants to elaborately express their views and interact with each other, while perceptions and opinions of our study population have not been previously investigated. Focus group discussions were conducted with whole blood donors, collection staff and physicians between February and July 2021. To ensure a geographically spread sample of the Dutch donor population, donors were selected from four blood collection locations: Amsterdam, Maastricht, Groningen and Den Bosch. In an effort to include donors from various age groups, we invited an equal number of donors below and above 50 years of age. Donors were eligible to participate if they had donated at least five times. Based on these inclusion criteria, 200 donors were selected in the donor database and invited by email. Sixteen out of the 200 invited donors indicated to be willing to participate in the study. Donors were provided with an overview of three potential dates to participate. The number of donors included in each focus group was based on the participants' availability on each particular date, with a maximum of

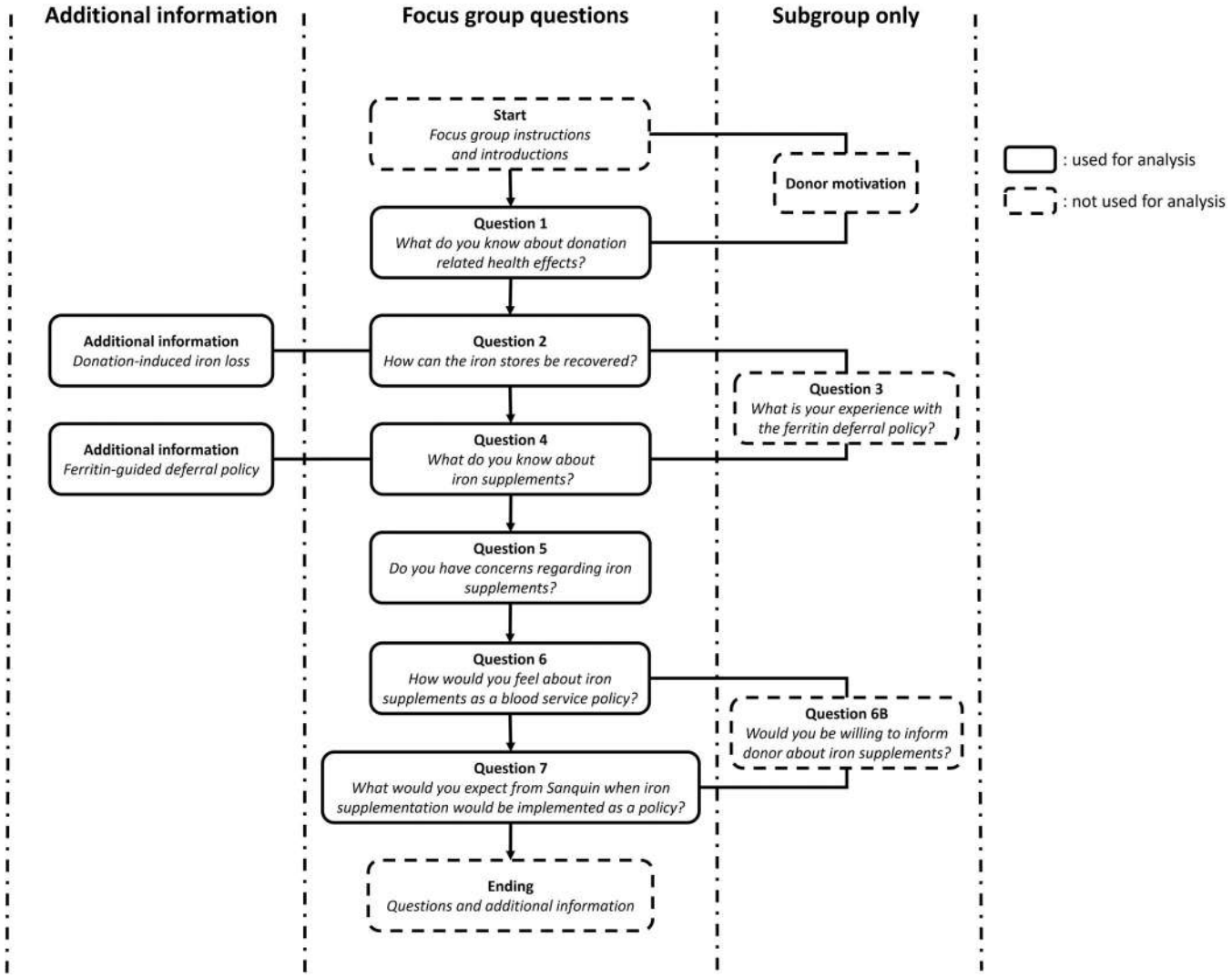

FIGURE 1 Schematic overview of the focus group discussion flow and guiding questions.

six participants per focus group. Focus groups with donors continued until no new topics emerged, other than those discussed in previous focus groups. Staff and physicians were recruited through an internal Sanquin employee newsletter. Due to a low response rate, the number of focus groups with collection staff and physicians was limited, and all respondents were included in the study. The invitational email contained information on what a focus group discussion entails and what was expected of the participants. The information stated that the topic of the focus group discussion concerned current and potential new blood service policies, thereby not specifying the topic focus on iron supplementation as a blood service policy. All participants had to be fluent in Dutch to be able to participate.

Focus group discussions were carried out online using Zoom Video Communications and Microsoft Teams. Due to COVID-19 restrictions, live focus group sessions were not allowed to take place. All online focus groups were conducted by a facilitator. Besides the facilitator, an observer was present at each focus group to take notes and observe non-verbal participant interactions. During the focus group discussions, open-ended questions were presented in a Powerpoint slideshow (PowerPoint®, version 2211, Microsoft®). These questions were used to guide the discussion using a structured questioning approach. When new information was no longer shared or when the discussion stopped. Guiding of the focus group discussions differed slightly for donors compared to collection staff and physicians. For example, the focus groups with donors started with a question about their motivation to donate, which was not the case for the collection staff and physicians. A schematic overview of the focus group discussion flow is presented in Figure 1. To establish an equal knowledge level amongst all participants, information regarding Sanquin's ferritin-guided donation interval policy and donation-induced iron loss was provided after the discussions regarding these topics had ended. Here, incorrect information shared by the participants regarding these topics was also rectified. Only during the focus group discussions with the collection staff, information shared by the participants regarding the lack of effect of iron supplements on the iron

stores was corrected. Each focus group discussion lasted until no new information was shared, which took approximately 60 min.

Analysis

All focus group recordings were manually transcribed for analysis. Transcripts were analysed using qualitative analysis software MAXQDA 2022/2020 (VERBI Software, 2021). Prior to analysis, each transcript was proofread in its entirety by the researchers involved. At every level of data analysis, at least two researchers were involved. A grounded theory approach was used for the analysis, consisting of three phases. In the first phase, a combination of deductive and inductive coding methods was used. A deductive approach was used to start with an initial set of codes, with the focus group guiding the questions. Next, inductive coding was used to generate codes for the most important constructs within the initial set of codes [21]. These codes were grouped into topic categories and assessed by the involved researchers together to identify semi-overlapping and entirely overlapping codes. Codes that were mismatched were reclassified, decategorized or excluded. For the second phase we used axial coding. This consisted of constructing higher order themes from the topic categories and identifying potential connections. The final phase consisted of selective coding: assessing overlapping higher order themes, opinions and concepts to determine the final themes. The final themes were designed to be non-overlapping, and each category was appointed to a distinct theme. After the researchers agreed on the total number and definition of the themes, a final codebook was designed with main themes and separate sub-themes.

RESULTS

In total, 28 individuals participated in this study, with 11 donors (43.8% female), 8 collection staff members (87.5% female) and 4 physicians (100% female), as displayed in Table 1. The analysis of the focus group discussions resulted in four main themes: (1) donor health, (2) donation-induced iron loss, (3) iron supplements and (4) iron supplementation as an iron management policy. The main findings per theme and corresponding sub-themes are summarized in Table 2 for donors, collection staff and physicians, separately. To support the results and illustrate the findings, participant quotes are presented.

TABLE 1 Participant characteristics.

Group type	Focus group	Sex Females (%)	Age (years) Mean (SD)
Donors	I ($n = 5$)	1 (20%)	43.0 (10.5)
	II ($n = 5$)	4 (80%)	42.8 (17.7)
	III ($n = 6$)	2 (33.3%)	27.4 (12.3)
Collection staff	I ($n = 4$)	3 (75%)	52.3 (7.1)
	II ($n = 4$)	4 (100%)	47.0 (5.0)
Physicians	I ($n = 4$)	4 (100%)	42.3 (7.3)

Donor health

Donation-related health effects

Most (i.e., the vast majority) individuals involved in blood donation were aware of donation-related health effects. Decreased ferritin levels, or iron deficiency, was one of the most mentioned donation-related health effects by donors. Some donors (i.e., less than half of the participants) mentioned that they had been informed about low ferritin levels in the past. A few of these donors indicated that this was the first time that they became aware of the increased risk of iron deficiency through donation.

> I was not aware of health effects of donation, but since I have been deferred due to low ferritin, I know how my health is affected by donating.
>
> (*Male donor*)

However, the difference between Hb and ferritin was not clear to all donors and both terms were often interchanged. This lack of knowledge in donors was also mentioned by the collection staff. They indicated that donors often ask questions regarding donation-induced iron deficiency, especially after having been deferred because of low ferritin levels.

> Donors often ask about how their iron stores can be low while their Hb levels are sufficient.
>
> (*Male blood collection employee*)

Other health effects that were mentioned ranged from decreased physical fitness and increased fatigue after donation to fainting during or after donation. The collection staff and physicians indicated that donors often mentioned to have experienced such health effects. Only a few participating donors had experienced these negative donation-related health effects, mainly fatigue.

> I don't recognize these effects at all, I could run a marathon afterwards, as a matter of speaking of course.
>
> (*Male donor*)

This quote illustrated an opposing view of one of the donors in response to the discussion regarding donation-related fatigue. Donors discussed that donation is a non-time-consuming and relatively simple altruistic act for them, which makes them feel good about themselves. Donors also described that potential disadvantageous health effects did not affect their willingness to donate because the positive feeling they gain from donation outweighs such effects.

> The advantages and good feeling which I get through donation would for sure make up for such effects.
>
> (*Female donor*)

Two donors mentioned that they saw blood donation as an opportunity to get a free health check by getting their blood pressure and

TABLE 2 Summarized overview of the main thematic outcomes per group type.

Main themes	Sub-themes	Donors	Collection staff	Physicians
Donor health	Donation-related health effects	Aware of and experience with negative and positive donation-related health effects. Poor understanding of the effect of donation on iron stores. Disadvantageous effects of donation did not affect willingness to donate, as this is compensated by the positive feeling after donating.	Observe a lack of knowledge in donors regarding donation-induced iron loss and iron deficiency.	Often encounter donors with negative health effects due to donation.
Donation-induced iron loss	Ferritin-guided deferral	Aware of the ferritin-guided deferral policy but surprised when informed about their low ferritin levels. Deferral increased awareness of the health effects of donation.	Often encounter disappointed and upset donors with low ferritin levels.	Often encounter disappointed and upset donors with low ferritin levels.
	Iron store recovery	Knowledge of iron store recovery through diet. No active change in diet pre- or post-donation to enhance their iron stores.	Provide donors with information on iron store recovery through diet.	Refer donors to The Netherlands Nutrition Centre for dietary information regarding iron store recovery.
Iron supplements	Awareness and experience	Familiar with iron supplements and aware of its availability. Open towards iron supplementation under certain conditions.	Poor understanding of the effect of iron supplements on iron stores.	Aware of iron supplements and potential side effects.
Iron supplements as a blood service policy	Perception and concerns	Positive view on iron supplements as a policy, as it increases accessibility and improves donor health. Aware that this could potentially be perceived as unethical by other donors.	Opposed to iron supplementation as a policy, as this would lead to exploitation of donors. Opinion shifted when informed about the effect of iron supplements on ferritin levels, and the current ferritin-guided donor deferral was provided.	Mainly positive about iron supplementation as a policy to enhance donor health.
	Expectations	Voluntary, more elaborate iron store monitoring, clear information and involvement of a donor physician.	Voluntary, more elaborate iron store monitoring, and elaborate information resources and instructions.	Voluntary, more elaborate iron store monitoring and involvement of a donor physician.

blood levels checked. This health check was one of the main reasons for them to continue to donate. Furthermore, the health check was later mentioned by the collection staff as a positive aspect of donation, which they inform the donors about.

> When I started, I didn't have a deep motivation to donate, but then I realized that it is beneficial for my health and a convenient check-up. From that moment I became a committed donor.
>
> (*Male donor*)

Donation-induced iron loss

Ferritin-guided deferral

During the discussion on donation-induced iron deficiency, most donors indicated being aware of the ferritin-guided deferral policy. Moreover, some donors had been deferred because of low ferritin levels at least once. These donors were surprised when they were informed that their ferritin level was too low to return to donate at the regular donation interval.

> I have always been under the assumption that I was fit and healthy, but upon the first (ferritin) measurement I already fell through.
>
> (*Male donor*)

Some donors who had been deferred indicated that their deferral made them more cautious about the effect of donation on their health. Some of these donors considered switching to plasma donation instead of whole blood donation or quit donating completely, to prevent further iron depletion.

> If my ferritin level is not improved after this deferral period, then I will reconsider if donating blood is something for me.
>
> (*Male donor*)

Previously deferred donors expressed their disappointment about not being able to donate for an extended period. Disappointment due to ferritin-based deferral was also acknowledged by the collection staff and physicians. They mentioned that donors often express their frustration about being deferred, especially donors with a long history of frequent donations. Donors often indicate not to understand why they are deferred, while this never happened prior to the implementation of the ferritin-guided donation interval policy. Staff and physicians indicated that many donors (i.e., more than half of the participants) were surprised to hear about their low ferritin levels, and they often asked about potential health implications.

> I often hear from donors that it is hard for them to accept that their ferritin levels are low. It gives them a feeling of being unhealthy or that there is something wrong with their health.
>
> (*Female donor physician*)

Iron store recovery

Although knowledge of donation-induced iron deficiency was limited, most donors did know about iron store recovery through dietary iron intake. Donors were able to name iron-rich dietary products, and some were even aware of the inhibitory and stimulating effect on iron uptake of other dietary products such as dairy and vitamin C [22]. Although providing dietary advice is not part of Sanquin's standard policy, many of the collection staff members indicated that they provide donors with dietary advice. This is done either by referring donors to The Netherlands Nutrition Centre when asked about iron store recovery (physicians), or by directly providing dietary advice (collection staff).

> We explain to donors that their iron stores can be improved through diet, by eating a lot of vegetables and meat.
>
> (*Male blood collection employee*)

Many of the donors did not actively change their diet to increase their iron levels before or after donation. Some of the donors mentioned that they would take dietary adjustments into account now that they were made aware of the potential positive effect on their iron levels. Donors who did adjust their diet to account for iron loss during donation mentioned changes like eating spinach or iron-rich apple syrup. However, the collection staff noted that donors are not always aware of the ineffectiveness of some dietary interventions in improving their iron stores.

> Donors think that by eating one sandwich with apple syrup prior to their donation their iron levels will be improved, but that is not how it works.
>
> (*Female blood collection employee*)

Iron supplements

Awareness and experience

When asked about familiarity with iron supplements, all included donors were aware of iron as a dietary supplement and that it is readily available at drugstores in the Netherlands. However, only a few donors had ever used it or considered using iron supplements. Most donors had not felt the need to start using iron supplements because they never experienced any health complaints related to low iron stores. Some donors indicated that they prefer to get iron from their diet as long as iron supplements are not urgently needed to improve their iron stores.

> I am aware of the side effects of iron supplements, and because I didn't have any health complaints due to low iron stores, the benefits did not outweigh the negative effects.
>
> (*Female donor*)

All donors who had used iron supplements in the past indicated that these were prescribed by their general physician. Supplements were provided when donors were anaemic or to prevent anaemia after pregnancy. None of them had suffered from iron-supplementation-related side effects. Most donors indicated that they would be open to using iron supplements if advised by a physician.

> I wouldn't be against iron supplementation if a physician would tell me that it would be beneficial for my health to take it. I think that I would take the advice.
>
> (*Male donor*)

The collection staff and physicians were all aware of iron supplements as a dietary supplement and its potential side effects. However, about half of the interviewed collection staff members had a poor

understanding of the anticipated effects of iron supplementation on iron stores.

> Iron supplementation does not help in increasing the ferritin levels of course, it only affects hemoglobin.
>
> (*Female blood collection employee*)

Iron supplementation as an iron management policy

Perception and concerns

Opinions regarding iron supplementation as a blood bank policy varied amongst the different focus groups with donors and between collection staff and physicians. Many donors and all physicians indicated that iron supplementation for iron-deficient donors would be a positive development. They mentioned that that iron supplementation would be more accessible if Sanquin would provide iron supplements, instead of donors getting a prescription from their general practitioner or having to buy iron supplements themselves.

> Sanquin also has their own physicians, so I trust that their advice will be reliable. I don't have any doubts about that.
>
> (*Female donor*)

Donors indicated that iron supplementation as a policy felt like a convenient way for donors to be able to continue to donate. Donors mentioned that donating is important to them and that they would be prepared to take measures, including iron supplementation, in order to be able to continue to donate. These donors preferred iron supplementation over extended donation intervals.

> I was just told that after my deferral my eligibility to donate would be reevaluated. However, I would rather have been informed about how I could improve my recovery, and if iron supplements would help then I would have been open towards it.
>
> (*Female donor*)

Most donors indicated that iron supplementation as a policy would not have a negative effect on their willingness to donate if this would reduce iron-deficiency-related symptoms or improve their health.

> I decided to switch to plasma donation instead of whole blood. However, if this would not have been possible and iron supplements would help me to be able to continue to donate, then I would seriously consider taking them.
>
> (*Female donor*)

However, not all donors agreed with this viewpoint. Two donors, from the same focus group, mentioned that they would be unsure about continuing to donate if iron supplementation was necessary to donate regularly without health complaints. Another donor was hesitant about using iron supplements, as he preferred to recover his iron stores through dietary iron intake.

> I would certainly not start using iron supplements when I would experience negative health effects from donating. I would rather stop donating than start using iron supplements only to keep donating.
>
> (*Female donor*)

Even though the majority of donors were open to iron supplementation as a blood bank policy, they also indicated that this question felt like an ethical dilemma. Donors were positive about Sanquin prioritizing the donors' health by providing iron supplements and that this would lower the threshold to use iron supplements. Yet, during the discussion it was also hypothesized that it could be perceived that iron supplements would be provided to make sure donors can donate more often to benefit Sanquin. However, donors discussed that when it became clear in the communication to donors that iron supplementation as a policy is implemented to improve donor health, donors would not feel like they are being exploited by Sanquin to increase their donation frequency.

> I do not expect that donors would feel like Sanquin is only trying to take advantage of their donors by using them as "milk cows" for their blood.
>
> (*Female donor*)

Contrarily, most collection staff members mentioned that ethical concerns were the main reason to oppose iron supplementation as a blood bank policy. They felt that if it became necessary for donors to take iron supplements prior to their next donation, blood donation would feel less voluntary. Therefore, the collection staff suggested alternative interventions such as lower donation frequencies and dietary advice. If these interventions are found ineffective, donors should be regarded as unsuitable for further donation. However, after the collection staff was provided with information about the ferritin-guided donation interval policy, the voluntary nature of iron supplementation as a policy only for donors with low ferritin levels and the actual effect of iron supplements on ferritin levels, their opinion shifted towards a more positive view on iron supplementation as a policy.

> Are we not demanding too much of the donors if we start asking them to take iron supplements?
>
> (*Female blood collection employee*)

Physicians were predominantly positive about iron supplementation as a blood bank policy and did not see this as an ethical dilemma. They felt that it would be beneficial if Sanquin would guide and support their donors with their post-donation iron store recovery to prevent iron deficiency through iron supplementation, thereby

preventing donors from taking iron supplements without any professional support. The physicians were aware that iron supplementation is already used by some international blood banks as an iron management policy. Therefore, they expected that donors would be open and positive towards iron supplements if this would prevent deferral.

> I think we should be glad if we could support donors, instead of them having to search for supplements themselves at the drugstore and use it without any professional help.
>
> (*Female donor physician*)

Expectations

The most important requirement for iron supplementation as a policy, as mentioned in all focus group discussions, was the freedom of choice of the donor. Donors should always be able to decide for themselves whether to accept iron supplements, even if this would result in a significantly longer donation interval. Additionally, the importance of an emphasis on the voluntary basis in the communication to donors was mentioned.

> Iron supplementation would feel more accessible if it was part of Sanquin's policy. However, this should be without any obligation of course, which must be clearly communicated.
>
> (*Female donor*)

Additional ferritin and Hb measurements were also mentioned as a requirement for implementing iron supplementation as a policy. Donors felt that Sanquin would be responsible for monitoring their iron levels more regularly during the iron supplementation period and expected to be informed about the results.

> I feel that it is Sanquin's responsibility to monitor the iron levels of the donors very closely when iron supplements are going to be provided.
>
> (*Male donor*)

Donors expected that information about donation-induced iron deficiency, as well as guidance and instructions regarding iron supplementation, would be provided prior to receiving iron supplements. The information and instructions have to be clear, transparent and substantiated. Furthermore, it was stated by donors that the involvement of a donor physician is important.

> I expect monitoring and a clear explanation by one of the donor physicians with an accompanying information letter, or something like that.
>
> (*Female donor*)

In turn, the physicians indicated that they expect themselves to be responsible for informing the donors, rather than the collection staff. Furthermore, the physicians also suggested that donors should be able to contact Sanquin about potential side effects, rather than referring them to their general practitioner.

> If we are going to provide supplements, then this should be the medical responsibility of the donor physicians.
>
> (*Female donor physician*)

The collection staff and physicians expected clear information and unambiguous instructions regarding iron supplementation as a blood bank policy. Moreover, the collection staff felt a need for tangible resources in the form of a booklet or brochure. Resources should contain background information regarding the policy and standard operation procedures. It was emphasized that the information should be universal for all blood collection locations and collection staff, to prevent confusion amongst employees and donors.

> I think that we as blood collection staff would really appreciate an unambiguous policy description to be provided on paper.
>
> (*Female blood collection employee*)

DISCUSSION

This study conducted focus group interviews to gain more insight into the awareness of donation-induced iron deficiency and perceptions on iron supplementation as an iron management policy in individuals, donors and staff, involved in blood donation. This study shows that donors and physicians are predominantly positive towards iron supplementation as an iron management policy, whereas the collection staff had a more opposing view. Furthermore, although all groups were aware of potential health effects of donation, donors had a poor understanding of ferritin, as they did not know how it differed from Hb. Furthermore, the collection staff lacked knowledge on the effects of iron supplementation on iron stores.

Throughout the focus group discussions, it emerged that being able to continue to donate on a regular basis is of great importance for donors, mainly because this altruistic act made them feel good. This positive feeling, or warm glow, has previously been described by Ferguson et al. as an important motivator for donors and is a strong predictor of donation intention and behaviour [23]. Moreover, donors in this study indicated that warm glow compensates for potential negative health effects related to donation, including symptoms associated with donation-induced iron deficiency. Being able to continue to donate on a regular basis was also mentioned by donors as an important reason to be willing to use iron supplements when provided by the blood service after donating. This was not unexpected because deferral has been associated with disappointment, a feeling of social

rejection and annoyance due to wasted time amongst donors, which can threaten their sense of belonging to the donor community [24, 25]. As a likely result, it has been shown that deferral has a detrimental effect on donors' motivation to donate and actual donor return [25, 26].

Donors, as well as physicians and collection staff, stated that iron supplementation as an iron management policy felt like an ethical dilemma: enhancing donor health on one hand, and asking too much of the donors to benefit the blood service on the other. The collection staff strongly felt that donors would feel exploited by the blood service. However, the collection staff underestimated the donors' dedication to continue to donate when discussing iron supplements as a policy, by mentioning that donors could quit donating completely when were deferred because of low ferritin levels. Even though this view was not shared by donors, they did hypothesize that iron supplementation could potentially be perceived by other donors as unethical if more frequent donations was the main purpose of the policy. This was one of the reasons why donors emphasized that iron supplementation as a policy should be completely voluntarily and that rejecting iron supplements should not have a negative effect on their donorship other than extended deferral.

Physicians saw iron supplementation as a useful strategy to mitigate iron deficiency and anaemia. Yet, none of the donors had previously considered using iron supplements as they had not experienced any health complaints related to low iron stores due to donation, which was mentioned as a necessity to start using iron supplements. This is consistent with findings from Pajor et al., who showed that, for the Dutch general population not using dietary supplements, there must be a health necessity to start using dietary supplements [27]. Similarly, Goldman et al. showed through interviews that donors were not inclined to start iron supplementation after donation at their own initiative and that they would only consider iron supplements when they felt the need for it [28]. However, knowledge on the effect of donation on ferritin levels, and in turn donor health, was lacking in donors. As a result, deferral due to low ferritin levels was often unexpected, led to great disappointment and was associated with a feeling of being unhealthy amongst donors. This is in line with results from the INTERVAL study in which British donors during interviews indicated to be 'shocked' when informed about their low iron levels [29]. Furthermore, donors indicated during interviews to have started taking iron supplements after donation when informed about their low iron levels. Moreover, participants in the STRIDE study who were informed of the influence of donation and iron supplementation on their iron levels, and subsequently on deferral, were five times more likely to take iron supplements compared to those who did not receive any information [30]. These findings suggest that it is essential that the awareness of donors regarding potential health effects of donation-related iron deficiency, potential health benefits of iron supplementation and iron management policies should be improved for successful implementation of iron supplementation as a policy.

Collection staff believed that iron supplements are effective in improving Hb levels but not ferritin levels. Therefore, the collection staff did not see any added value in providing iron supplements to donors with low ferritin levels. These findings suggest that the knowledge level of the collection staff regarding iron metabolism, and in particular ferritin, is limited. Greenhalgh et al. highlighted that acceptance by professional staff is one of the single most important factors for successful implementation of a healthcare service [31]. It is therefore essential to improve the medical training of the collection staff in terms of iron metabolism and the effect of iron supplements on the iron stores in case of the implementation of post-donation iron supplementation as a policy.

Donors trusted the good intentions of the blood bank and felt that donor health is a high priority. As a result, donors had confidence in the expertise of physicians to provide iron supplements. This is in line with results from a study by van der Schee et al., showing a high level of confidence in healthcare providers' professional expertise amongst the Dutch general population [32]. Since trust in the healthcare system is associated with shaping health-related behaviours and the importance of involvement of physicians when implementing iron supplementation was stated as important by donors, physicians should play a key role in informing the donor about the use and potential health and side effects of iron supplements [33]. In turn, this could improve the acceptability of iron supplementation as a blood service policy amongst donors.

Taking into account the lack of knowledge amongst donors and collection staff, informing those involved in donation about donation-related iron loss and the potential beneficial health effects of iron supplements could have a large effect on the donors' willingness towards post-donation iron supplementation. Furthermore, in the communication to donors it should be emphasized that post-donation iron supplementation would be completely voluntarily and that rejecting supplementation would not have a negative effect on their donorship. All in all, considering the predominantly open view of donors, physicians and, to a lesser extent, the collection staff towards iron supplements in this study, iron supplementation as a policy could be well perceived by those involved in blood donation when presented as an alternative to deferral.

Qualitative research on the awareness of donation-induced iron depletion and perceptions on iron supplementation as an iron store management strategy amongst individuals involved in blood donation is limited. However, this approach is important for the detection of potential barriers and facilitators to the successful implementation of an iron supplementation policy [31]. The diversity of the study population and the qualitative set-up, which facilitated interaction between participants and allowed for elaborate input, resulted in a comprehensive overview of the different perspectives of both donors and the collection staff. Moreover, selection bias was minimalized, as participants were invited to participate in focus group discussions on Sanquin's policies and donor health rather than iron suppletion. Yet, because of the small sample size, especially of the physicians, the results should be interpreted with caution. The focus group discussions were digital because of COVID-19 restrictions in the Netherlands, potentially reducing the interaction between participants. However, previous research has shown that participants are more prone to share personal experiences in digital compared to in-

person focus group discussions [34]. Furthermore, digital focus group discussions enabled us to include a more geographically diverse group of participants, allowing for a more representative sample of the Dutch donors, collection staff and physicians.

In conclusion, individuals involved in blood donation, both donors and staff, were predominantly positive towards iron supplementation as a blood bank policy on a voluntary basis. Awareness of the effects of donation and of iron supplements on iron stores was limited in donors and the collection staff, but not physicians. Important conditions for successful implementation of iron supplementation are sufficient training of the collection staff, as well as the provision of sufficient information, guidance and iron status monitoring to donors by physicians.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ORIGINAL ARTICLE

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Factors related to the development of high antibody titres against SARS-CoV-2 in convalescent plasma donors from the ConPlas-19 trial

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Abstract

Background and Objectives: The efficacy of COVID-19 convalescent plasma (CP) associates with high titres of antibodies. ConPlas-19 clinical trial showed that CP reduces the risk of progression to severe COVID-19 at 28 days. Here, we aim to study ConPlas-19 donors and characteristics that associate with high anti-SARS-CoV-2 antibody levels.

Materials and Methods: Four-hundred donors were enrolled in ConPlas-19. The presence and titres of anti-SARS-CoV-2 antibodies were evaluated by EUROIMMUN anti-SARS-CoV-2 S1 IgG ELISA.

Results: A majority of 80.3% of ConPlas-19 donor candidates had positive EUROIMMUN test results (ratio ≥1.1), and of these, 51.4% had high antibody titres (ratio ≥3.5). Antibody levels decline over time, but nevertheless, out of 37 donors tested for an intended second CP donation, over 90% were still EUROIMMUN positive, and nearly 75% of those with high titres maintained high titres in the second sample. Donors with a greater probability of developing high titres of anti-SARS-CoV-2 antibodies include those older than 40 years of age (RR 2.06; 95% CI 1.24–3.42), with more than 7 days of COVID-19 symptoms (RR 1.89; 95% CI 1.05–3.43) and collected within 4 months from infection (RR 2.61; 95% CI 1.16–5.90). Male donors had a trend towards higher titres compared with women (RR 1.67; 95% CI 0.91–3.06).

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Conclusion: SARS-CoV-2 CP candidate donors' age, duration of COVID-19 symptoms and time from infection to donation associate with the collection of CP with high antibody levels. Beyond COVID-19, these data are relevant to inform decisions to optimize the CP donor selection process in potential future outbreaks.

Keywords

anti-SARS-CoV-2, convalescent plasma, COVID-19, donors, passive immunotherapy

Highlights

- More than 80% of convalescent plasma (CP) donors in the ConPlas-19 trial had positive EUROIMMUN antibody results, and of these, >50% had high antibody titres. Those older than 40 years of age, with over 7 days of COVID-19 symptoms and collected within 4 months from infection have a greater probability of developing high titres of anti-SARS-CoV-2 antibodies.
- Antibody levels decline over time, but most donors who were tested for a potential second CP donation remained positive and maintained high antibody titres.
- Beyond COVID-19, these data are relevant to inform decisions to optimize the CP donor selection process in potential future outbreaks.

INTRODUCTION

The safety and efficacy of convalescent plasma (CP) as a therapeutic option for patients with COVID-19 has been thoroughly examined in multiple studies, clinical trials and meta-analyses [1–6]. Emerging from this evidence, current recommendations are for CP to be used in early stages of the disease, in high-risk patients and in those who have not developed an appropriate immunological response, and for CP units to be chosen based on high antibody titres to improve efficacy [7–9]. However, very few studies have focused on CP donors and investigated their characteristics that may associate with the development of antibody titres and efficacy. ConPlas-19 (NCT04345523), a multi-centre randomized open-label clinical trial of CP in hospitalized patients with COVID-19 pneumonia showed a treatment benefit in preventing disease progression or death at 28 days after CP treatment [6]. Here, we now aim to analyse the characteristics of the CP donors enrolled in ConPlas-19 and their potential association with the collection of higher quality CP with high antibody titres.

MATERIALS AND METHODS

Study design

This is an ad hoc retrospective sub-study of the multi-centre open-label randomized ConPlas-19 clinical trial (NCT04345523) [6, 10]. The clinical trial was approved by the Research Ethics Committee of the Hospital Universitario Puerta de Hierro Majadahonda in Madrid, Spain (PI57-20 from 23 March 2020). This ad hoc study was approved by the Research Ethics Committee of the Hospital Universitario Puerta de Hierro Majadahonda in Madrid, Spain (PI 92/22 from 26 April 2022) and by the Research Ethics Committee of the Universidad Autónoma de Madrid, Spain (CEI-125-2561 from 15 July 2022). Informed consent was obtained from all donors.

CP donors

ConPlas-19 enrolled CP donors from 30 centres in Spain, including hospitals and regional transfusion centres, between April and November 2020. Potential donors were identified using local registries of convalescent COVID-19 patients. In the context of the pandemic, an initial assessment of their eligibility was made by telephone. Donors eligible for screening were assessed according to EU requirements and the Spanish regulations for plasma donation (RD 1088/2005) [11, 12]. EUROIMMUN anti-SARS-CoV-2S1 ELISA IgG assays (EUROIMMUN, Luebeck, Germany) were performed in all screened donors prior to donation to confirm the presence of anti-SARS-CoV-2 antibodies. Donors selected had a laboratory-confirmed SARS-CoV-2 infection, had been asymptomatic for at least 14 days prior to CP collection and were positive for anti-SARS-CoV-2 IgG (EUROIMMUN ratio ≥1.1). In keeping with FDA recommendations, high antibody titres were defined by EUROIMMUN ratios ≥3.5 [13]. Donors could undergo more than one CP apheresis process as long as they continued to fulfil criteria and had confirmed SARS-CoV-2 antibodies in a new sample. Donors' characteristics included in this sub-study were age, gender, ABO blood group, symptom duration and time to donation between symptom onset and antibody testing. Further details of ConPlas-19 have been reported [6, 10].

Data management and monitoring

The screened CP donors were registered using a web-based electronic Case Report Form performed with ORACLE clinical. Remote data

monitoring was performed by dedicated staff, independent of the site investigators, with source data verification performed for donors recruited for critical data points that were previously established in the monitoring plan.

Statistical analysis

A detailed statistical analysis plan for ConPlas-19 has been already reported [6]. The current statistical analysis to describe and analyse retrospectively donors' characteristics and their association with CP titres in the different analyses has used Student's *t*-test, Fisher's exact test, analysis of variance, and simple linear and multinomial logistic regression analyses. *p*-values <0.05 were considered statistically significant. The statistical analysis was conducted with STATA/IC 16.1 version (StataCorp, College Station, TX, USA).

RESULTS

Donors' characteristics

ConPlas-19 enrolled 400 CP donors between April and November 2020 (Figure 1 and Table 1). Three additional donor candidates were initially screened and selected, but did not sign the informed consent. They were mostly men (78.8%), with a median age of 40.5 years (IQR 30.5–50.5). Male donors were a median of 4 years older than female donors (42.0 vs. 37.9; $p = 0.003$). The distribution of donors' ABO blood type matched that of the Spanish population [14]: 44.6% group A, 39.4% group O, 8.8% group B and 7.2% group AB, with the commonest types being A positive (36.8%) and O positive (33.2%). Donors' COVID-19 diagnosis was confirmed by RT-PCR in 292 cases (73%). Nearly 30% of donors, during the early phase of the pandemic, when there was not a wide availability of serological tests, had a high clinical suspicion of infection despite a confirmatory SARS-CoV-2 RT-PCR being either not performed or negative. All these donors had a confirmed serological diagnosis of SARS-CoV-2 infection prior to collection. Donors' COVID-19 severity was not recorded in the study protocol. Their median duration of symptoms of SARS-CoV-2 infection was 13 days (IQR 7–19), and it increased with donor age at a rate of 0.17 days for each year increase in donor age (95% CI, 0.09–0.25; $p < 0.001$). We did not find an association between symptom duration and donor sex or ABO blood type.

Overall EUROIMMUN ELISA test results

An initial EUROIMMUN ELISA test was carried out in 392 of the 400 donors recruited for the study (98%). Median time from COVID-19 symptoms onset to the first ELISA to be assessed as CP donors was 50 days (IQR 39–62). A majority of 315 (80.3%) donor candidates had a positive anti-SARS-CoV-2 EUROIMMUN result in the first serological testing, while 63 (16.1%) were negative and 14 (3.6%) had an indeterminate result (Figure 2). There were no differences in donors' serological status depending on having a microbiological confirmation of SARS-CoV-2 infection by RT-PCR: 80.9% of donors with versus 78.8% of those without a confirmed SARS-CoV-2 diagnosis by RT-PCR did have anti-SARS-CoV-2 antibodies ($p = 0.668$). Donors selected based on a high clinical suspicion without RT-PCR had overall a similar profile and characteristics to those with a confirmed diagnosis by RT-PCR. Median antibody level of donors with a positive screening ELISA test result was 3.53 (IQR 2.29–5.39). We did not find any factor (age, sex, ABO blood group, duration of symptoms and time between symptom onset and

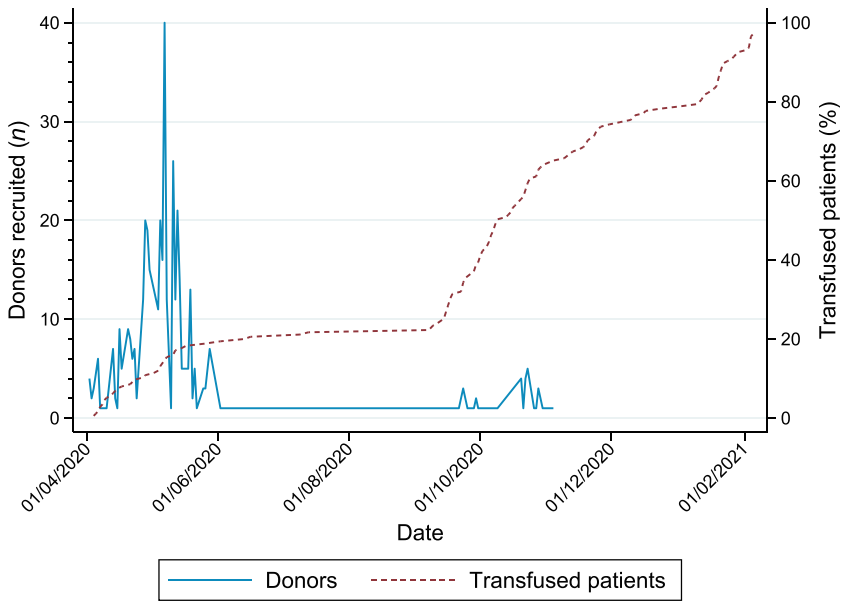

FIGURE 1 Donors' recruitment and convalescent plasma transfusion over time.

TABLE 1 Donors' basal characteristics.

Characteristic	All ($N = 400$)[a]
Sex, *n* (%)	
Male	315 (78.8%)
Female	85 (21.2%)
Age, median (IQR), years	40.5 (30.5–50.5)
ABO blood group, *n* (%)[b]	
Group A	173 (44.6%)
Group O	153 (39.4%)
Group B	34 (8.8%)
Group AB	28 (7.2%)
Period of inclusion, *n* (%)	
First wave	359 (89.8%)
Second wave	41 (10.2%)
SARS-CoV-2 RT-PCR at diagnosis, *n* (%)	
Positive	292 (73%)
Negative	7 (1.8%)
Not performed	74 (18.5%)
Unknown	27 (6.7%)
Duration of COVID-19 symptoms, median (IQR), days	13 (7–19)
Donor's antibodies at screening, *n* (%)[c]	
Positive	315 (80.3%)
Negative	63 (16.1%)
Indeterminate	14 (3.6%)
Time from symptoms onset to first antibody test, median (IQR), days	50 (39–62)

[a]There were three additional donors who were registered in the clinical trial but did not sign informed consent.
[b]There are 12 (3.0%) donors whose ABO blood group is unknown.
[c]There were eight (2%) donors to whom serological tests were not performed.

EUROIMMUN determination) related with not developing IgG anti-SARS-CoV-2 antibodies.

Donors with higher antibody levels

With a median EUROIMMUN test result of 3.53, over half of all positive donors (162 out of 315, 51.4%) met the criteria for antibody high titres, as pre-defined by EUROIMMUN ratios ≥3.5 [13]. These donors with high titres had median levels of 5.33 (IQR 4.26–6.66). Several donor factors are associated with the probability of having high titres (Table 2). Donors with a duration of COVID-19 symptoms >7 days were nearly twice as likely to have high titres (RR 1.89; 95% CI 1.05–3.43; $p = 0.037$), and those who had passed COVID-19 within the previous 4 months from testing for CP collection were 2.6 times more likely to have high titres (RR 2.61; 95% CI 1.16–5.90; $p = 0.021$). In addition, donors older than 40 years of age had a higher probability of developing high titres (RR 2.06; 95% CI 1.24–3.42; $p = 0.005$) than younger donors, and male donors showed a statistical

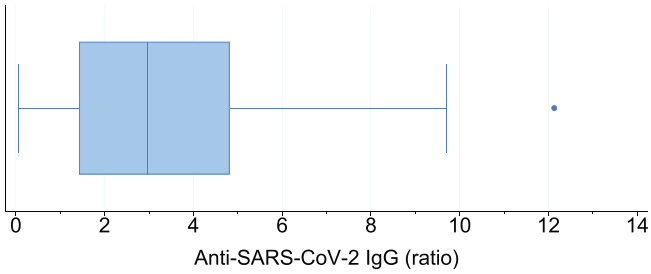

FIGURE 2 Distribution of antibody titres among all recruited donors. Box-and-whiskers diagram showing the distribution of antibody titres in all donors ($n = 392$; minimum: 0.06; p25: 1.43; p50: 2.98; p75: 4.82; maximum: 12.13).

trend towards higher titres of anti-SARS-CoV-2 antibodies than women (RR 1.67; 95% CI 0.91–3.06; $p = 0.098$). ABO blood groups were not associated with antibody levels.

Antibody level testing for subsequent CP donations

Thirty-seven donors with detectable anti-SARS-CoV-2 antibodies (11.7%) were tested at a second time point for a potential second CP donation. These samples were obtained 82 days after the onset of COVID-19 symptoms (IQR 68–195) and 27 days after the first donation (IQR 13–113). A vast majority of 34 of these donors (91.9%) maintained detectable antibodies (EUROIMMUN ratio ≥1.1) in serum in the second analysis. In addition, 17 out of 23 of these donors who had initially high titres (73.9%) maintained high titres in the second sample. Nevertheless, for each month elapsed between donations, antibody levels decreased in 0.17 units (95% CI 0.03–0.35; $p = 0.016$) (Figure 3). Overall, there was median of 1.10 lower EUROIMMUN ratio units in the second sample compared with the first one (95% CI 0.63–1.56; $p < 0.001$), and this reduction was more pronounced in donors with high titres, in whom the decline between first and second donation was of 1.59 units (95% CI 1.04–2.15; $p < 0.001$).

DISCUSSION

This is to our knowledge one of the few studies of CP for COVID-19 focused on the donors enrolled in a clinical trial and on their characteristics that associate with the collection of CP with high anti-SARS-CoV-2 titres. Thus far, research has concentrated primarily on the efficacy of CP in patients, and less information is available about the donors from whom CP is obtained, from which the final efficacy results arise. Available data on anti-SARS-CoV-2 CP donors are mainly descriptive and come from regional centres of blood donation and national CP programmes. This study analyses the CP donors enrolled in the multi-centre, randomized, open-label, clinical trial ConPlas-19 and identifies several donor factors that associate with the collection of higher quality CP with high antibody titres.

TABLE 2 Biological and clinical factors associated with high titres.

	Multinomial logistic regression			
Variable	**RR**	***p***	**95% CI**	
Age				
<40 years (reference group)	1			
≥40 years	2.06	0.005	1.24	3.42
Sex				
Women (reference group)	1			
Men	1.67	0.098	0.91	3.06
ABO blood group				
Group O (reference group)	1			
Group A	1.04	0.883	0.61	1.77
Group AB	0.69	0.456	0.26	1.81
Group B	1.24	0.648	0.49	3.11
Symptom duration				
<7 days (reference group)	1			
≥7 days	1.89	0.037	1.05	3.43
Time from onset to EUROIMMUN testing				
≥4 months (reference group)	1			
<4 months	2.61	0.021	1.16	5.90

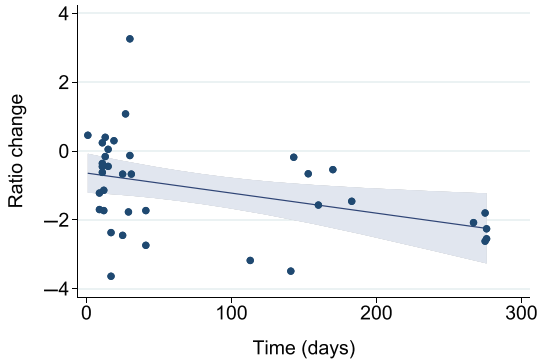

FIGURE 3 Change in antibody titres in donors considered for a second convalescent plasma donation. The horizontal axis represents the time in days between the first and second EUROIMMUN test values in donors considered for a second donation ($n = 37$). Dots represent the individual change of antibody levels in each donor between donations; the dark line represents the fitted values and the grey area represents the 95% CI of the mean.

Donors with a duration of COVID-19 symptoms of more than 7 days had CP with higher anti-SARS-CoV-2 titres than donors with shorter duration of symptoms. Patients with severe forms of COVID-19 are known to develop higher antibody levels [15], although some studies suggest that these patients do not develop effective humoral and cellular immune responses [16]. Unfortunately, beyond duration of symptoms, our clinical trial protocol did not collect the severity of COVID-19 in CP donors. The CAPSID trial has studied its CP donors, and although duration of symptoms was not evaluated, the authors found that the number of symptoms was associated with higher antibody titres [17].

Our study suggests that CP donors collected within 4 months from the beginning of COVID-19 symptoms are more likely to have high anti-SARS-CoV-2 antibody titres. A decline in antibody levels with time from infection was clearly identified in our series, and it appeared more pronounced in donors with high titres. Other studies have shown similar results. Chen et al. reported that most cases show a decrease in anti-SARS-CoV-2 IgG antibodies in the third month since recovery from COVID-19 [18]. Prus et al. also showed that the decrease was more marked after 12 weeks and recommended an earlier CP collection between 4 and 8 weeks from recovery from COVID-19 [19]. Of note, our experience with a subgroup of 37 donors who were tested for an intended second CP donation is nevertheless reassuring, as it shows that a vast majority of 92% of those donors still had anti-SARS-CoV-2 antibodies at a median of 24 days from the first CP donation, and 74% of those with high titres maintained high titres in the second sample.

In our series, donors over 40 years of age were more likely to have CP with high titres. Age has been described as a factor associated with antibody titres in several studies [20–23], including some large multivariate regression analyses that found a positive correlation with age even when adjusting the model by hospitalization history [24], and showed that the rate of antibody decline was significantly slower for donors over 55 years of age [25]. The reasons underlying the impact of age on anti-SARS-CoV-2 immune response are not clear [26], but some authors suggest that it may be linked to immunesenescence and the generation of immunoglobulins with lower antigen specificity in these patients [27]. In addition, our data found a statistical trend towards male donors having higher anti-SARS-CoV-2 antibodies. The literature on this topic is less clear. Some studies have shown that women have higher antibody titres, but also a faster rate of decline with time, compared with men [25]. Others, on the contrary, concur with our findings of higher titres in males, albeit in association with a more severe presentation of COVID-19 in these patients [28]. Finally, our findings do not support an association between antibody levels and ABO blood group. There is a clear association of blood group A with a higher risk and severity of COVID-19 [29–31], but the evidence of an association between ABO blood group and antibody levels are not clear in the literature [20, 21, 23].

This study has some limitations. Although it stems from a prospective randomized clinical trial, this ad hoc study is retrospective and carries intrinsic limitations of such a design. In addition, we have not analysed neutralizing antibodies (NAbs) anti-SARS-CoV-2. While current guidelines specifically recommend CP treatment with high NAb titres [9], the complexity and technical specifications of neutralization assays, in particular during the first wave of the pandemic when most of our donors were recruited, prevented us from including these results in this manuscript. Furthermore, an analysis of total IgG anti-SARS-CoV-2 (EUROIMMUN assay) provides broader information on the role of the CP, including antiviral activities that depend on the integrity of the Fc region, like complement-dependent cytotoxicity, antibody-dependent cell-phagocytosis and antibody-dependent cell cytotoxicity [5]. More recently, the so-called hybrid plasma or Vax-plasma, obtained from convalescent donors who were subsequently

vaccinated seems to present much higher antibody titres (10-fold to 100-fold) than conventional CP and may be preferable for the treatment of immunocompromised patients [32, 33]. Unfortunately, our study conducted during the early phase of the pandemic, before vaccines were developed, cannot address this comparison. Finally, even though the severity of infection was not an exclusion criterion to select candidate CP donors, in the complex medical scenario of the early pandemic, there was a bias towards recruiting as donors COVID-19 convalescent patients who were younger, with a healthier medical background and who had not suffered a severe COVID-19. Now, we know that some of those characteristics had a negative impact on the antibody titres of the CP collected. Nevertheless, even with such potential bias, 92% of our candidate CP donors were positive for anti-SARS-CoV-2 antibodies, and of these, more than half had high antibody titres. Even without a confirmed microbiological diagnosis by RT-PCR, a high clinical suspicion of past clinical infection was enough in our clinical trial to recruit suitable donors. This strategy of recruitment was successful and proved particularly useful in the early phases of a pandemic where diagnostic tests are neither fully developed nor widely available.

In conclusion, our study suggests that basic demographic and clinical information such as duration of SARS-CoV-2 infection over 7 days, donations performed within the first 4 months since the onset of symptoms and donor's age above 40 years associate with the collection of CP with high antibody levels. Beyond its relevance in the context of COVID-19, we hope that these data will be of relevance and inform decisions to be made in the planning to optimize the CP donor selection process in potential future outbreaks.

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C.A.-S., J.L.B.C., R.F.D. and A.R.-M. conceived the clinical trial study. I.R.M., J.L.B.C. and R.F.D. contributed to the design of this study. I.R.M., J.L.B.C., C.V.D.R., A.B.L, J.A.G.E., M.I.G.F., L.G.D., I.V.A., J.A.V., V.P.G.R., E.C., S.U.U., A.L.P.H., T.J.-M., A.M.O.P. and J.L.A.R. contributed to CP collection, qualification and release. A.V.-I. coordinated study activities. M.P.-O. performed and interpreted serology assays. I.R.M., J.L.B.C. and R.F.D. designed and supervised statistical analyses. I.R.M. wrote the first version of the manuscript. R.F.D. corrected the final draft version of the manuscript. All authors contributed to critical revision of the manuscript and approved its final version.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

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The value of genetic data from 665,460 individuals in managing iron deficiency anaemia and suitability to donate blood

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Abstract

Background and Objectives: Although the genetic determinants of haemoglobin and ferritin have been widely studied, those of the clinically and globally relevant iron deficiency anaemia (IDA) and deferral due to hypohaemoglobinemia (Hb-deferral) are unclear. In this investigation, we aimed to quantify the value of genetic information in predicting IDA and Hb-deferral.

Materials and Methods: We analysed genetic data from up to 665,460 participants of the FinnGen, Blood Service Biobank and UK Biobank, and used INTERVAL ($N = 39{,}979$) for validation.

We performed genome-wide association studies (GWASs) of IDA and Hb-deferral and utilized publicly available genetic associations to compute polygenic scores for IDA, ferritin and Hb. We fitted models to estimate the effect sizes of these polygenic risk scores (PRSs) on IDA and Hb-deferral risk while accounting for the individual's age, sex, weight, height, smoking status and blood donation history.

Results: Significant variants in GWASs of IDA and Hb-deferral appear to be a small subset of variants associated with ferritin and Hb. Effect sizes of genetic predictors of IDA and Hb-deferral are similar to those of age and weight which are typically used in blood donor management. A total genetic score for Hb-deferral was estimated for each individual. The odds ratio estimate between first decile against that at ninth decile of total genetic score distribution ranged from 1.4 to 2.2.

Conclusion: The value of genetic data in predicting IDA or suitability to donate blood appears to be on a practically useful level.

Keywords

genetic risk, GWAS, Hb-deferral, iron deficiency anaemia, PRS, statistical inference

Highlights

- Genetic information could be as valuable as age and weight to blood donation management.

A full list of FinnGen members and their affiliations appears in the Supporting information.

- The value of genetic information in predicting iron deficiency anaemia (IDA) and haemoglobin deferral in donors is unclear.
- Genetic variation predisposing significantly to IDA seems to be a small subset of genetic variation affecting haemoglobin and ferritin.

INTRODUCTION

Iron deficiency (ID) is a substantial contributor of global disease burden, and the World Health Organization (WHO) has set its reduction as a global health priority [1]. ID can lead to iron deficiency anaemia (IDA). Especially in 15–49-year-old women, anaemia prevalence is found to vary from 7.3% to 66.1% globally, of which half was estimated to be IDA, according to WHO statistics of 2019 [2]. Although IDA is most common in Southern Asia and Central and Western Africa, it has been reported to significantly decrease overall survival and health-related quality of life in Dutch older than 60 years [3]. The WHO defines anaemia as blood haemoglobin (Hb) below 130 g/L in men and 120 g/L in women. To separate IDA from other anaemias, various molecular markers have been developed. Serum or plasma ferritin, although difficult to interpret as it is elevated by inflammation, is commonly used with a WHO cut-off of <15 μg/L to signify ID [4].

Blood donation and availability of blood products is a critical part of modern healthcare. However, blood donation can lead to anaemia due to iron loss if not properly managed [5]. Pre-donation point-of-care Hb measurement is a common prerequisite for blood donation. In Europe, an Hb level of ≥125 g/L for women and ≥135 g/L for men is typically required before donation. If the donor's Hb is below that level, they are deferred from donating due to low Hb (in short, 'Hb-deferred'). Deferral is demotivating for blood donors and costly for both the donor and blood establishment [6]. Sufficient minimum donation intervals and iron supplementation have been found to be effective ways to keep Hb-deferral rates of blood services low [7]. As Hb thresholds for anaemia and Hb-deferral are very similar, they are expected to be closely related. In addition to the minimum Hb requirement, a minimum weight of 50 kg and minimum age of 18 years is required in the European Union [8].

FinnGen is a public–private partnership project combining electronic health record (EHR) data from six regional and three national Finnish biobanks [9]. FinnGen aims to collect EHR data and genotypes for ∼0.5 million Finns, of whom >10% will be active blood donors from the Blood Service Biobank. Genotyping in FinnGen is carried out with an array of ∼0.5 million genetic variants, after which up to 17 million variants were imputed. The isolated nature of Finnish population allows identification of novel protective or harmful genetic variants. In FinnGen, genome-wide association studies (GWASs) were carried out for ∼2000 clinical endpoints, including IDA. The UK biobank [10] provides a similar resource.

From GWAS results, a polygenic risk score (PRS) can be calculated for all individuals. PRS is the weighted sum of the number of alternative alleles of each genetic variant included in the PRS. The weights are the effect sizes of the variants' individual association with the phenotype of interest, for example, IDA. In this paper, PRS represents the total effect of a person's genotype on the risk of exhibiting the said phenotype, for example, risk of developing anaemia [11]. The clinical value of PRSs of blood donation traits is still under active research [12]. The heritability explained by currently known genetic variants is typically smaller than that estimated by family-based studies. However, variant discovery by sequencing and increased sample size is expected to make PRSs far more powerful in future [13].

EHRs often contain Hb measurements, and hence multiple well-powered GWASs have been carried out for Hb recently [14–16]. For ferritin, the summary statistics of one well-powered meta-analysis are publicly available [17]. Variants in or near the human homeostatic iron regulator protein (HFE) and transmembrane protease, serine 6 (TMPRSS6) genes have been associated with Hb in several studies [18] and recently also with ferritin [17]. HFE is thought to regulate iron absorption through an interaction with the transferrin receptor in the cellular iron import pathway. TMPRSS6 inhibits the production of the iron regulatory protein hepcidin [19]. Numerous other genes reported to be associated with Hb and ferritin at genome-wide significant level cover additional biological pathways involved in iron homeostasis, such as iron sensing and storage, inflammation and blood clotting, intestinal iron absorption, iron recycling, erythropoiesis and menstruation.

Although GWASs of IDA have been reported earlier, embedded in large-scale analyses [20, 21], we present, to our knowledge, the first dedicated analysis. In addition, using large-scale electronic healthcare and biobank data, we quantify for the first time the value of genotyping data at genome-wide scale in assessing the blood donation suitability of a donor.

METHODS

The FinnGen data release 6 has 230,000 participants from Finnish hospital biobanks and 30,000 blood donors from the Finnish Red Cross Blood Service biobank (see Table 1 for subgroup counts). The basic information of each participant comprises the individual's age, sex, smoking information, weight and height (see Figures S1 and S2, Tables S1–S3 for the variable distributions). In addition to the genome-wide single-nucleotide polymorphism (SNP) genotyping data of 16.7 million variants, we have access to the EHRs of the participants. In this paper, we have only extracted the IDA events (defined by the ICD-10 code D50; information partially available since year 1969 and fully available since 1998) from the EHR database, and the follow-up ends at the end of year 2019. The D50 code is given in Finland when the laboratory measurements indicate low Hb (below 130 g/L in men and 120 g/L in women) and the treating medical doctor decides that the patient has signs of ID, for instance, low ferritin and/or high transferrin receptor. Our models for IDA utilize FinnGen

TABLE 1 FinnGen participant subgroup sizes.

Group	*n*	Male	Pre-menopausal female	Post-menopausal female
FinnGen	260,405	113,344	37,750	109,311
Blood donors	28,901	11,807	9242	7852
Non-blood donor	231,504	101,537	28,508	101,459

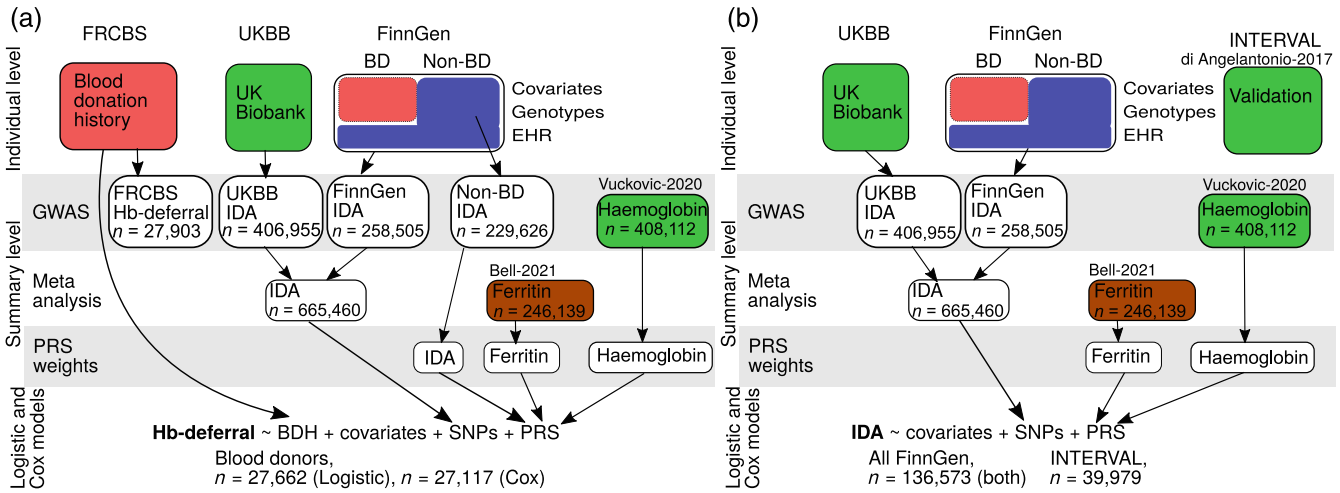

FIGURE 1 Initial and derived datasets were used to fit the models. Boxes filled with red denote blood donors, with blue the rest of FinnGen cohort, with green external and white boxes are results computed in this paper. (a) Dataflow of the haemoglobin (Hb)-deferral models. (b) Dataflow of the iron deficiency anaemia (IDA) models. BD, blood donor; BDH, blood donation history; EHR, electronic health record; FG, FinnGen; FG Basic, FinnGen basic data which includes weight, height and smoking status of donors; FRCBS, Finnish Red Cross Blood Service; GWAS, genome-wide association studies; PRS, polygenic risk score; SNP, single-nucleotide polymorphism.

data only. Since weight, height and smoking information is not available for all individuals, we are left with 136,168 individuals with full information.

For 28,901 blood donors of the Finnish Red Cross Blood Service (FRCBS), we have also at our disposal all the blood donation histories stored in the eProgesa database (MAKSYSTEM, Paris, France) between years 2000 and 2020. For every donation event, information about the Hb value (pre-donation point-of-care capillary finger-prick sample), time of day, donation location, type of donation and whether it was successful is included. This dataset was subsequently preprocessed to derive new variables (described in Tables S1, S4 and S5, Figures S3 and S4) that are used in our models. Importantly, our second endpoint, Hb-deferral, is defined as whether the pre-donation Hb measurement is below a threshold (125 g/L for females and 135 g/L for males), and hence the donation is Hb-deferred.

These two datasets along with the external data utilized in this study, namely ferritin meta-analysis from Bell et al. [17] and Hb GWAS from Vuckovic et al. [15], are shown in Figure 1 and Table 2. Note that, currently, due to regulations and contracts, we can only combine the eProgesa information with genotype and basic data (weight, height and smoking status) of the blood donors, but not with the EHR data. We develop our models for Hb-deferral on this combined dataset for blood donors.

We use Saige [22] for finding genetic variants that are associated with IDA and Hb-deferral. As covariates we use sex, age (age at last donation for Hb-deferral; age at first event, or at death, or at the end of follow-up, whichever comes first, for IDA), weight, height, smoking status and the first 10 principal components of the genetic relationship matrix (and the genotyping batch when available). Fixed-effect inverse-variance IDA meta-analysis of FinnGen and UKBB [9] and the Hb-deferral GWASs on blood donors were used in selecting important SNP variants as predictors in our models. To allow the use of genetic information outside these selected SNPs as well, we derive PRSs for three related endpoints: IDA, ferritin and Hb. The PRS of IDA was based on GWASs for FinnGen participants who are not blood donors. To avoid overlap with the selected four SNPs, we excluded areas of ±3 Mb around them from the 16.7 million variants while performing the PRS weight computation with the PRS-CS [11] software. The cohorts used to derive the PRS weights were essentially separate from the cohorts for which we computed the polygenic scores, and the IDA PRS was not used as a predictor in the IDA models, to avoid overfitting.

We use logistic regression models to evaluate the value of genetic variables in predicting IDA and Hb-deferral. For Hb-deferral, we predict the most recent donation attempt using the most recent covariate values. For IDA, we predict whether a donor has ever been diagnosed with IDA using baseline covariate values, except age which is defined as in GWASs. We also fitted Cox proportional hazards (Cox PH) models for both endpoints. For Hb-deferral, we predict the time to event using time-dependent values for the Hb and donation count covariates, and baseline values for other covariates. For IDA, the Cox

TABLE 2 Number of individuals in each GWAS or meta-analysis.

Phenotype	*n*	Cases	Controls	Cohorts
Haemoglobin	408,112			UKBB (UK)
Ferritin	246,139			deCODE (Iceland), INTERVAL (UK) and Danish Blood Donor Study (Denmark)
IDA	665,460	18,076	647,384	UKBB and FinnGen
Hb-deferral	27,903	6714	21,189	FinnGen blood donors

Abbreviations: GWAS, genome-wide association studies; Hb, haemoglobin; IDA, iron deficiency anaemia.

model uses only baseline covariate values. The details of model fitting are given in the Supplemental Methods, and the source code used in this research is publicly available at https://github.com/FRCBS/anemia_and_hb_deferral_prediction.

RESULTS

The Manhattan plots of the external Hb and ferritin GWASs that were used in building the PRS weights for the respective phenotypes are shown for completeness in Figure 2a,b, respectively. To have more detection power, we carried out a meta-analysis of IDA with the FinnGen data and the UK Biobank data (18,076 cases, 647,384 controls). The Manhattan plot of this IDA meta-analysis, shown in Figure 2c, reveals four genome-wide significant SNPs, which are independent by SuSiE fine-mapping analysis [9]. We selected these four SNPs, listed in Table 3, to be included in our models for IDA and Hb-deferral. We used the minor allele in FinnGen as the effect allele. Note that this is different from the alternative allele in the case of SNP rs199138 in chromosome 15. The Manhattan plot of the Hb-deferral GWAS (6714 cases, 21,189 controls) is shown in Figure 2d. Even though the GWAS found only one significant locus (lead SNP rs199598395), there were several peaks nearly reaching the significance threshold of $p < 5e{-}8$. The respective quantile–quantile plots and the genomic inflation factors of the four GWAS or meta-analyses are shown in Figure S5. Detailed genotype distributions of the four SNPs by the case–control status for both Hb-deferral and IDA are shown in Figures S6 and S7, Tables S10 and S11.

We compared the similarity of association peaks in IDA meta-analysis to Hb and ferritin GWASs and found that only rs6025 and rs199138 were clearly associated with ferritin and rs3129761 to Hb (see Supplemental Results).

After the PRSs were computed for IDA, ferritin and the Hb phenotypes, we fitted multivariable logistic models for IDA (events ever) and Hb-deferral (latest event), and multivariable Cox PH models for time to first IDA or Hb-deferral events. Note that even though the IDA and Hb phenotypes are partly related, we still included the PRSs of both in the Hb-deferral model as they are not highly correlated according to Figure S9; also the Manhattan plots look dissimilar. The odds ratios (ORs) of logistic models and hazard ratios of Cox PH models with their 95% confidence intervals (CIs) are shown in Figure 3 and Tables S13–S16, and the Kaplan–Meier plots of Hb-deferral and IDA are shown in Figures S14 and S15. Overall, the CIs for predictors are smaller in the Cox PH models, suggesting greater power of the Cox analysis. Subsequently, we considered the predictors whose CI does not cross 1 to have an effect in the model in question (Figure 3). When a predictor was available for both phenotypes, the signs of the effect seemed to agree, except for the weight and smoking variables. These differences are most likely explained by the interactions between weight and smoking with the 'Is blood donor' variable. At least for women, in the Cox PH model of IDA the hazard ratio of the interaction with weight is statistically significant, see Figure 3 and Table S16. The fact that being blood donor seems to prevent IDA is due to inverse causation: individuals with low Hb are not allowed to donate.

Although the effect sizes of genetic data had similar directions of effect, their magnitudes varied between models. The largest effect of the genetic data in the IDA model was by the SNP in chromosome 17 for pre-menopausal females (2.9 [2.1–4.0]), and for Hb-deferral again by the same SNP for pre-menopausal females (3.3 [2.0–5.3]). In addition to individual variants, ferritin PRS was found to be inversely associated with IDA in females according to the Cox model and in pre-menopausal females according to the logistic model, while Hb PRS was found to be inversely associated with Hb-deferral in both sexes by the Cox PH model.

We then used the INTERVAL cohort [23] to validate our findings about the importance of genetic predictors. We were able to fit only the multivariable logistic regression model of IDA because of the lack of similar response or predictor variables. Some of the four SNPs and both the ferritin and Hb PRSs were found to be significant predictors of IDA (for details, see Figure S17 and Supplemental Results).

As an initial attempt to get a more quantitative measure for the total genetic effect on Hb-deferral, we computed for each donor the weighted sum of genetic variables (SNPs and IDA, ferritin and Hb PRSs), here called the total genetic score, using the Bayesian logistic regression and the Cox PH model for the Hb-deferral (see the Supplemental Results for the formula). The medians of the genetic variables in each bin defined by the deciles of the total genetic score in the Cox PH model with time-dependent covariates for Hb-deferral are shown in Figure S21 (logistic model omitted for brevity). Even though the effect size of the SNP in chromosome 17 is high, the SNP in chromosome 15, for instance, is far more important, since it is more common. Next, we compared the total genetic score at the first decile against that at the ninth decile. This gave us an estimate for the lower limit of ORs of Hb-deferral between individuals who are genetically ill-suited for donation versus those who are well-suited for donation. This lower limit for the OR ranged from 1.4 to 2.2 over different model types and demographic groups (see Table S17).

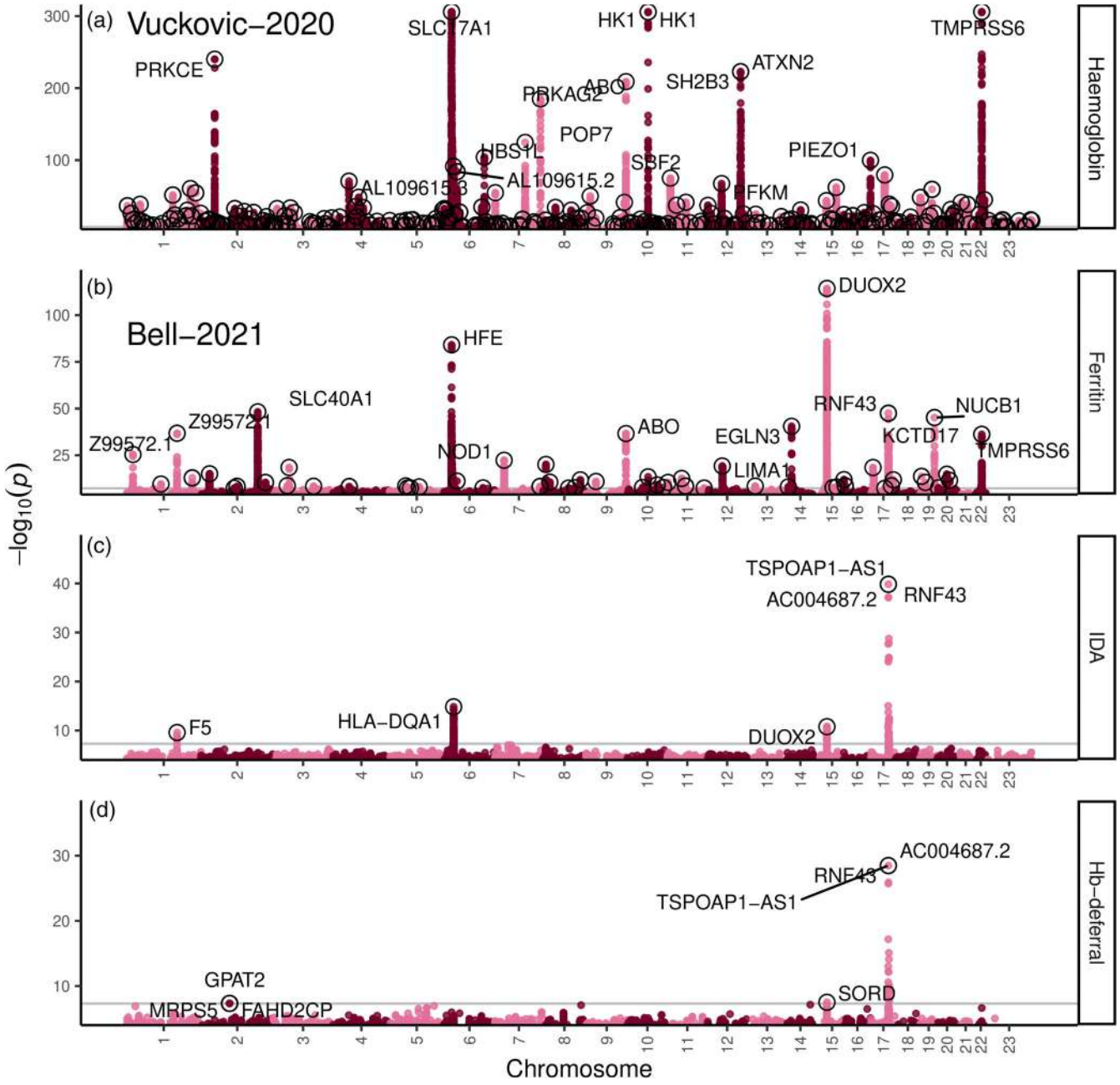

FIGURE 2 Manhattan plots of four phenotypes obtained from genome-wide association studies (GWAS) or meta-analysis on different datasets. The lead single-nucleotide polymorphisms are marked with a black circle, and their corresponding gene names (given by variant effect predictor [26]) are shown where space permits. Only *p*-values smaller than 1e−4 are plotted for performance reasons. (a) GWAS of haemoglobin ($n = 408{,}112$) [15]. (b) Meta-analysis of ferritin ($n = 246{,}139$) combining GWAS results from Iceland, the United Kingdom and Denmark [17]. (c) Meta-analysis of iron deficiency anaemia (IDA) (18,076 cases, 647,384 controls) combining GWAS results from FinnGen R6 and UKBB cohorts. (d) GWAS of haemoglobin (Hb)-deferral phenotype on FinnGen blood donors (6714 cases, 21,189 controls).

DISCUSSION

The Manhattan plot for Hb from [15] (Figure 2a) shows 388 genome-wide significant peaks ($p < 5e{-}8$) and for ferritin from [17] (Figure 2b) 58 peaks. In contrast for IDA, with 18,076 cases and 647,384 controls we could detect four peaks and for Hb-deferral with 6714 cases and 21,189 controls 1 peak. Most likely, this is due to the lack of power in IDA and Hb-deferral GWASs relative to the Hb and ferritin GWASs. However, we had enough power to detect more peaks of the Hb GWAS in the IDA GWAS, but we did not detect any (Figure S22). This might indicate that the SNPs detected for Hb, but not for IDA, are more related to higher iron values. Because for each peak the causal gene is not known, we compared the set of genes around in each peak (see Supplemental Results), and it appears that the genetic variation predisposing significantly to IDA is a small subset of both the genetic variation affecting the levels of ferritin and Hb.

TABLE 3 The four genome-wide significant lead SNPs from the IDA meta-analysis.

CHR	POS	SNP	REF	EA	Nearest gene	FG EAF	FG OR	FG *p*-value	UKBB EAF	UKBB OR	UKBB *p*-value	All OR	All *p*-value
1	169549811	rs6025	C	T	F5	0.02	0.74	1.01E−06	0.02	0.82	2.44E−05	0.79	2.54E−10
6	32617727	rs3129761	G	C	HLA-DQA1	0.48	1.07	9.40E−05	0.46	1.10	4.61E−12	1.09	5.32E−15
15	45095352	rs199138	A	G	DUOX2	0.93	0.87	2.69E−05	0.92	0.87	1.39E−07	0.87	1.65E−11
17	58358769	rs199598395	C	T	RNF43	0.01	3.00	1.49E−40				3.00	1.49E−40

Abbreviations: CHR, chromosome; EA, effect allele; EAF, effect allele frequency; FG, FinnGen; IDA, iron deficiency anaemia; OR, odds ratio; POS, position; REF, reference allele; SNP, single-nucleotide polymorphism; UKBB, UK Biobank.

The TMPRSS6 and HFE genes have been shown to be associated with markers of iron metabolism many times [17, 24]. Curiously, these were not detected in our Hb-deferral GWAS and IDA meta-analysis from Finnish and UK populations. The HFE SNPs rs1800562 and rs179945 and the TMPRSS6 SNP rs855791 were detected in our own GWAS for blood donor Hb (not shown), suggesting that their lack of association with IDA is not specific to the Finnish population. The reason these three SNPs were not significantly associated with either IDA or Hb-deferral is either due to lack of power or because the SNPs are more related to iron overload, that is, haemochromatosis instead of low iron. In particular, FinnGen haemochromatosis GWASs detect only the HFE gene, whose effect size is much larger than in the IDA, see Figure S23. Accordingly, even though Mast et al. showed that TMPRSS6 and HFE SNPs are associated with iron-related variables, they did not find any significant associations in their donation tolerance GWAS [24].

We assumed that the effect of smoking and weight on IDA may vary by blood donor status, and hence we included interactions between these (see Supplemental Methods for details). This highlights the difference between healthy blood donors and the rest of the FinnGen participants, who are hospital patients. The rs199598395 (17_58358769_C_T) SNP in the RNF43 gene has a larger effect than age or weight. According to Open Targets [25], it has an allele frequency of 0.013 in Finnish population and 0.0034 in Non-Finnish European population, that is, it is Finnish-enriched. According to the variant effect predictor (VEP) [26], rs199598395 is a missense variant in gene RNF43 (ENSG00000108375.13) and AC004687.2 (ENSG00000285897.1) and intron variant in TSOAP1-AS1 (ENSG00000265148.6). Although this Finnish-enriched SNP has not been previously reported to be associated with IDA, in the 1-Mb area around RNF43, ferritin associations have been reported in genes MRPS23 [27] in Dutch blood donor population, MTMR4 in the UK, Danish and Icelandic population [17] and TEX14 in European populations [28]. Bell et al. performed [17] a literature review that showed the gene MTMR4 to be associated with hepcidin. In addition, their pQTL study revealed a variant in gene MTMR4 to be associated with hepcidin levels. Associations with various blood counts have been reported to this same locus [29]. Genetically isolated populations such as Finns have been thought to provide special opportunities to discover rare causal variants [9, 30]; hence the missense variant in gene RNF43 could represent the true causal variant behind all these associations, but further molecular analysis is required to resolve this.

We quantified the effect of various explanatory variables on the risk of becoming anaemic or Hb-deferred with two different methods, multivariable Bayesian logistic regression and the Cox PH models, and get very similar results. Overall, Cox PH models appear to be more powerful in quantifying effects as exemplified by their smaller CIs. Irrespective of a person's blood donation or their previous pre-blood-donation Hb measurement, genetic effects are found to be larger than or in a similar range as the other variables available to us. Age and weight are typically used for donor selection. Considering our results, genetic information could be of equal value for blood

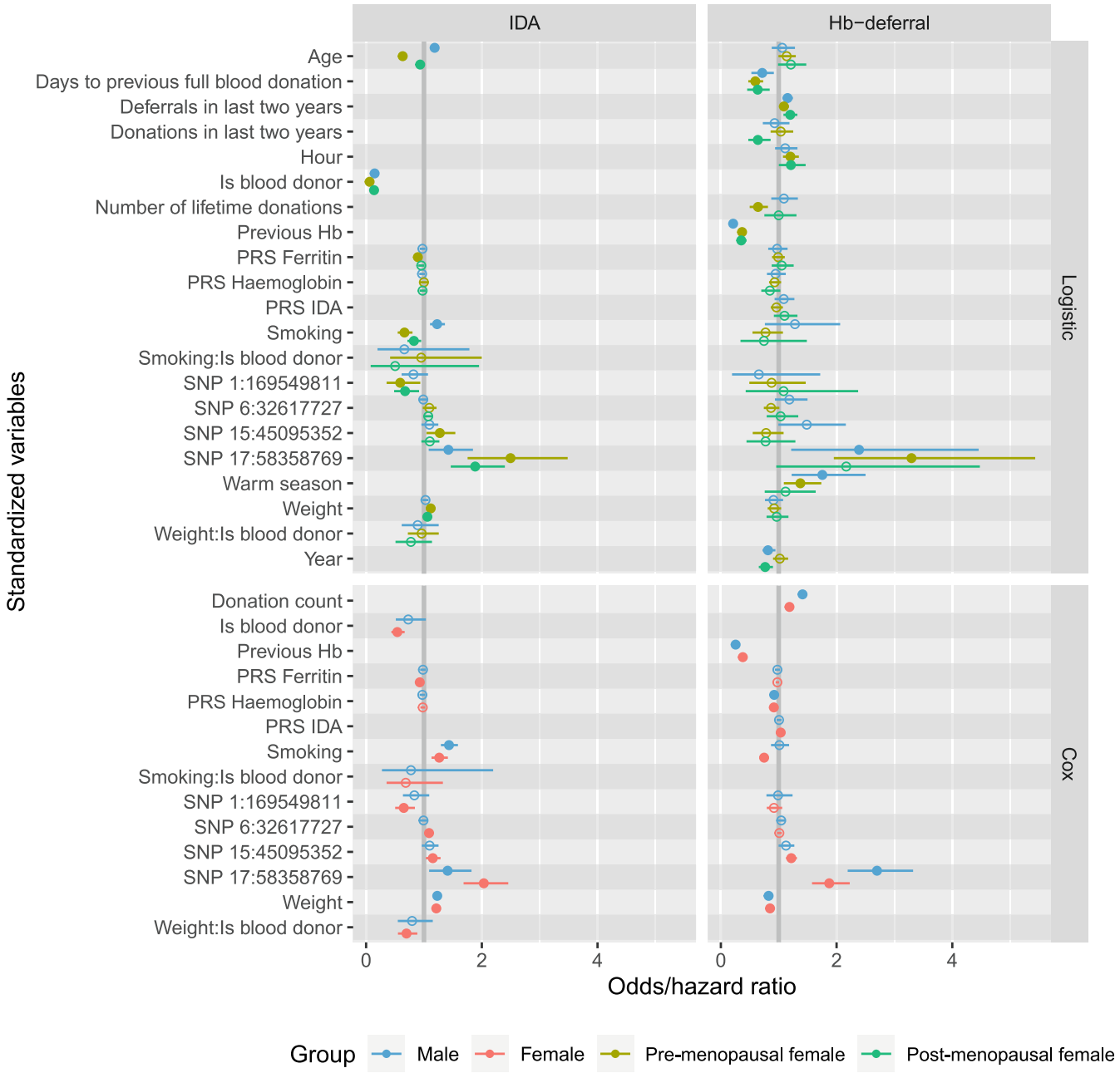

FIGURE 3 Odds ratios of the multivariable Bayesian logistic regression and hazard ratios of the multivariable Cox proportional hazards regression for both iron deficiency anaemia (IDA) and haemoglobin (Hb)-deferral. The explanatory variables, with the exception of binary variables and the allele dosages from the four single-nucleotide polymorphisms (SNPs), were standardized to zero mean and standard deviation of 1. The horizontal bars indicate the 95% confidence intervals of the odds or hazard ratio estimates. The estimates are drawn as hollow points if the odds/hazard ratio 1 is contained in the corresponding confidence interval. In the Cox proportional hazards (Cox PH) models, females were not split into pre- and post-menopausal groups, as that would have meant changing the strata at the age of 45. In the Cox PH model of Hb-deferral, the variables 'Donation count' and 'Previous Hb' were modelled as time-dependent variables. PRS, polygenic risk score.

donor management. The potential value of genetic data is confirmed by our INTERVAL cohort validation, although the exact effect sizes can differ, possibly due to differences in genetics, donation policies and availability of data.

Both ferritin and Hb PRSs were found to have significant effects. Their effect sizes might reflect the fact that much of their heritability is yet to be discovered ($R^2 = 0.03$ for variance of Hb explained by Hb PRS).

The cost of array genotyping varies but can be currently expected to be (for institutional use) tens of euros. A genotyping result is valid for a lifetime and allows blood group and HLA imputation as well, whereas, for example, a ferritin measurement would be expected to cost several euros per measurement.

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J.T. performed the analysis and wrote the first draft of the manuscript; E.A. performed the validation of the IDA logistic regression on the INTERVAL cohort; FinnGen performed the meta-analysis of iron deficiency anaemia; J.C. helped in defining the phenotypes and offered medical expertise throughout the research; E.d.A. supervised the validation on the INTERVAL cohort; M.A. supervised the research and participated in writing the first draft; all authors reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The Hb-deferral GWAS results are available at GWAS catalogue under accession number GCST90104113. The iron deficiency anemia GWAS results are available through the website https://www.finngen.fi/en/access_results. The PRS weights are available at the PRS catalog under ID PGS003426 for ferritin and PGS003425 for hemoglobin.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Donor pregnancies and transfusion recipient mortality: A role for red blood cell storage?

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Abstract

Background and Objectives: Donor characteristics have been implicated in transfusion-related adverse events. Uncertainty remains about whether sex, and specifically pregnancy history of the blood donor, could affect patient outcomes. Whether storage duration of the blood product could be important for patient outcomes has also been investigated, and a small detrimental effect of fresh products remains a possibility. Here, we hypothesize that fresh red blood cell products donated by ever-pregnant donors are associated with mortality in male patients.

Materials and Methods: We used data from a cohort study of adult patients receiving a first transfusion between 2005 and 2015 in the Netherlands. The risk of death after receiving a transfusion from one of five exposure categories (female never-pregnant stored ≤10 days, female never-pregnant stored >10 days, female ever-pregnant stored ≤10 days, female ever-pregnant stored >10 days and male stored for ≤10 days), compared to receiving a unit donated by a male donor, which was

stored for >10 days (reference), was calculated using a Cox proportional hazards model.

Results: The study included 42,456 patients who contributed 88,538 person-years in total, of whom 13,948 died during the follow-up of the study (33%). Fresh units (stored for ≤10 days) from ever-pregnant donors were associated with mortality in male patients, but the association was not statistically significant (hazard ratio 1.39, 95% confidence interval 0.97–1.99). Sensitivity analyses did not corroborate this finding.

Conclusion: These findings do not consistently support the notion that the observed association between ever-pregnant donor units and mortality is mediated by blood product storage.

Keywords

blood donor, erythrocyte transfusion, mortality, RBC storage lesion

Highlights

- We hypothesize that the transfusion of fresh red blood cell products donated by ever-pregnant donors to male patients might increase mortality.
- The association between exposure, via transfusion, to ever-pregnant donor units and mortality in young men may be modified by product storage.
- Studying parameters related to blood product haemoglobin requires careful consideration of statistical methods.

INTRODUCTION

Although transfusions can be a necessary life-saving medical intervention, they are also associated with adverse events [1]. Some of these are attributable to certain donor characteristics, such as the passive infusion of leucocyte and neutrophil antibodies in transfusion-related acute lung injury (TRALI) [2] and the transfer of plasma containing IgA and IgE antibodies in allergic transfusion reactions [3]. Nevertheless, the influence of blood donor characteristics on long-term patient outcomes is incompletely understood. Uncertainty remains about whether sex and pregnancy history of the blood donor could influence recipient outcomes, beyond an increased risk of TRALI. In two earlier large-scale cohort studies, we identified an association between transfusions of red blood cells from female donors and increased mortality in male recipients under 50 years of age [4, 5]. The association was shown to be limited to female donors with a history of pregnancy, with an estimated impact of one death per day [5, 6]. In contrast, another large cohort study on this topic did not support these findings [7]. This lack of agreement between studies could be explained by differences among country-specific production methods, patient populations and statistical methods. Although these studies constitute observational research, associations are interpreted causally [8].

Whether 'fresh' or 'old' red blood cell transfusions are better for clinical outcomes has long been a subject of debate, a question complicated by the widely varying ways this contrast has been defined in the transfusion research field. A systematic review and meta-analysis including evidence from randomized controlled trials up to 2017 did not find any benefit of using fresh red blood cell products in hospitalized patients, combining evidence from studies using different definitions of fresh and old red blood cell transfusions [9]. However, the authors could not exclude a small detrimental effect of fresh blood products on mortality, as confidence intervals (CIs) included the potential for 1%–2% benefit and up to 9% harm. Our research group previously investigated the association between storage time and mortality, and found that, when comparing blood products that were stored for <10 days with products stored for >24 days, longer stored blood was associated with a lower risk of mortality (hazard ratio [HR] 0.56, 95% CI 0.32–0.97) [10].

Here, we quantified the association between storage time of the red cell product, donor sex and pregnancy history, and mortality of patients in a large observational cohort in the Netherlands. We hypothesize that mortality will be highest in male patients who received fresh units from ever-pregnant donors.

METHODS

Source database

In this observational cohort study, analyses were performed as a post hoc analysis on a combined cohort that has previously been described in the publications by Middelburg et al. and Caram-Deelder et al. [4, 5, 11]. The cohort includes adult (≥18 years) first-ever transfusion recipients from six hospitals in the Netherlands between 2005 and

2015. Information was collected on donor, product and patient characteristics. Data have been collected for the 'R-FACT study' (CCMO-NL29563.058.09; clinicaltrials.gov: NCT01616329), and the study design for the cohort has been previously described [5, 12, 13]. The statistical analysis plan was specified prior to data analysis and was reviewed and approved by the Scientific Committee of the Department of Clinical Epidemiology, Leiden University Medical Center (LUMC). The database is available at the Department of Clinical Epidemiology at the LUMC. All analyses were performed in Stata [14].

Statistical analysis

We quantified the association between product characteristics and mortality using a Cox proportional hazards model. As can be seen in Figure 1, patients were classified as either having received blood products from *ever-pregnant*, *never-pregnant* or *male* donors, and storage was defined as *fresh* or *old* (Figure 1). Results were stratified by patient sex to be consistent with previous publications, where no association between mortality and previous pregnancy of the donor was observed in female patients [5].

We defined *fresh* products as red cell products stored for 1–10 days and compared those to *old* products, with a storage duration of 11–36 days. Results for exposure defined as 0–7 days for *fresh* products, and *old* products defined as products stored for 8–36 days, are provided in the Supplementary Materials to be consistent with the initial study protocol, which was adapted to allow for more balanced comparison groups.

Exposure categories were further defined according to the sex and pregnancy history of the donors, sourced from the questionnaire about pregnancy status since the last donation at the time of donation at the blood bank. For this study, the patients receiving units donated by never-pregnant female donors act as a 'negative control'. The reference category constitutes old units donated by male donors, unless otherwise specified. We hypothesize that female patients are not affected by blood products from ever-pregnant donors, and thereby view this patient group as a negative control for the research question. HRs were estimated to quantify the risk of mortality per transfused unit from the exposure category, compared with receiving a unit from the reference category.

Reference and exposure were included in the model as the time-varying cumulative number of units. For all analyses, HRs were not presented if a subgroup experienced less than five events [15]. Follow-up in all analyses was limited to a maximum of 15 transfusions to maintain a homogeneous population of patients. Follow-up was accordingly defined as the time from inclusion up until the 16th transfusion (after which follow-up was censored), the first subsequent transfusion from an exposure category other than the categories included in the comparison (after which follow-up was censored), death or administrative censoring due to reaching final hospital follow-up date.

Confounding

As sex and pregnancy history of the donor are unknown, at the time a blood product is requested or transfused by the patient's treating physician—this exposure can be considered randomly distributed. Yet, the storage duration of red blood cell products is known. In neonates and younger patients who require massive transfusion, transfusion of fresh products (i.e., ≤5 days stored) is indicated. Also, irradiation (of predominantly fresh products) is indicated following intra-uterine transfusion, in premature neonates and in patients with severe combined immunodeficiency syndrome [16, 17]. Therefore, in this patient group, short storage duration is associated with poorer clinical outcomes. For this reason, only adult patients were included in the cohort.

Additionally, the probability of exposure with respect to storage is tied to the cumulative number of transfusions received and blood product distribution factors. Based on these considerations, the following confounders for the study research question were identified and included in the models: number of transfusions (time varying); calendar year (time varying); blood group (fixed); donor age (time varying); and hospital (fixed). Additional information about confounders can be found in the Supplemental Methods (Figure S2). A restricted cubic spline with five knots was used for the time-varying cumulative number of transfusions. An interaction term for hospital and cumulative number of transfusions (time varying) was included in the model to account for differences in transfusion practices between hospitals.

Primary analysis

The primary analysis was performed in the cohort of all patients, stratified by recipient sex, and this analysis is referred to as the *full cohort*.

Here, follow-up was limited to the time during which the patient received units from the concerned exposure category and reference category only; the patient's follow-up was censored as soon as they received units from a different exposure category. This means a patient could receive units from both the exposure and reference category without being censored, with this patient then contributing follow-up time to both arms [18]. However, the patient's follow-up is censored upon receiving transfusions from another category. For example, after any other exposure than *male*, *old* and *ever-pregnant fresh* for the comparison *male old* versus *ever-pregnant fresh*, the patients's follow-up time is no longer included (see Figure S1 for visual representation of this example).

Sensitivity analyses

Four sensitivity analyses were performed:

i. **No mixture**: In the full cohort, more than one product category (exposure and reference) can be attributed to a single patient, which we expect might result in the underestimation of the association. Thus, we performed a sensitivity analysis where patients

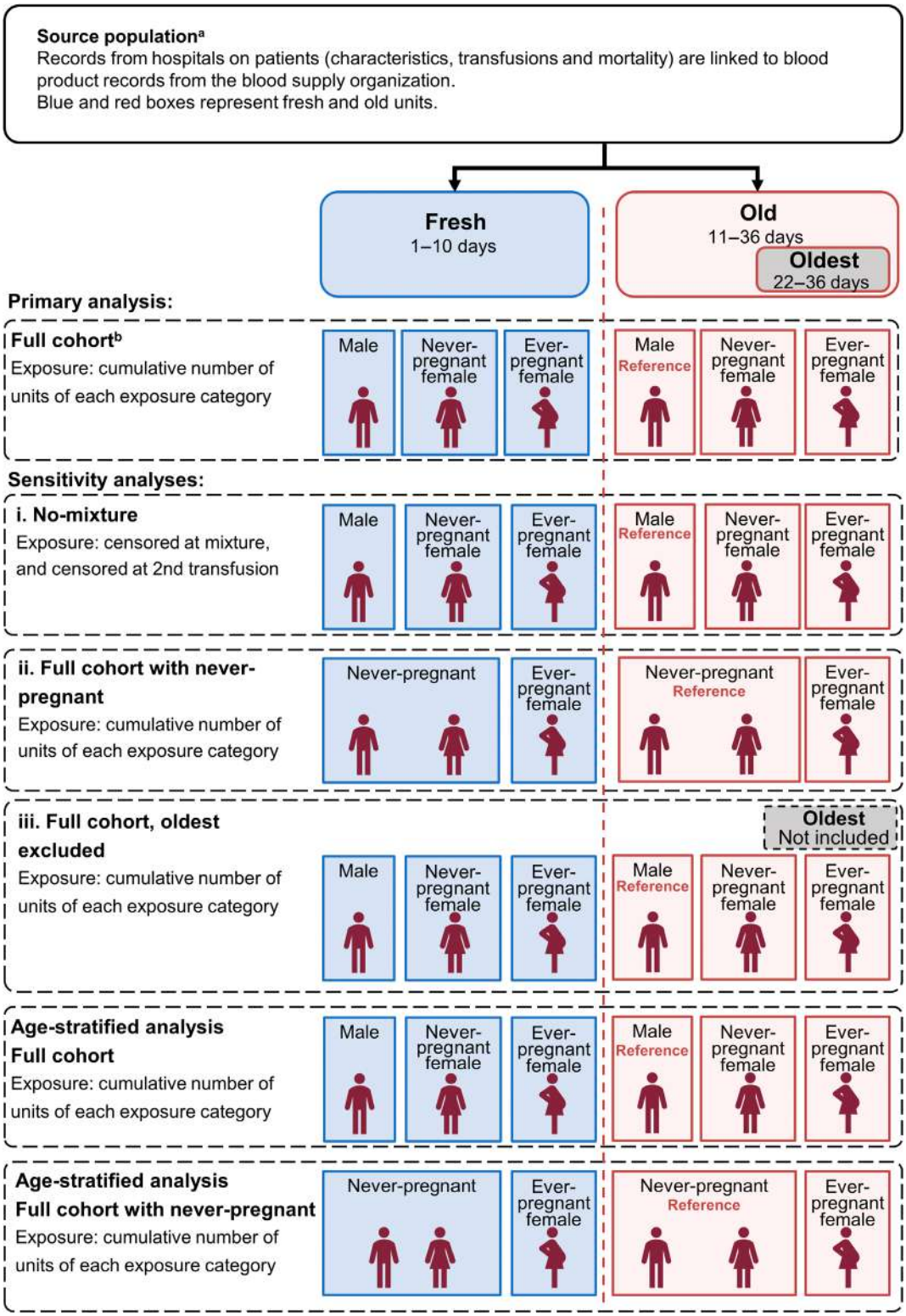

FIGURE 1 The figure contains a visual representation of the different exposure and reference groups for the primary and sensitivity analyses. [a]Products donated by female donors with unknown pregnancy history were not assessed in this analysis. [b]For sensitivity analysis (iv), the same exposure and reference groups were used.

were censored upon receiving a transfusion from a different exposure category (*no mixture*) and where patients who received multiple transfusions were censored at their second transfusion (*single transfusion*). Although censoring at the moment a product from a different exposure category is received is a type of informative censoring, a no-mixture analysis can be used to study the effect of transfusion exposures when patients receive multiple transfusions[18].

ii. **Full cohort with reference group of never-pregnant donors**: To increase the subgroup size, within the full cohort, an alternative reference category was introduced by combining all male and never-pregnant female donors into the category *never-pregnant donors*. The reference category for this analysis therefore constitutes both female and male donor products.

iii. **Full cohort oldest excluded**: This sensitivity analysis was performed in the full cohort, and a comparison was made between fresh (less than or equal to 10 days of storage) and intermediate (between 11 and 21 days of storage) products. The cut-off of 21 days was chosen to rule out a possible detrimental effect of long storage, which could then have concealed associations in our comparisons. These storage-induced blood product changes, such as haemolysis, oxidative stress and micro-vesicle formation, are collectively called the red blood cell storage lesion [19]. Units in the fourth and last week of storage are still generally considered safe, but evidence for the safety of end-of-storage (stored for 28–36 days) red blood cell units is limited, as is evidence for use in vulnerable patient populations [20–22].

iv. **No mixture first exposure only**: This sensitivity analysis was performed in the no-mixture cohort and only the first exposure was used, after which the complete follow-up was included in the analysis. Patients for whom it was not possible to determine which transfusion was their first (i.e., patients who received multiple transfusions on their first transfusion day) were excluded. This analysis was performed to assess potential misspecification of the models that censored patients upon receiving multiple transfusions.

Age-stratified analysis

The *primary analysis* and *sensitivity analysis* (ii) were stratified by patient sex and age to study the effect measure modification by age [5, 7]. Age categories were defined as 18–50, 51–70 and over 70 years of age. Effect measure modification was formally quantified by adding an interaction term for patient age to the final model (p-value for interaction trend between patient age and exposure) as described previously [5].

RESULTS

Population

Patient and transfusion characteristics for three cohorts included in the primary and sensitivity analyses (*full cohort*, *no mixture* and *single transfusion*) are presented, stratified by recipient sex (Table 1). In total, 42,456 patients contributed 88,538 person-years. From the total population, 53% ($n = 22,412$) were female patients. During follow-up, 13,948 (33%) patients died, with a median follow-up of 405 days (IQR 36–1269) for the total population. The median age of all patients was 68 (IQR 55–77) years. The study population received a total of 127,687 transfusions, with a median of 2 transfusions per patient (IQR 2–4). The large majority of red cell products were stored for >10 days. When the storage cut-off of 7 days was used, fewer patients could be included for the product categories ever-pregnant, *fresh*; never-pregnant, *fresh*; and male, *fresh* (see Table S1).

Primary analysis

A total of 42,456 patients were included in this analysis—22,412 female and 20,044 male patients (Figure 2). No statistically significant associations between exposure categories and mortality were observed among male patients. Male patients receiving fresh blood from ever-pregnant donors may have had higher mortality after transfusions, but this association was not statistically significant (HR 1.39, 95% CI 0.97–1.99). No association was present when the units donated by ever-pregnant female donors were old (HR 1.05, 95% CI 0.99–1.12).

All HRs for female patients were around or below 1, suggesting a smaller risk when compared to the reference category of old male units. Receiving fresh units from ever-pregnant donors was not associated with mortality in female patients (HR 0.83, 95% CI 0.52–1.30). For female patients, receiving fresh male units was associated with a small survival benefit (HR 0.86, 95% CI 0.79–0.93).

Due to small sample size, the HR for exposure to ever-pregnant units stored for a short duration could not be shown when the cut-off of 7 days was used in both male and female patients (Table S2).

Sensitivity analyses

We only present sensitivity analyses with implications for the interpretation of the primary analysis here, so refer to the Supplementary Materials for further information (Tables S3 and S4).

In sensitivity analysis (iv) (**no mixture, no censoring**, Table S3), which is the analysis where follow-up was not censored, results differed from the primary analysis in both direction and magnitude of the effect of exposure. The HR was 0.87 (95% CI 0.54–1.42) when comparing fresh ever-pregnant donor red blood cell units with the reference group (male, stored >10 days) for male patients. For female patients, the HR was 0.78 (95% CI 0.47–1.28) for ever-pregnant donor red blood cell units that were fresh compared to units that were stored >10 days and donated by male donors.

Age-stratified analysis

For the comparisons stratified by age, for male patients, the number of included patients was small (Table 2). Therefore, the analysis was only carried out for the **full cohort** and the full cohort with the combined category of male donors and never-pregnant female donors **(full cohort with never pregnant)**.

TABLE 1 Patient and transfusion characteristics.

	Full cohort		No-donor mixture cohort[a]		Single-transfusion cohort[b]	
	Male patients	Female patients	Male patients	Female patients	Male patients	Female patients
Characteristics						
Number of patients	20,044	22,412	13,319	14,925	6473	6978
Number of deaths (%)	7465 (37%)	6483 (29%)	2155 (16%)	2096 (14%)	655 (10%)	604 (9%)
Follow-up, median (IQR), days[c]	282 (22–1098)	514 (59–1400)	91 (5–937)	309 (11–1303)	8 (2–547)	15 (2–744)
Person-time, sum in years	37,037	51,501	21,561	30,746	7519	9546
Age of patients, median (IQR), years	68 (58–76)	68 (52–79)	69 (59–77)	69 (54–79)	70 (60–77)	71 (57–80)
18–50 years	7889 (13%)	5202 (23%)	1665 (13%)	3276 (22%)	702 (11%)	1309 (19%)
51–70 years	15,877 (44%)	7097 (32%)	5762 (43%)	4654 (31%)	2660 (41%)	2148 (31%)
≥71 years	18,690 (43%)	10,113 (45%)	5892 (44%)	6995 (47%)	3111 (48%)	3521 (50%)
Transfusions of red blood cell units per patient, median (IQR)	2 (2–4)	2 (2–3)	2 (1–2)	2 (1–2)	1 (1–1)	1 (1–1)
Red blood cells transfusions, *n* (%)						
Total	63,837	63,850	26,032	28,626	6473	6978
Female donor, never-pregnant, fresh	581 (1%)	632 (1%)	73 (1%)	120 (1%)	48 (1%)	86 (1%)
Female donor, never-pregnant, old	8646 (14%)	8380 (13%)	1378 (5%)	1419 (5%)	863 (13%)	860 (12%)
Female donor, ever-pregnant, fresh	601 (1%)	665 (1%)	82 (1%)	115 (1%)	49 (1%)	75 (1%)
Female donor, ever-pregnant, old	8850 (14%)	8369 (13%)	1463 (6%)	1461 (5%)	903 (14%)	876 (13%)
Male donor, fresh	3501 (5%)	3852 (6%)	1416 (5%)	1736 (6%)	286 (4%)	539 (8%)
Male donor, old	41,658 (65%)	41,952 (66%)	21,620 (83%)	23,775 (83%)	4324 (67%)	4542 (65%)

Note: Storage time definition: *fresh* refers to storage from 0 to 10 days; and *old* refers to storage from 11 to 36 days.
Abbreviation: IQR, interquartile range.
[a]Consists of all the follow-up time during which patients either received all their red blood cell transfusions exclusively from one exposure category: male donors (fresh or old) and female donors without a history of pregnancy (never-pregnant donors, fresh or old); or from female donors with a history of pregnancy (ever-pregnant donors, fresh or old).
[b]Consists of patients with only a single red blood cell transfusion during the period in which they were followed up. Follow-up time was censored at the time this inclusion criterion was violated.
[c]Median follow-up time is defined as the longest time any patient is in one of the comparisons. Exposure categories are as follows: female donors without a history of pregnancy (never-pregnant donors, fresh or old), female donors with a history of pregnancy (ever-pregnant donors, fresh or old) and male donors (fresh or old).

For the full cohort analysis, the HR for the age group of 18–50 years was not shown due to the low number of events, and the HR for the age group of 51–70 years was 1.36 (95% CI 0.77–2.40) for the ever-pregnant, fresh in comparison with male, old. The HR for the age group of 71 years and older could not be computed due to zero events in this age group after exposure to fresh red blood cell units from ever-pregnant donors. The *p*-value for the trend for the interaction between age and exposure was 0.316. The low event numbers suggest considerable uncertainty regarding the interaction between age and exposure. The interaction between age and exposure was significant in other comparisons (never-pregnant female, old; never-pregnant female, old; and male, fresh).

The results for fresh ever-pregnant units, now compared to the reference of the combined category of male donors and never-pregnant female donors (stored for >10 days; old) for male patients, were similar to those presented above (Table 2; 18–50 years, HR not shown; 51–70 years, HR 1.38, 95% CI 0.85–2.23; and 70 and older, HR 1.32, 95% CI 0.82–2.14), with no significant interaction with patient age ($p = 0.179$).

No noteworthy associations were present between product characteristics and mortality in female patients in the stratified analysis, with effect sizes around 1 for all comparisons, and small group sizes (Table S5).

Results for the storage cut-off of 7 days can be found in the Tables S6 and S7.

DISCUSSION

In this study, a large database of patient and transfusion data was used for an in-depth analysis of multiple aspects of the 'transfusion continuum', namely sex and pregnancy history of the donor and storage of blood products [23]. Although these parameters have been studied in great detail separately, blood product storage has not yet been studied together with sex of the donor and whether the donor

Full cohort[a]	Deaths/Recipients (exposure)	Deaths/Recipients (reference)[b]	HR per unit[c]
Male patients			
Ever-pregnant female old	922/4560	2551/13,078	1.05 (0.99–1.12)
Never-pregnant female old	908/4420	2561/13,025	1.05 (0.98–1.12)
Male fresh	174/1049	1840/10,506	0.93 (0.86–1.01)
Ever-pregnant female fresh	18/101	1783/10,232	1.39 (0.97–1.99)
Never-pregnant female fresh	9/93	1779/10,239	0.61 (0.33–1.11)
Female patients			
Ever-pregnant female old	784/4664	2424/14,569	0.99 (0.92–1.06)
Never-pregnant female old	820/4759	2461/14,655	0.95 (0.89–1.02)
Male fresh	187/1410	1846/11,905	0.86 (0.79–0.93)
Ever-pregnant female fresh	13/140	1764/11,545	0.83 (0.52–1.30)
Never-pregnant female fresh	11/150	1760/11,544	0.68 (0.42–1.11)

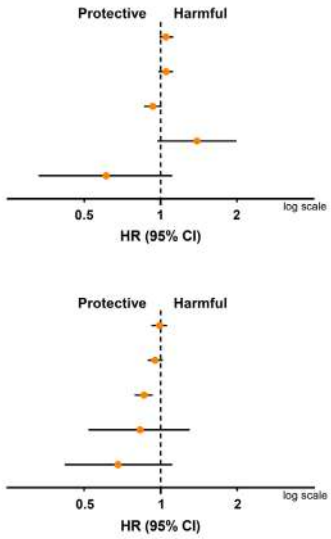

FIGURE 2 Forest plot containing the hazard ratios (HRs) from the primary analysis, stratified by sex. Reference category consists of patients exposed to units donated by male donors, stored for >10 days (old). HRs are shown as orange dots, along with 95% confidence intervals. [a]All models adjusted for calendar year, blood group (ABO-RhD), age of donor, hospital, cumulative number of transfusions and an interaction term for hospital and cumulative number of transfusions. [b]Recipients in the full cohort could receive mixed blood from both the exposure of interest and the reference category; therefore, the number of recipients receiving blood from male donors (old) is different for the different comparisons (see also Supplemental Methods). [c]HRs per transfused unit compared with receiving a stored unit from a male blood donor (reference group: male, old).

was previously pregnant. The findings did not consistently support the notion that storage plays a role in modifying the association between donor characteristics and patient survival.

Recent publications have rightly criticized aspects of previous work investigating the effect of sex (and pregnancy history) of the donor, specifically that Cox regression may not be appropriate [24, 25]. Bias due to treatment-confounder feedback could lead to biased HRs obtained with Cox regression. Female donors have lower haemoglobin concentrations and this could lead to more, or earlier, additional transfusions. This issue could be further exacerbated by looking at 'fresh' and 'older' units, as storage also affects red blood cell viability and subsequent haemoglobin measurements. However, the small subgroup sizes for the various storage contrasts did not allow for data-intensive approaches like g-methods. Alternatively, we performed an analysis in which patients were studied according to their first transfusion independent of additional transfusions, thereby avoiding the problem of treatment-confounder feedback. The results of the latter analysis did not corroborate the results from the primary analysis, suggesting that the observed association did not reflect a causal effect.

Furthermore, we did not have access to the indication of the transfusion or disease severity of the patient. The indication of the transfusion is associated with both the number of transfusions a patient will receive and the risk of mortality, but is not directly associated with the probability of receiving transfusions with certain donor and product characteristics. However, transfusion indication could still be an effect modifier, with subpopulations of patients potentially being 'sensitive' to an effect of exposure. Exploring outcomes of subgroups of patients could be a way to help us understand the biological mechanisms of harm when an effect is present [26, 27]. It is also important to note that patients who are transfused at a young age are inherently different from adults with regard to blood product distribution policy and prognosis. For neonates and young children, units stored shorter than 5 days are prescribed to decrease the exposure to blood products with an increased potassium and decreased 2,3-diphosphoglycerate (2,3-DPG) content. Because we do not know which patients were prescribed these fresh units, all children were excluded from the study (see Supplemental Methods) [16]. Importantly, blood products are frequently irradiated and subsequently administered in the first week of storage [16]. The inclusion of irradiated products potentially biases the effect estimates because irradiated products are more likely to be prescribed to patients with a poor prognosis. These products are not only requested for preterm neonates but are also prescribed for other immunologically impaired patients. We postulated previously that the associations between transfusion of products from ever-pregnant donors and mortality are mediated by a cellular component [28]. If lymphocyte proliferation-dependent effects are inhibited by irradiation in a subset of products included in this study, the estimates could be an underestimation of the effect of exposure, although these patients tend to have a poor prognosis. It is therefore difficult to predict the direction and magnitude of confounding by the request of irradiated products.

TABLE 2 Mortality hazard ratio (HR) of male patients exposed to fresh or old red blood cell transfusions from female ever-pregnant donors versus male donors in the full cohort, stratified by patient age.[a]

Donor category	18–50 years			51–70 years			≥71 years			*p*-value for interaction[c]
	Deaths	Recipients	HR (95% CI)[b]	Deaths	Recipients	HR (95% CI)[b]	Deaths	Recipients	HR (95% CI)[b]	
Full cohort										
Male, old (reference)[d]	161	1632	1 (reference)	949	5631	1 (reference)	1441	5815	1 (reference)	0.000
Ever-pregnant female, old	73	572	1.38 (1.09–1.74)	363	1996	1.02 (0.93–1.13)	486	1992	1.02 (0.93–1.12)	
Male, old (reference)[d]	160	1659	1 (reference)	922	5603	1 (reference)	1479	5763	1 (reference)	0.000
Never-pregnant female, old	62	618	0.97 (0.75–1.26)	327	1919	1.01 (0.90–1.12)	519	1883	1.08 (0.99–1.18)	
Male, old (reference)[d]	100	1244	1 (reference)	642	4393	1 (reference)	1041	4595	1 (reference)	0.316
Ever-pregnant female, fresh	2	16	-	7	45	1.36 (0.77–2.40)	9	38	1.36 (0.81–2.27)	
Male, old (reference)[d]	100	1245	1 (reference)	639	4394	1 (reference)	1040	4600	1 (reference)	0.069
Never-pregnant female, fresh	1	19	-	3	46	-	5	37	1.01 (0.46–2.25)	
Male, old (reference)[d]	103	1294	1 (reference)	674	4531	1 (reference)	1063	4681	1 (reference)	0.000
Male, fresh	12	193	0.94 (0.68–1.32)	87	512	0.96 (0.85–1.07)	75	344	0.96 (0.85–1.09)	
Full cohort with never pregnant										
Never pregnant, old (reference)[d]	273	2320	1 (reference)	1507	7691	1 (reference)	2245	7739	1 (reference)	0.000
Ever-pregnant female, old	123	845	1.18 (0.99–1.41)	594	2758	1.03 (0.95–1.11)	771	2650	1.00 (0.93–1.07)	
Never pregnant, old (reference)[d]	163	1756	1 (reference)	972	5988	1 (reference)	1562	6102	1 (reference)	0.179
Ever-pregnant female, fresh	3	23	-	10	54	1.38 (0.85–2.23)	11	42	1.32 (0.82–2.14)	
Never pregnant, old (reference)[d]	174	1835	1 (reference)	1040	6208	1 (reference)	1606	6242	1 (reference)	0.000
Never-pregnant female, fresh	22	275	0.93 (0.74–1.17)	138	724	0.97 (0.89–1.06)	109	489	0.91 (0.83–0.99)	

Note: Storage time definition: *fresh* refers to storage from 0 to 10 days; and *old* refers to storage from 11 to 36 days.

Abbreviation: CI, confidence interval.

[a]All models are adjusted for calendar year, blood group (ABO-RhD), hospital, age of donor, cumulative number of transfusions, and an interaction term for hospital and cumulative number of transfusions.

[b]HRs per transfused unit compared with receiving a unit from the reference category.

[c]For the trend in interaction across the continuous-variable patient age.

[d]Recipients in the full cohort could receive mixed blood from both the exposure of interest and the reference category; therefore, the number of recipients receiving blood from male donors (old) or never-pregnant donors (old) is different for the different comparisons (see also Supplemental Methods).

Assessing the exposure of interest in context with other conditions where an effect should be absent (negative controls, e.g., never-pregnant exposure or female patients) alleviates this relevant concern. Lastly, as the data collection for this study spanned several years, minor changes were implemented regarding blood product processing and transfusion guidelines during the study period [29, 30]. However, during this period, no changes were made to leucoreduction filter types.

In summary, blood products from ever-pregnant donors stored for a short duration were associated with increased mortality in male patients in the primary analysis of this study, but this was not corroborated in sensitivity analyses. The validity of studies on donor- and blood-product characteristics relies on strong assumptions about the data, which should be thoroughly verified, especially when treatment-confounder feedback is suspected.

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S.J.V., C.C.-D., R.A.M. and J.G.v.d.B. designed the study. D.E., K.M.K.d.V., D.v.d.K., M.J.W., N.C.V.P., F.H., J.J.Z. and J.G.v.d.B. collected the data. S.J.V., C.C.-D. and J.G.v.d.B. analysed and interpreted the data and wrote the manuscript. D.d.K. and L.M.G.v.d.W. provided subject-specific content knowledge. All authors revised and approved the final manuscript. We thank the Scientific Committee at the Department of Clinical Epidemiology of the LUMC for their methodological support. A conference abstract has previously been published on the same dataset as described here [11].

CONFLICT OF INTEREST STATEMENT

J.J.Z. is in the scientific advisory council of Novartis/Amgen/Sanofi and received a speaker's fee. The other authors declare that they have no conflict of interest relevant to the work presented in this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available for inspection upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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ORIGINAL ARTICLE

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The impact of an antibody investigation algorithm emphasizing specificity on reducing potential false-positive warm autoantibody detection at a Canadian tertiary care centre

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Abstract

Background and Objectives: To reduce potential false-positive warm autoantibody (WAA) by solid-phase red cell adherence assay (SPRCA), our centre implemented a new antibody investigation algorithm (AIA) by classifying cases with panreactive SPRCA but negative saline-indirect antiglobulin test as 'antibody of undetermined significance' (AUS) after excluding clinically significant antibodies. We assessed the effects of the new AIA and subsequent alloantibody formation in patients with AUS.

Materials and Methods: Samples from patients with positive SPRCA screens between 1 September 2017 and 31 August 2021 were selected for the study. Frequencies of antibodies classified by the old and new AIAs were compared using Fisher's exact test. Patient demographics, transfusion history and antibody formation in cases of AUS were collected.

Results: A significant reduction in potential WAA frequencies from 127/1167 (11%) to 53/854 (6%) was observed ($p < 0.001$) when compared between the old and new AIAs among 2021 positive SPRCA antibody screens. While no patients with AUS later transitioned to potential WAA using the new AIA, four patients developed alloantibodies, including anti-E, anti-C, both anti-C and anti-E, and anti-Wr^a.

Conclusion: A significant reduction in the frequencies of potential false-positive WAA detection at our centre was observed after implementing the new AIA, leading to less resource and phenotypically matched red blood cell (RBC) use. Some patients still developed subsequent RBC alloimmunization, so clinically relevant alloantibodies should be carefully excluded before determining AUS, taking forming or evanescent antibodies into consideration.

Keywords

antibody investigation, antibody of undetermined significance, AUS, panreactivity, saline-indirect antiglobulin test, solid-phase red cell adherence assay, warm autoantibody

Highlights

- Solid-phase red cell adherence assay (SPRCA) has increased red blood cell (RBC) antibody detection sensitivity that leads, like other sensitive methods, to non-specific reactivity detection and increasing costs by prompting further investigation and potentially delaying RBC transfusion.
- A significant reduction in frequencies of false-positive cases defined as potential warm autoantibodies was observed using a new antibody investigation algorithm using a saline-indirect antiglobulin test (SIAT). Patients with panreactive SPRCA but negative SIAT in the new antibody investigation algorithm were classified as having 'antibody of undetermined significance' (AUS).
- When determining AUS, clinically significant alloantibodies should be carefully excluded to avoid missing low-titre antibodies as seen in some patients in this study.

INTRODUCTION

The solid-phase red cell adherence assay (SPRCA) was developed to improve sensitivity in red blood cell (RBC) antibody detection and identification using standardized and automated technology. With the higher sensitivity, non-specific reactions such as panreactivity are frequently observed in SPRCA testing [1, 2]. This higher sensitivity is also seen in other automated methods, such as gel agglutination column methods [3]. Reactivity with all RBCs tested or panreactivity by SPRCA can be due to warm autoantibody (WAA), alloantibody to high prevalence antigen, multiple alloantibodies to common antigens, therapeutic substances—anti-CD38 monoclonal antibody, and other non-specific reactions. Panreactivity from WAA commonly interferes with RBC antibody detection, causing incompatible crossmatch and obscuring underlying alloantibodies, possibly increasing the risk of haemolytic transfusion reaction due to incompatible RBC transfusion. Further specialized testing, such as RBC adsorption and recipient RBC antigen phenotyping or genotyping, is often required to exclude alloantibodies and provide antigen-matched RBCs for patients. As this specialized testing may only be available at either external reference immunohematology laboratories or large transfusion medicine services, RBC provision in patients with possible WAA is often delayed. Overdiagnosis of WAA may also lead to patients being inappropriately treated with immunomodulatory therapy if associated with anaemia, given a false diagnosis of warm autoimmune haemolytic anaemia.

While accurate detection of WAA is important, the differential diagnosis of panreactive antibody screen includes an antibody of undetermined significance (AUS). AUS is commonly used to report unexplained reactions when antibodies against specified RBC antigens have been ruled out. AUS interferes with pre-transfusion testing by reacting similar to WAA, but unlike WAA detection, it is unlikely to be clinically significant to the patient and does not warrant further investigation. Chemicals in testing reagents, enhancement media, drugs and other non-RBC antibodies producing anomalous agglutination are responsible for AUS. Liu et al. described a high frequency of AUS at 18% using gel technology, but the data on AUS frequency by the SPRCA platform are still lacking [4].

Vancouver General Hospital (VGH) is a quaternary care centre in Vancouver, British Columbia, Canada, which performs approximately 46,000 pre-transfusion tests and transfuses 25,000 RBC units per annum. VGH provides multiple subspecialty medical services, including haematology–oncology and bone marrow transplant, subspecialty surgical services, including cardiac surgery and organ transplantation, and an adult Level 1 trauma service. In modern transfusion medicine services that serve a large patient population, a high-throughput automated RBC antibody screening method is required to serve patients promptly. After SPRCA was implemented as a primary RBC antibody screening and identification platform in 2014, panreactive antibody screens were frequently observed, leading to a high volume of further antibody investigations to exclude RBC alloantibodies.

To distinguish AUS from potential WAA, saline-indirect antiglobulin test (SIAT) via manual tube testing was introduced into the antibody investigation algorithm (AIA) to increase the specificity of findings. SIAT determines the reactivity of the antibodies at 37°C in saline without enhancement media, whereas a low ionic strength solution (LISS) is used in SPRCA. We hypothesize that panreactivity present in SPRCA alone, but not in SIAT, should be considered false-positive reactivity or AUS. After implementing the new AIA integrating the SIAT results, the frequencies of potential WAA, AUS and other RBC antibodies at our centre were evaluated, comparing the old and new AIAs. Historical and subsequent antibody results and transfusion history of patients with AUS were also reviewed, focusing on RBC alloantibody development.

SAMPLES AND METHODS

A retrospective observational assessment examining frequencies of RBC antibody results before and after implementation of the old and new AIAs between 1 September 2017 and 31 August 2021 was completed, with the implementation occurring on 1 September 2019.

In patients with AUS, the following variables were collected: patient demographics, previous and subsequent antibody investigation results, auto- and alloantibody formation, transfusion history and direct antiglobulin test (DAT) results. A review by the University of

British Columbia Research Ethics Board determined that this was a quality improvement initiative involving antibody investigation findings that may not be necessarily generalizable to other centres or patient populations. Only demographic and laboratory findings were utilized without patient outcomes.

Institutional antibody screening and identification methods

RBC antibody screening was performed using one of two SPRCA automated instruments (Echo and Neo; Immucor, Norcross, GA). Both instruments utilized the two- or three-cell antibody detection test plates (Capture-R Ready-Screen, Immucor). The automated instruments interpreted and graded the antibody results, and were confirmed by medical laboratory technologists as negative, weak+, 1+, 2+, 3+ or 4+. Samples positive at the screening by SPRCA (weak+ to 4+) were typically re-tested using a trio panel PEG-IAT additive tube method (Gamma PEG, Immucor).

After a confirmed positive antibody screen by PEG-IAT, further antibody identifications were performed using the SPRCA 14-cell panels (Capture-R Ready-ID, Capture-R Ready-ID Extend I and Extend II; Immucor). If needed, the testing was supplemented by a PEG additive tube method using the previously described grading strategy. We used double-dose antigen-positive RBCs for the exclusion of clinically significant antibodies.

All samples positive at screening by SPRCA and confirmed by PEG-IAT were investigated further by SIAT screening, DAT using polyspecific antibodies followed by anti-IgG and anti-C3 if indicated. When DAT was positive in patients who received RBC transfusion in the past 90 days, an eluate from patient RBCs was tested with an antiglobulin (Gamma ELU-KIT II, Rapid Acid Elution, Immucor).

For patients with a known history of anti-CD38 monoclonal antibody therapy within the past 6 months and the antibody screen by SPRCA or PEG-IAT showed panreactivity, no further investigation would be required if the phenotypically matched RBC units had been transfused. However, if the patient was transfused with non-phenotypically matched RBC units, the antibody screen would be repeated monthly, and the specimen would be submitted to the reference laboratory for further investigation if the screen was positive.

Samples with panreactive SPRCA and PEG-IAT screens were assessed for cold agglutinin by step (1), cold screen, where the patient's plasma and three-screen cells were incubated at room temperature for 30 min. In cases of the positive cold screen, step (2), a pre-warmed PEG screen, would be performed for confirmation [5].

Development of our new institutional AIA

The general principle of developing our new AIA was to add the SIAT to assess the reactivity of antibodies at 37°C without adding enhancement media [6]. SIAT is performed to eliminate non-specific reactions that may have led to false-positive determinations of potential WAA previously and to rule out clinically significant alloantibodies on the antibody panel, starting by using three screening cells in SIAT after a positive PEG-IAT antibody screen.

Before 1 September 2019, a panreactive SPRCA on the antibody panel and confirmed positive PEG-IAT screen was considered potential WAA, regardless of DAT, elution study and SIAT findings. The definition of panreactive screen by SPRCA, PEG-IAT and SIAT shows no difference in the reactivity strength on each test cell greater than 1+ to exclude potential specificity. This algorithm resulted in frequent cases with possible WAA. From 1 September 2019, SIAT results were integrated into the new RBC AIA to distinguish AUS from potential WAA. In the new algorithm, panreactivity in SPRCA antibody panels where clinically significant antibodies could be ruled out with negative reactions on SIAT is considered AUS rather than potential WAA. Reinvestigation with a complete antibody identification will be performed if there is an increase in reactivity strength greater than 1+ compared to the previous results.

If there is a positive reaction that was not panreactive, the antibody is considered an unidentified antibody to denote a potentially clinically significant antibody may exist, but specific clinically significant RBC alloantibodies on the panels and autoantibodies have been ruled out. Reinvestigation will be performed on the next admission or if serological reactions change. If the reaction is panreactive with a positive SIAT, regardless of the DAT results, it is considered a potential WAA. We do not routinely refer all the specimens with potential WAA to the immunohematology reference laboratory to exclude the alloantibody of high-prevalence antigen but opt to perform RBC phenotyping locally and provide extended phenotypically matched RBC units to the patient, considering the referral process and the turnaround time generally takes approximately 24–48 h, which is impractical for our service.

A standardized approach for providing RBCs and reinvestigating potential WAA was also adopted. As fewer cases were predicted to have potentially false-positive WAA, a policy to provide patients with potential WAA prophylactic extended phenotype-matched RBC units (D, C, c, E, e, K, Jk^a, Jk^b, Fy^a, Fy^b, S, s) rather than units that were phenotypically matched for Rh and Kell only (D, C, c, E, e, K) was discussed and agreed upon with the jurisdictional Canadian Blood Services' blood supplier medical team (Canadian Blood Services acts as the national blood supplier in Canada except for Quebec). Reinvestigation will then be performed for potential WAA every 3 months if the patient has been transfused with extended phenotype-matched RBC unit(s) or monthly if the patient has been transfused without or partially extended phenotype-matched RBC unit(s). Given that our centre does not perform autoadsorption or alloadsorption, further investigations would be referred to the immunohaematology reference laboratory if the patient received non-phenotypically matched RBCs. For AUS, reinvestigation will be performed every 3 months if the patient has been transfused. Crossmatch-compatible RBC units by any of the following methods, SPRCA, PEG–indirect antiglobulin test (PEG-IAT) and SIAT, will be provided for patients with unidentified antibodies or AUS; if no crossmatch-compatible units can be found, least-incompatible units will be provided with consultation between

the transfusion medicine and clinical service. Figure 1 illustrates the key comparison between old and new AIA.

Statistical analysis

R-software (version 4.1.1) was used for statistical analysis [7]. Count data were analysed using Fisher's exact probability method, and continuous variables were analysed using the Mann–Whitney *U* test. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 2485 antibody investigation tests were performed among 1926 patients in the 4-year period. There were 464 repeat tests performed on the same patients using the same algorithm, which was considered duplications. After removing duplicate test results, 2021 antibody results remained in the analysis, with 1167 and 854 obtained using the old and new AIA, respectively.

In this study, PEG-IAT confirmed 1135 alloantibodies (56%), 195 unidentified antibodies (10%), 180 possible WAA (9%), 112 cold agglutinins (5%), 93 passive anti-D (4%), 57 AUS (3%), 37 combined

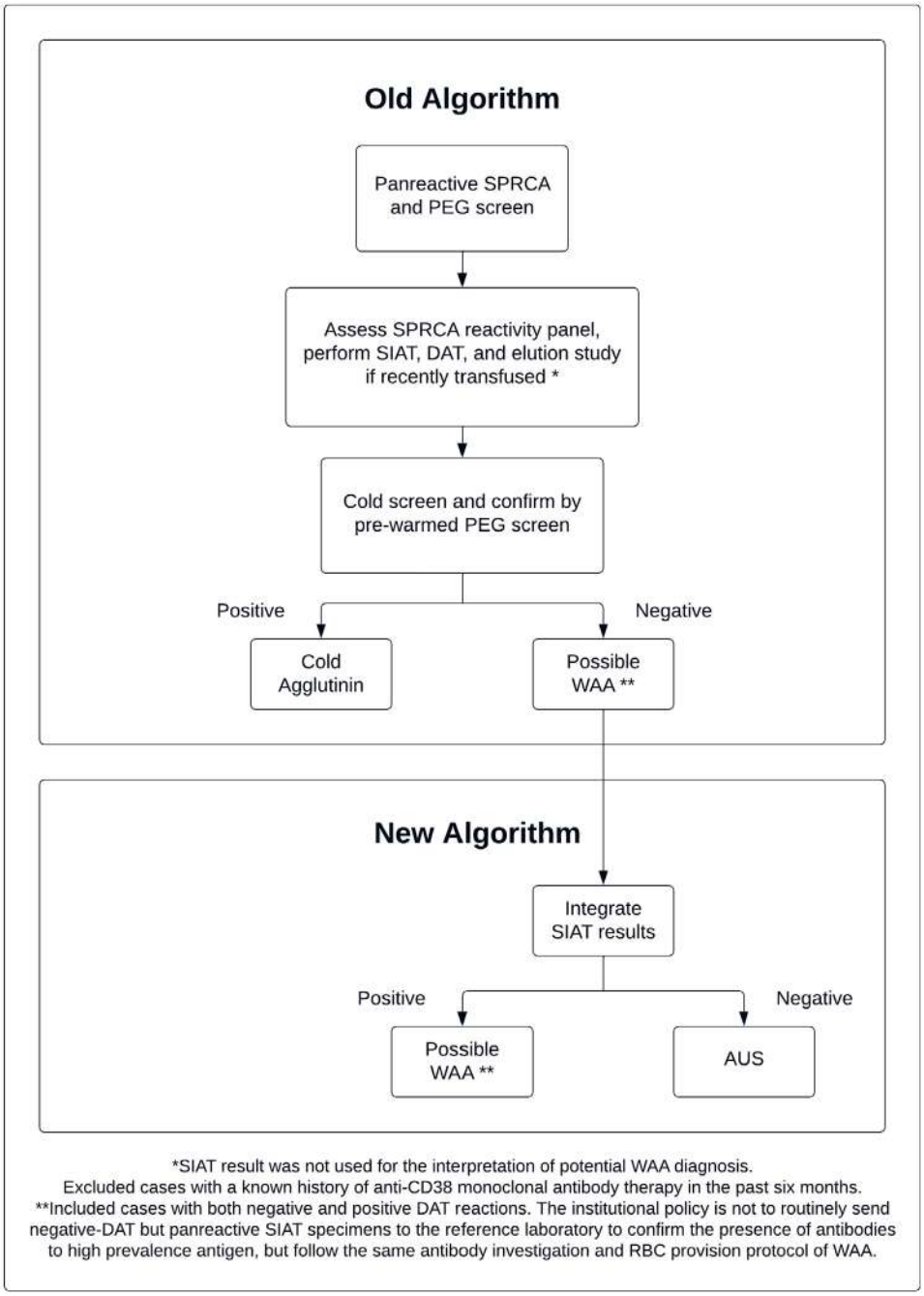

FIGURE 1 Flowchart of the old and new antibody investigation algorithms and their relationship with workflow.

possible auto- and alloantibodies (2%), 16 drug-related antibodies (1%) and 196 negative tests (10%).

Among 1135 alloantibodies, anti-E was the most prevalent antibody detected in 409 patients (36%), followed by anti-K in 206 (18%), anti-Jk^a in 108 (10%), anti-D in 100 (9%) and anti-c in 73 cases (6%). The remaining alloantibodies, including anti-C, anti-C^w, anti-Jk^b, anti-Fy^a, anti-Fy^b, anti-S, anti-M, anti-Kp^a and anti-Wr^a, were less common with a prevalence of under 5%.

Frequencies of the categorized antibodies affected by the new AIA compared before and after implementing the new AIA with the odds ratios and *p*-values are included in Table 1. A significant reduction of potential WAA frequencies from 127 (11%) to 53 tests (6%) was observed (*p*-value <0.001). Frequencies of passive anti-D were also significantly reduced from 68 (6%) to 25 (3%) after implementing the new AIA ($p = 0.002$). There was no significant difference in frequencies of alloantibodies (648 [56%] vs. 487 [57%]; $p = 0.525$), unidentified antibodies (109 [9%] vs. 86 [10%]; $p = 0.594$), combined possible auto- and alloantibodies (26 [2%] vs. 11 [1%]; $p = 0.132$), drug-related antibodies (9 [1%] vs. 7 [1%]; $p = 1.000$) and cold agglutinin (67 [6%] vs. 45 [5%]; $p = 0.694$) using the old and new AIA.

Laboratory features of patients with AUS

Among 57 AUS cases, other RBC antibodies were previously identified in 21 patients as shown in Table 2, including 14 with potential WAA, 3 with combined cold agglutinin and potential WAA, 2 with cold agglutinin and 2 with unidentified antibodies. Twenty-four patients with AUS previously received RBC transfusions. While 16 patients had confirmed transfusion records with a median interval of the most recent transfusion of 4 days (IQR 4–127), 8 patients recalled RBC transfusions but with uncertain dates or documentation. PEG-IAT showed panreactivity with +w reaction in 43 (75%) and 1 to 2+ in 14 patients (25%). Polyspecific DAT was positive in 40 patients (70%), showing +w to 2+ and 3 to 4+ in 29 and 11 cases, respectively. IgG was positive in 32 cases, with +w to 2+ and 3 to 4+ in 29 and 3 cases, respectively. C3d was +w to 2+ in 16 patients. Elution studies were performed in 11 cases, showing panreactivity in 10 cases and a negative reaction in 1 case, respectively.

While no patients with AUS later transitioned to potential WAA using the new AIA, four patients developed specific RBC alloantibodies, anti-E, anti-C, both anti-C and anti-E and anti-Wr^a, at a median time of 3 months post-AUS (IQR 0.9–4.8) with detailed transfusion histories described in Table 3.

DISCUSSION

SPRCA detects RBC antibodies by capturing their adherence to lysed RBC membranes immobilized on the polystyrene micro-wells [8]. Despite its high sensitivity, excellent throughput and less manual work for the initial antibody screen and panel, SPRCA appears to produce more frequent non-specific reactivity than tube methods. Dwyre et al. reported a 34.5% false-positive rate using SPRCA, Capture-R, to detect RBC antibodies compared to PEG methods as a gold standard [9]. The higher false positives led to a higher cost of additional investigations. Bunker et al. described 10.9% of inconclusive results from positive manual SPRCA over negative PEG. While manual SPRCA screening identified the most clinically significant RBC alloantibodies with 80.7% sensitivity and 96.7% specificity at the lowest reagent and labour cost, sequential identification methods using the automated and manual SPRCA of positive screens followed by PEG also had the lowest hands-on time [10]. The differential sensitivity between SPRCA or PEG-IAT and SIAT can also impact the WAA-positive rates because of enhancement media used in addition to automated versus manual tube testing, such as LISS in SPRCA and PEG.

When specificity of the autoantibody exists, WAA is most often directed against highly prevalent antigens such as the Rhesus blood group, Kp^b and U at the optimal temperature of 37°C [11].

TABLE 1 Frequencies of categorized antibodies impacted by the new antibody investigation algorithms with the odds ratio (OR) and *p*-values from Fisher's exact test for two proportions.

Results impacted by AIA change	Category	Total (%)	Using old AIA (%)	Using new AIA (%)	OR (95% CI)	*p*-value
Yes (24%)	Potential WAA	180 (9%)	127 (11%)	53 (6%)	0.54 (0.38, 0.76)	<0.001
	Unidentified antibodies	195 (10%)	109 (9%)	86 (10%)	1.09 (0.80, 1.48)	0.594
	AUS	57 (3%)	0 (0%)	57 (7%)	∞ (23.5, ∞)	<0.001
	Combined potential WAA and alloantibodies	37 (2%)	26 (2%)	11 (1%)	0.57 (0.25, 1.21)	0.132
No (76%)	Alloantibodies	1135 (56%)	648 (56%)	487 (57%)	1.06 (0.89, 1.28)	0.525
	Cold agglutinin	112 (5%)	67 (6%)	45 (5%)	0.91 (0.60, 1.37)	0.694
	Passive anti-D	93 (4%)	68 (6%)	25 (3%)	0.49 (0.29, 0.79)	0.002
	Drug-related antibodies	16 (1%)	9 (1%)	7 (1%)	1.06 (0.34. 3.22)	1.000
	Negative	196 (10%)	113 (9%)	83 (10%)	1.00 (0.74, 1.37)	1.000
Total		2021 (100%)	1167 (100%)	854 (100%)		

Abbreviations: AIA, antibody investigation algorithm; AUS, antibody of undetermined significance; CI, confidence interval; WAA, warm autoantibody.

TABLE 2 Antibody results of patients with AUS who previously had RBC antibodies and the transfusion histories.

Before AUS	Type of RBC antibodies	Interval of previous RBC antibodies and AUS	Previously received RBC transfusions/interval of most recent transfusion and AUS detection	Follow-up results/interval in months
Case 1	WAA	3 months	No/NA	No/NA
Case 2	WAA	3 months	Yes/unknown	No/NA
Case 3	WAA	96 months	Yes/3 months	No/NA
Case 4	WAA	40 months	Yes/4 days	AUS/5 months
Case 5	WAA	1 month	Yes/4 days	No/NA
Case 6	WAA	13 months	Yes/4 days	No/NA
Case 7	WAA	24 months	No/NA	No/NA
Case 8	WAA	5 months	No/NA	No/NA
Case 9	WAA	72 months	No/NA	No/NA
Case 10	WAA	60 months	Yes/47 months	No/NA
Case 11	WAA	7 months	Yes/4 days	No/NA
Case 12	WAA	48 months	Yes/unknown	No/NA
Case 13	WAA	42 months	No/NA	No/NA
Case 14	WAA	10 months	Yes/4 days	No/NA
Case 15	WAA/cold agglutinin	36 months	Yes/34 months	AUS/3 months
Case 16	WAA/cold agglutinin	5 months	Yes/4 months	Unidentified antibodies/22 months
Case 17	WAA/cold agglutinin	3 months	Yes/unknown	No/NA
Case 18	Cold agglutinin	18 months	No/NA	No/NA
Case 19	Cold agglutinin	2 weeks	No/NA	No/NA
Case 20	Unidentified antibodies	1 month	No/NA	AUS/3 months
Case 21	Unidentified antibodies	1 week	No/NA	No/NA

Abbreviations: AUS, antibody of undetermined significance; NA, not available including no follow-up results; RBC, red blood cell; WAA, warm autoantibody.

TABLE 3 Development of RBC antibodies after AUS identification and the transfusion histories.

After AUS	Type of RBC antibodies	Transfused pre-AUS detection/interval of most recent previous RBC transfusion	Interval of AUS and other RBC antibody formation	Transfused post-AUS detection/interval of RBC transfusion post-AUS and other RBC antibody formation	Follow-up results/interval in months
Case 22	Anti-E	No/NA	2 weeks	No/NA	No/NA
Case 23	Anti-C	Yes/4 days	2 weeks	No/NA	No/NA
Case 24	Anti-c, anti-E	Yes/1 month	3 months	Yes/1 month	No/NA
Case 25	Anti-Wr^a	No/NA	3 months	Yes/4 months	Anti-Wr^a/3 months

Abbreviations: AUS, antibody of undetermined significance; NA, not available including no follow-up results; RBC, red blood cell.

When RBCs are heavily coated with IgG in WAA, a panreactive antibody screen is often observed with no exception of antibody investigation methods. In the presence of WAA, underlying alloantibodies are often unable to be excluded, which may lead to an inadvertently haemolytic transfusion reaction if patients are transfused with antigen-positive blood. Patients with WAA are also at risk of developing RBC alloimmunization with a prevalence of 10%–53% [12, 13].

Avoiding exposure to highly immunogenic antigens, such as the Rhesus and Kell antigens, through transfusing RBCs that are negative for antigens that the patient also lacks, referred to as prophylactic antigen matching, can mitigate the complexity of the pre-transfusion testing [14]. Selecting RBC units that are phenotypically or genotypically matched to the patient can prevent alloimmunization, reduce the cost of investigations and avoid delay of RBC provisions in patients who require ongoing transfusion support [15, 16]. As serological RBC

antigen determination is unreliable in patients with recent transfusions, genotyping to predict recipient RBC antigens plays a significant role in guiding RBC selection, though turnaround times in emergency situations currently limit its usefulness in that setting.

For the above reasons, panreactivity was previously interpreted as potential WAA but is now classified as AUS and will not require unnecessary serological investigations, RBC phenotyping and genotyping. To mitigate the non-specific reactions in SPRCA, Dwyre and Bunker had previously shown that adding a second antibody screening method alleviated false positives [9, 10]. The use of SPRCA and PEG as primary and secondary methods for antibody screening since 2014 at VGH has shown similar rates of panreactivity, leading to a large number of cases that required further investigations. Many cases with non-specific reactions remain inconclusive even after excluding the antibodies with a known impact on transfusion, where complete antibody determination may not be warranted if crossmatch-compatible RBC units can be provided.

SIAT is still considered the gold standard technique used to detect in vitro sensitization of RBCs by IgG to detect clinically significant IgG antibodies in the serum or plasma of the patient and for crossmatching. Integrating a negative SIAT to help exclude potential WAA in cases of panreactive SPRCA and/or PEG-IAT can simplify the pre-transfusion investigations and prevent transfusion delay. As shown in our study, the frequencies of potential WAA detection were significantly decreased using a new AIA, including the SIAT method. The reduction in potential WAA frequencies could be attributed to several factors, such as the prevalence of WAA in particular periods and the reasons for antibody screening, such as routine pre-operative testing or in patients with haemolytic features. However, since the antibody results had to be issued promptly and only one diagnosis could be made, it is impossible to simultaneously perform a direct comparison using old and new AIAs. It is also not feasible or cost-effective to refer all specimens with potential WAA, which may have included both true WAA and alloantibodies to high prevalence antigens or AUS, to the reference laboratory for confirmation.

To monitor the patients with AUS for new antibody formation, complete antibody investigations were repeated every 3 months if the blood samples were available. However, if there were significant changes in the antibody screen reactivity by SPRCA, (greater than 1+ of increased reactivity strength on each test cell than the previous findings), reinvestigations would be performed before 3 months. Seventeen patients previously had potential WAA from the old algorithm, and are now classified as AUS when using the new AIA. Among these, only three patients had follow-up antibody investigations based on pre-transfusion testing ordered by clinicians, and all remained as AUS at the follow-up time of 3 and 5 months. One case of AUS later became an unidentified antibody at a follow-up time of 22 months. Despite the different diagnostic criteria between AUS and unidentified antibodies where the latter's reactions are not panreactive, the policy to provide crossmatch-compatible RBC units is the same.

Four patients were initially identified as having an AUS but later developed specific RBC alloantibodies; two received RBC transfusions between 4 days and 1 month. With the short intervals between AUS identification and subsequent specific alloantibody formation, these scenarios may raise concerns about missing either existing or newly formed antibodies using the new AIA. For two patients for whom anti-E and anti-Wr^a were identified without RBC transfusion events, it is possible that the new AIA had missed a low level of antibodies from a previous pregnancy in the first case, or was not able to detect the pre-existing anti-Wr^a because the Wr^a antigen-positive cells were not used for the SIAT screen in the second case.

A similar phenomenon of panreactive SPRCA accompanied by a negative autocontrol and a lack of specificity but later acquired alloantibodies was described by Olofson et al., suggesting higher rates of both transfusion reactions and alloantibody development at 2% and 11%, respectively, compared to those without SPRCA panreactivity [17]. Liu et al. reported approximately 18% of the gel-based antibody screens as AUS. With follow-up, 15% of the patients with AUS had a subsequent clinically significant alloantibody detected by additional testing [4]. However, Liu's inclusion criteria differed from ours, which included exclusively panreactive SPRCA and PEG and did not include cases with differential reactions between each test cell.

We chose the term AUS or 'antibody of undetermined significance' instead of 'antibody of unknown specificity', which was described in the literature aiming to capture only non-specific reactions that appeared panreactive and were most likely not associated with haemolysis risk [4, 18, 19]. Our aim was to eliminate non-specific solid-phase reactions frequently regarded as likely clinically insignificant. In contrast to other literature, we considered the non-specific reactions that are non-panreactive as unidentified antibodies which have potential clinical significance and could indicate a historical antibody in low titre under the minimal threshold of detection. Most of the reactions observed by PEG in AUS were weak and challenging to interpret in our centre, which can overlap between AUS and unidentified antibodies. Because of these limitations, RBC provision strategies for both types of antibodies are the same.

At our centre, we did not perform an autocontrol with fully automated SPRCA because the autologous RBCs cannot be lysed and immobilized for testing by the manufacturer, but we used reflex DAT to investigate the panreactivity further. Elution studies were not commonly used in the setting of panreactivity given no additional information would be elucidated. Judd et al. previously described a lower predictive value of DAT in detecting early-forming antibodies post-transfusions than autocontrol [20]. However, Lollie et al. recently described a comparable positivity rate of both methods and suggested replacing autocontrol with reflex DATs to reduce the cost of testing [21].

In our study, polyspecific DAT was positive in 40 of 57 patients with AUS (70%). Although a positive DAT enables the diagnosis of WAA, associated with autoimmune haemolytic anaemia, DAT alone does not define WAA since it can be positive in up to 15% of hospitalized patient specimens [22]. Positive DAT in AUS can be explained by non-specific RBC agglutination in many conditions, such as drugs, antiphospholipid antibodies, infections and interferences from IgG unassociated with haemolysis [23]. The elution study findings were also panreactive in 10 of 11 patients with AUS (91%), ensuring no antibodies of common specificity coating the RBCs.

Limitations of our study were that our retrospective analysis would not have been able to determine any clinical impact of our new AIA. Changes in reinvestigations when comparing our old and new AIAs could not be properly abstracted from our laboratory information system. As our centre is not a reference laboratory and certain immunological techniques are less commonly performed in Canada, we did not perform either enzyme- or chemical-treated RBCs, nor autoadsorption or alloadsorption. These tests are often referred to an immunohematology reference laboratory through the Canadian Blood Services as they often do not aid transfusion management in a timely fashion [6]. Patients' diagnoses and transfusion indications should also be carefully reviewed when handling AUS results, where special patient populations, such as those with haemoglobinopathies, will be at a higher risk for alloimmunization and delayed haemolytic transfusion reactions. Thus, we suggest considering the local patient population for additional investigations and prophylactically matched RBC units in some transfusion-dependent patient populations.

In conclusion, we observed a significant reduction in the frequencies of potential WAA detection post-implementation of the new AIA integrating SIAT results. This approach helped mitigate unnecessary further investigations and phenotypically matched RBC use. However, some patients still developed subsequent RBC alloimmunization, prompting careful evaluation of clinically relevant alloantibodies before determining AUS. Serial antibody investigation tests are also warranted considering forming or evanescent antibodies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

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Comparison of two apheresis systems for granulocyte collection without hydroxyethyl starch

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Abstract

Background and Objectives: Granulocyte transfusion (GTX) is a treatment option for severe infections in patients with neutropenia. In previous studies, hydroxyethyl starch (HES) was used to enhance red blood cell sedimentation for granulocyte collection (GC). However, there are safety concerns about HES, and HES is not readily available in some countries. Therefore, we compared the granulocyte counts and GC efficiency achieved by two apheresis systems without HES.

Materials and Methods: All consecutive GC procedures performed between July 2011 and March 2018 at our hospital were analysed. COBE Spectra was used until 5 February 2016, and Spectra Optia was used afterwards. HES was not used.

Results: Twenty-six GC procedures were performed, including 18 performed using COBE Spectra and 8 using Spectra Optia. When Spectra Optia was used, $>1 \times 10^{10}$ neutrophils were collected from seven of the eight (88%) procedures. Although there was no significant difference in the granulocyte yield between COBE Spectra-based and Spectra Optia-based GC procedures, the collection efficiency of Spectra Optia was significantly higher than that of COBE Spectra ($p = 0.021$). Furthermore, the granulocyte yields of Spectra Optia-based GC tended to be more strongly correlated with the peripheral blood neutrophil count on the day of apheresis than those of COBE Spectra-based GC.

Conclusion: Our results suggest that Spectra Optia achieves greater GC efficiency than COBE Spectra, even without HES. GTX may be a therapeutic option for severe neutropenia, even in places where HES is not available.

Keywords

apheresis, collection efficiency, granulocyte collection, hydroxyethyl starch

Highlights

- The collection efficiency of Spectra Optia was significantly higher than that of the COBE Spectra apheresis system, even when hydroxyethyl starch (HES) was not used.
- The granulocyte yields obtained using Spectra Optia tended to be more strongly correlated with the peripheral blood neutrophil count on the day of apheresis than those obtained using COBE Spectra.

- Even when HES is not used, Spectra Optia could collect 1×10^{10} granulocytes from >80% of donors, making granulocyte transfusions a treatment option in settings where HES is not available.

INTRODUCTION

Haematological disorders may be cured with intensive therapy such as chemotherapy and haematopoietic stem cell transplantation (HSCT). The most significant potential issue during these therapies is infections that arise in patients with neutropenia. Although antibiotics have evolved, granulocytes play an important role in eradicating microbes from hosts. Granulocyte transfusion (GTX) is a treatment option for patients with neutropenia who develop severe infections that are unresponsive to antimicrobial or antifungal therapies [1–4]. However, there is no established method for collecting granulocytes from healthy donors [4].

Although there are no proven advantages of receiving higher doses of granulocytes for recipients, a granulocyte count of at least 1×10^{10} cells is considered necessary for GTX to be effective [4, 5]. The administration of granulocyte colony-stimulating factor (G-CSF) makes it easier to collect at least 1×10^{10} neutrophils. Furthermore, the use of hydroxyethyl starch (HES) to remove red blood cells during apheresis has been reported to increase the efficacy of granulocyte separation and collection. However, there are safety concerns about HES. In addition, HES is not available in some countries, and it is not approved as a drug in Japan.

A cell separator system, Spectra Optia, which is the successor of COBE Spectra, has been reported to increase the yield of granulocyte collection (GC) [6, 7]. In these studies, HES was used during GC. At our institution, GC is performed without HES. It is not known whether Spectra Optia achieves more efficient GC than COBE Spectra, which is an older apheresis system, when granulocytes are collected without HES. Here, we examine the number of granulocytes collected when apheresis was performed without HES in the G-CSF era. The granulocyte counts and collection efficacy achieved during granulocyte apheresis performed using Spectra Optia were compared with those achieved during granulocyte apheresis performed using the COBE Spectra system.

METHODS

Donors

This was a single-centre, retrospective study. Written informed consent was obtained from all of the donors before the procedures. All consecutive GC procedures performed between July 2011 and March 2018 at our institution were analysed in this study. The donors were selected from blood relatives aged ≥18, according to the Japanese standard for platelet apheresis donors. All donors underwent a history check, a physical examination, complete blood count analysis, biochemical analysis, chest radiography and electrocardiography before the granulocyte mobilization. The donor eligibility criteria were as follows: ABO blood type compatibility or minor ABO blood type incompatibility, and negativity for infections such as hepatitis B and C viruses, human immunodeficiency virus, and human T-cell leukaemia and lymphoma virus type I. Subjects with a history of G-CSF allergy, thrombosis, coronary heart disease, cerebrovascular disease, interstitial pneumonia, haematological disease or cancer were excluded through a history check. Those with cardiovascular, pulmonary, renal or hepatic disease, diabetes that required treatment and those with poorly controlled hypertension or hyperlipidaemia were also ineligible to be donors. GC was performed twice from each donor. Informed consent was obtained from all donors prior to the procedures. This study was approved by the institutional ethics committee of Wakayama Medical University (approval number: 1310).

Granulocyte mobilization and apheresis

All donors received 500 μg of lenograstim subcutaneously (off-label use) and 8 mg of dexamethasone orally 12 h before GC. Granulocytes were collected with a blood cell separator via peripheral venous access. COBE Spectra (Caridian BCT, Lakewood, CO, USA) was used for GC until 5 February 2016. After that, Spectra Optia (Terumo BCT, Tokyo, Japan) was used. Acid-citrate-dextrose fluid was used to prevent coagulation in the blood circuit. The target inlet-to-anticoagulant ratio was 10:1. HES was not used as an erythrocyte sedimentation agent in any case. During GC with COBE Spectra, the interface was monitored, and the plasma flow rate was adjusted by a specialized laboratory technician. The volume of whole blood processed was set at 100 mL/kg weight. The target volume of collected liquid was set to 250 mL. On the other hand, although the product colour was monitored, the apheresis procedures were carried out automatically during GC using Spectra Optia. The polymorphonuclear leukocyte collection program of Spectra Optia was used as an automated interface monitoring system. The target volume of collected liquid was set at 400 mL in Spectra Optia.

Laboratory analyses and calculation of collection efficiency

The collection efficiency of the granulocyte harvesting (CE2) was calculated as follows. The pre-apheresis granulocyte count was defined as the neutrophil count recorded in the morning of the day of granulocyte apheresis.

$$\mathrm{CE2}(\%) = \Big[\text{granulocyte yield(cells)}/(\text{pre} - \text{apheresis granulocyte count}(/\mu\mathrm{L}) \times \text{processed whole blood volume}(\mathrm{mL}) \times 10^3)\Big] \times 10^2.$$

The blood counts used for donor health check-up were obtained during the prior donor eligibility testing. The peripheral blood counts of the donor and recipient were analysed using the XE-5000 system (Sysmex Corporation, Kobe, Japan). All data are reported as the mean ± SEM, unless otherwise noted. In the assessment of the increases in the number of granulocytes seen in the recipients, paediatric recipients were excluded from the analysis because increases in the granulocyte counts of children are affected by body size.

Statistical analysis

The entry of the data relating to the GTX donors and recipients was performed using Microsoft Excel 365. Bivariate analyses of categorical variables were conducted using Fisher's exact test. Continuous variables were analysed using the Mann–Whitney *U*-test. The unpaired *t*-test was used to determine the significance of inter-group differences in the granulocyte count. *p*-Values of <0.05 were considered statistically significant. All statistical analyses were performed using Excel or GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Donor characteristics

During the study period, eight recipients received GTX. Twenty-six GC procedures were performed. Three donors underwent GC twice. The characteristics of the donors are shown in Tables 1 and 2, according to the apheresis system used (COBE Spectra or Spectra Optia). Tables 1 and 2 show the donor characteristics in terms of donor-based data and collection-procedure-based data, respectively. Eighteen and eight GC procedures were performed using COBE Spectra and Spectra Optia, respectively. One donor underwent one GC procedure with COBE Spectra and another with Spectra Optia. The median age of all donors was 36 years (Table 1). There were no significant differences in age or body weight between the two apheresis system groups. The mean neutrophil count at the time of the medical health check-up was 3.6×10^9/L. The mean neutrophil count just before neutrophil collection (after the administration of G-CSF and dexamethasone) was 36.0×10^9/L (Table 2). There were no significant differences in the neutrophil counts obtained at either the time of the medical health check-up ($p = 0.76$) or just before the apheresis ($p = 0.71$) between the two apheresis system groups.

GC and efficiency

The mean processed blood volume during apheresis was 6.5 and 5.6 L in the COBE Spectra and Spectra Optia groups, respectively (Table 2, $p = 0.21$). The mean granulocyte yield of apheresis performed with COBE Spectra was 1.7×10^{10} (range, 0.14–5.95) (Figure 1a). A neutrophil yield of 1×10^{10} was achieved in 13 of 18 (72%) patients. On the other hand, the mean granulocyte yield of apheresis performed with Spectra Optia was 2.6×10^{10} (range, 0.93–4.48). Although more than 1×10^{10} neutrophils were collected from seven of the eight donors (88%) in the Spectra Optia group, there were no significant inter-group differences in the percentage of donors from whom $>1 \times 10^{10}$ neutrophils were collected ($p = 0.63$) or in the granulocyte yield ($p = 0.12$). The mean collection efficiency was 7.2% (range, 1.3–24.2) and 14.4% (range, 4.8–24.0) in the COBE Spectra and Spectra Optia groups, respectively (Figure 1b). The efficiency of GC of the Spectra Optia system was significantly higher than that of COBE Spectra ($p = 0.021$).

TABLE 1 Donor characteristics before granulocyte collection.

Characteristics	Total ($N = 23$)	COBE Spectra ($N = 16$)	Spectra Optia ($N = 8$)	*p*-Value
Median age (range)	36 (19–62)	37 (19–53)	40.5 (30–62)	0.21
Male, *n* (%)	13 (57)	9 (56)	4 (50)	1.00
Body weight (kg)	61.7 ± 2.6	60.9 ± 3.3	60.5 ± 4.6	0.88
Blood count at donor health check-up				
WBCs ($\times 10^9$/L)	6.4 ± 0.3	6.2 ± 0.3	6.6 ± 0.7	0.53
Neutrophils ($\times 10^9$/L)	3.6 ± 0.2	3.6 ± 0.3	3.5 ± 0.3	0.76
Haemoglobin (g/dL)	14.7 ± 0.3	14.6 ± 0.4	14.6 ± 0.5	0.91
Haematocrit (%)	43.1 ± 0.7	42.5 ± 0.9	43.5 ± 1.2	0.52
Platelets ($\times 10^9$/L)	217 ± 8.2	218 ± 9.1	215 ± 16.1	0.87

Note: All data are based on the number of donors rather than the number of procedures. One donor underwent one granulocyte collection procedure using COBE Spectra and another with Spectra Optia. Data are shown as the mean ± SEM (except for age and sex). *p*-Values were calculated with the *t*-test, except for age. The *p*-value for age was calculated using the Mann–Whitney *U*-test.
Abbreviation: WBC, white blood cell.

TABLE 2 Apheresis-related donor characteristics.

Variables	Total ($N = 26$)	COBE Spectra ($N = 18$)	Spectra Optia ($N = 8$)	p-Value
Blood count on the day of collection				
WBCs ($\times 10^9$/L)	38.1 ± 2.1	38.3 ± 2.0	37.5 ± 5.4	0.87
Neutrophils ($\times 10^9$/L)	36.0 ± 2.0	36.5 ± 1.9	34.8 ± 4.9	0.71
Haemoglobin (g/dL)	14.7 ± 0.3	14.8 ± 0.4	14.4 ± 0.6	0.64
Haematocrit (%)	42.7 ± 0.8	42.8 ± 0.9	42.4 ± 1.5	0.78
Platelets ($\times 10^9$/L)	229 ± 8.1	227 ± 9.8	232 ± 15.1	0.81
Apheresis procedure				
Total processed volume (L)	6.2 ± 0.3	6.5 ± 0.4	5.6 ± 0.3	0.21
Product volume (mL)	313 ± 12.2	279 ± 7.3	389 ± 15.4	<0.05
Apheresis duration (min)	131 ± 3.3	133 ± 3.8	126 ± 6.7	0.27

Note: All data are based on the number of procedures rather than the number of donors. Data are shown as the mean ± SEM. p-Values were calculated with the t-test.
Abbreviation: WBC, white blood cell.

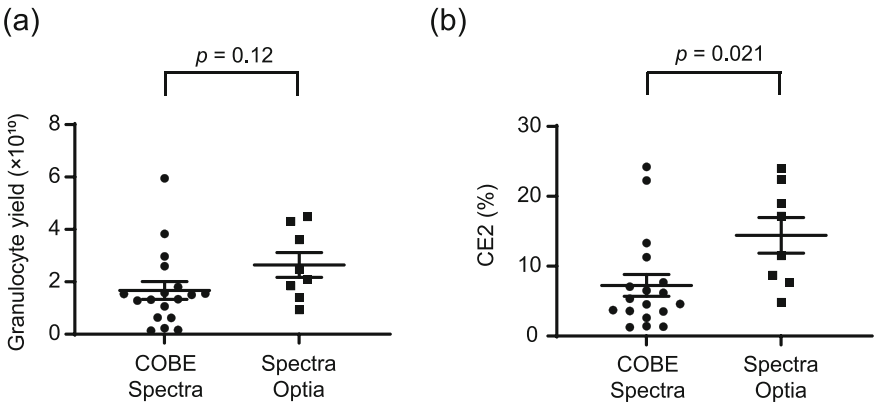

FIGURE 1 (a) Collected granulocyte yield and (b) collection efficiency (CE2) according to the apheresis system used. Each bar indicates the mean ± SEM.

There was no significant difference in the duration of apheresis between the COBE Spectra and Spectra Optia groups (Table 2). The mean product volume was higher when Spectra Optia was used than when COBE Spectra was used. Interestingly, the absolute number of platelets in the collection product was significantly lower when granulocytes were collected using Spectra Optia than when using COBE Spectra (Table 3, $p = 0.049$).

Correlation between the pre-apheresis neutrophil count and the neutrophil yield of the product

The correlation between the pre-apheresis neutrophil counts of the donors' peripheral blood and the neutrophil yields of the products was assessed. Although the inter-group difference in this correlation coefficient was not significant, the granulocyte yields achieved using Spectra Optia tended to be more strongly correlated with the peripheral blood neutrophil count on the day of apheresis than the granulocyte yields achieved using COBE Spectra ($r = 0.45$ for COBE Spectra vs. $r = 0.65$ for Spectra Optia, Figure 2a). The trend line for CE2 during neutrophil collection using Spectra Optia lay approximately 10 percentage points above that for CE2 during neutrophil collection using COBE Spectra (Figure 2b).

Increase in the number of neutrophils in the recipients

The neutrophil counts of the adult recipients were evaluated before GTX and the day after GTX. The increases in the numbers of neutrophils observed after the GTX were compared between the COBE Spectra and Spectra Optia groups (Figure 3). Although the difference was not significant, the increases in the peripheral blood neutrophil counts seen in the recipients transfused with Spectra Optia-collected granulocytes tended to be greater than those observed in patients transfused with COBE Spectra-collected

TABLE 3 Granulocyte collection product characteristics.

Variables	Total ($N = 26$)	COBE Spectra ($N = 18$)	Spectra Optia ($N = 8$)	p-Value
WBCs ($\times 10^{10}$)	2.7 ± 0.3	2.3 ± 0.4	3.4 ± 0.6	0.14
Neutrophils (%)	68 ± 3.7	64 ± 4.6	78 ± 4.9	0.089
RBCs ($\times 10^{11}$)	7.8 ± 1.1	5.4 ± 0.6	13.4 ± 2.1	<0.05
Platelet count ($\times 10^{11}$/L)	8.4 ± 1.0	10.2 ± 1.1	4.5 ± 0.6	0.0036
Absolute number of platelets ($\times 10^{11}$)	2.5 ± 0.3	2.9 ± 0.3	1.8 ± 0.3	0.049

Note: Data are shown as the mean ± SEM. p-Values were calculated with the t-test.
Abbreviations: RBC, red blood cell; WBC, white blood cell.

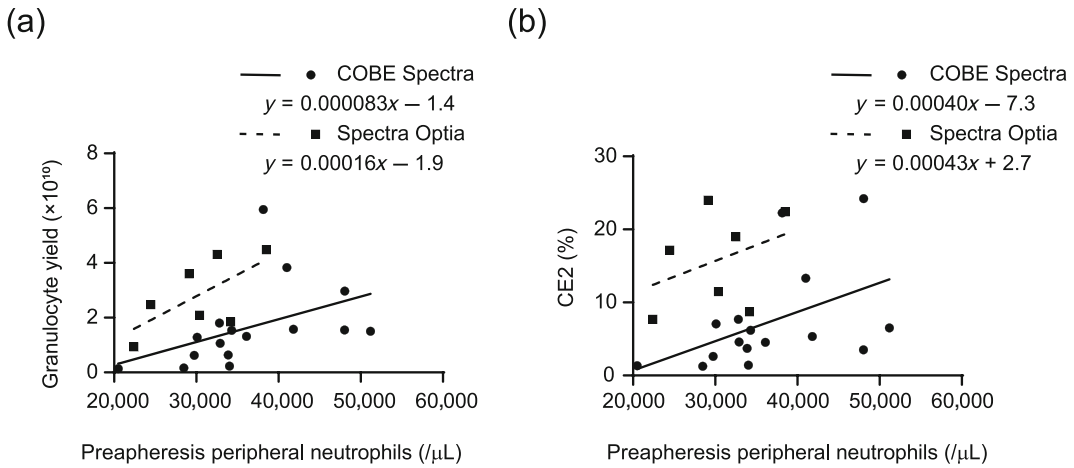

FIGURE 2 (a) Correlation between pre-apheresis peripheral neutrophil counts and granulocyte yields. (b) Correlation between pre-apheresis peripheral neutrophil counts and collection efficiency (CE2). Solid line: granulocyte collection using COBE Spectra; dotted line: granulocyte collection using Spectra Optia.

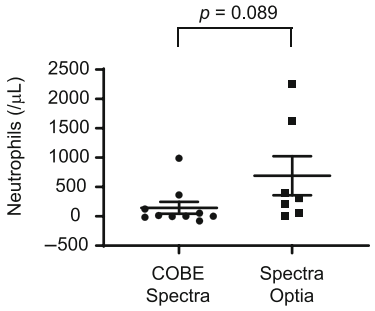

FIGURE 3 Increase in the recipients' peripheral blood neutrophil counts from before granulocyte transfusion (GTX) to the morning after GTX according to the apheresis system used.

granulocytes (mean: 144/μL in the COBE Spectra group vs. 690/μL in the Spectra Optia group, $p = 0.089$).

Alloimmunization to human leukocyte antigens (HLAs) and human neutrophil antigens can occur as an adverse reaction to GTX [4, 8]. Donor HLAs were not examined in this study. HLA antibodies cause refractoriness to leukocyte and platelet transfusions. Although no tests for HLA antibodies were performed, none of the patients showed diminished platelet transfusion efficacy within 6 months after GTX. Two patients had HLA antibodies prior to GTX and received HLA-matched platelet transfusions. In one recipient, HLA antibodies appeared 1 year after the GTX. Six of the eight recipients received GTX before HSCT or haematopoietic stem cell engraftment. Although alloimmunization to HLA in GTX may affect haematopoietic stem cell engraftment, all of our recipients achieved granulocyte engraftment after undergoing allogeneic HSCT [9].

DISCUSSION

GTX is a potential treatment for invasive infections that arise during severe neutropenia. However, the number of facilities that perform GTX is limited. In addition, there are no established GC methods. At our hospital, GC is conducted without HES because HES is not approved for use in Japan. Even in the absence of HES, Spectra Optia showed greater GC efficiency than COBE Spectra. Using Spectra Optia made it possible to collect more than 1×10^{10} granulocytes from seven of eight (88%) donors. Although there was no significant difference in the number of granulocytes collected, the collection efficiency of Spectra Optia was significantly higher than that of COBE Spectra. This study suggests that GC using Spectra Optia may be

more beneficial than that using COBE Spectra, even when HES is not used.

Although a higher granulocyte yield does not necessarily lead to a more effective GTX, it is considered that at least 1×10^{10} granulocytes are required for GTX to be effective [2]. The administration of G-CSF on the day before apheresis markedly increases the number of granulocytes collected. Using HES to enhance red blood cell sedimentation prior to GC can also increase the efficiency of GC [10, 11]. However, HES has been reported to have severe adverse effects, including kidney injuries and bleeding, in critically ill patients or patients with sepsis [12, 13]. During GC, donors receive a smaller dose of HES than is administered to critically ill patients. Therefore, the adverse effects of HES on granulocyte donors are minimal [14]. However, a previous study showed that the complete elimination of HES takes 7 weeks [15]. Another study suggested that using HES for repeated GC may cause lymphopenia in donors for up to 2 years after the GC procedure [16]. Although these concerns about the adverse effects of using HES for GC are relatively limited, HES is not easy to obtain in many countries and is not approved for use in Japan. In addition, sales of HES have been temporarily suspended in the European Union because of the possible adverse effects of the drug. In other countries, HES needs to be imported and stored for occasional GC. Many recent prospective or retrospective studies of GTX for adult patients have used HES [17–21]. Thus, in this study, we examined GC without HES in the G-CSF era. Our findings suggest that Spectra Optia could be used to collect more than 1×10^{10} granulocytes without HES. In addition, increased neutrophil counts, averaging 690/μL, were observed in the recipients' peripheral blood.

A previous study showed that only 26% of patients who met the indications for GTX actually underwent the procedure [22]. Apheresis is the standard cell-harvesting procedure employed at institutions that perform peripheral blood stem cell transplantation. On the other hand, stem cell harvesting does not require HES. GTX of granulocytes collected without HES may be available at more facilities, especially hospitals that perform HSCT.

COBE Spectra requires intermittent optical and manual input of the interface position by the operator [23]. The operator must visually fine-tune the interface position, which requires experience and skill. Spectra Optia is a newer apheresis system that enables real-time interface monitoring, interpretation and automatic plasma flow adjustment for interface stability relative to the collection port [24]. Thus, Spectra Optia is easier to use, as it automates the collection process.

Several studies have compared autologous or allogeneic peripheral blood stem cell collection efficiency between COBE Spectra and Spectra Optia systems [24–27]. The CD34-positive cell collection efficiency of Spectra Optia was found to be comparable to or better than that of COBE Spectra [24–27]. GC efficiency has also been compared between COBE Spectra and Spectra Optia systems [7, 28]. However, in these studies HES was used. Our study suggests that Spectra Optia increases the efficiency of GC even in the absence of HES. The correlation between the number of neutrophils in the donors' peripheral blood before GC and the number of granulocytes collected tended to be stronger for Spectra Optia than for COBE Spectra. Therefore, Spectra Optia may be more stable when collecting granulocytes from the peripheral blood, which may lead to higher CE2 values.

Controlled trials and a systematic review have failed to demonstrate the clinical benefits of GTX [29–31]. GTX is provided as an empirical treatment in some areas and at some facilities [1, 32]. A systematic review found no differences in all-cause mortality between the granulocyte dose subgroups [31]. In contrast, a controlled study found that a mean granulocyte dose per GTX of 0.6×10^9/kg tended to produce better outcomes [30]. The number of granulocytes collected may affect the effectiveness of GTX. A previous report found that using HES to collect more granulocytes was still advantageous when granulocyte apheresis was performed using Spectra Optia [11]. The number of granulocytes collected in our study was comparable to those collected in studies in which GC was carried out without HES but lower than those seen in previously reported studies involving the use of HES [21, 28, 33, 34]. Recently, HES of medium molecular weight was reported to be a safe and effective alternative to HES for granulocyte apheresis [35]. Alternative erythrocyte sedimentation agents may be an option in countries where HES is unavailable.

This study had several limitations. First, it was a single-centre retrospective study. Although it involved a small sample size, it is important to examine GC in settings where HES is not available. Second, COBE Spectra and Spectra Optia were used for GC in different periods. However, the collection methods and the staff performing the collection were the same. Third, the effectiveness of GTX at preventing or treating infections was not assessed because of the wide range of conditions that afflict recipients. GTX is indicated both prophylactically and therapeutically against infections that arise in patients with severe neutropenia in real-world clinical practice. Therefore, the effects of GTX could not be uniformly evaluated in this study.

In conclusion, our results suggest that the Spectra Optia system achieves higher GC efficiency than the COBE Spectra system even when HES is not used. Although the granulocyte yields for which GTX is effective have not been established, more than 1×10^{10} granulocytes can be collected using Spectra Optia. Therefore, GTX may be a therapeutic option for severe neutropenia, even in places where HES is not available.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest associated with this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SHORT REPORT

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Autoimmune anti-D in an RhD-positive young infant: Learning from a rare case

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Abstract

Background and Objectives: Anti-D is usually immune in nature and is formed in individuals lacking D antigen or having variants/altered D phenotypes. In the Indian population, 93.8% are RhD positive, and R_1R_1 is the commonest Rh phenotype. Here we report a rare and interesting case of autoimmune anti-D in an RhD-positive 3-month-old infant leading to warm autoimmune haemolytic anaemia.

Study Design and Methods: Auto-anti-D was detected serologically by immunohaematological techniques such as direct antiglobulin test, antibody detection and identification, dithiothreitol, enzyme treatment, antibody titration and elution. Molecular studies were performed to rule out genetic variants of RhD.

Results: Anti-D was confirmed in eluate and blood group post elution was B RhD positive. On genotyping using the Indian-specific *RHD* genotyping assay, the sample was found to be negative for the *RHD*01W.150* (most common RhD variant in Indians) but positive for *RHD exon 5* and *RHD exon 10* along with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The sample was further sequenced for *RHD exons 1–10* by Sanger sequencing and found to be a wild type, thus, ruling out the presence of an *RhD* variant.

Conclusion: This case is of interest because of the rare occurrence of autoimmune anti-D in an RhD-positive patient of such a young age (3 months). To the best of our knowledge, only two case reports have been published on autoimmune anti-D in infancy (in 1961 and 1964).

Keywords

blood groups, genotyping, immunohaematology, molecular testing, RBC antigens and antibodies

Highlights

- This case is of interest because it involves the rare occurrence of autoimmune anti-D in an RhD-positive patient aged only 3 months.
- On genotyping using the Indian-specific *RHD* genotyping assay, the sample was found to be negative for the *RHD*01W.150* (most common RhD variant in Indians) but positive for *RHD exon 5* and *RHD exon 10* along with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene.
- To the best of our knowledge, only two case reports (in 1961 and 1964) have been published on autoimmune anti-D in infancy.

INTRODUCTION

The Rh system is a highly polymorphic and complex blood group system comprising 56 antigens [1]. Rh antigens are significantly immunogenic, next only to the ABO system in importance with regard to transfusion medicine [2]. Rh antibodies are generally produced after exposure to foreign red cells and are clinically significant. In the Indian population, 93.8% are RhD positive, and R_1R_1 is the commonest Rh phenotype [3]. Here we report a rare and interesting case of autoimmune anti-D in an RhD-positive 3-month-old infant leading to warm autoimmune haemolytic anaemia (WAIHA).

CASE PRESENTATION

Blood requisition of a 3-month-old male child along with the mother's blood sample was received in the Department of Transfusion Medicine for a paediatric packed red blood cell (PRBC) unit. A brief review of the history of the child revealed that the child presented with pallor and jaundice for 3 days. There was no history of neonatal jaundice at birth. Laboratory reports revealed haemoglobin (Hb) as 7.1 g/dL, total leucocyte count 8920/μL, mean corpuscular volume 85.22 μm^3, mean corpuscular haemoglobin 30.87 pg, mean corpuscular haemoglobin concentration 36.22 g/dL and platelet counts of 2 lakhs. G6PD was within the normal range (31.5). Liver function test were deranged, with total serum bilirubin (TSB) 19 mg/dL with indirect component as 17.8 mg/dL, serum glutamic-oxaloacetic transaminase/serum glutamic pyruvic transaminase 71/20 and alkaline phosphatase 367. Peripheral blood smear showed a predominantly microcytic blood picture with reduced red blood cell (RBC) density and a few macro-ovalocytes.

Blood grouping of the patient showed B RhD positive without any discrepancy. Autocontrol was negative at immediate spin (room temperature) and positive at the anti-human globulin phase by the tube technique. Blood group of the mother's sample was AB RhD positive. One B RhD-positive paediatric PRBC unit (volume 90 mL) was crossmatched for the patient, which came out to be incompatible (3+) with the patient's sample but was compatible with the mother's sample. The direct antiglobulin test (DAT) was positive (3+) by LISS Coombs gel cards by column antiglobulin technique (CAT) with immunoglobulin G (IgG) specificity. Antibody screening and antibody identification (Bio-Rad, Cressier FR, Switzerland) by CAT confirmed the presence of anti-D. Further immunohaematology (IH) work-up included dithiothreitol (DTT) treatment of plasma, enzyme (papain) treatment of the RBCs (three cell antibody screening panel) and antibody titration, performed by CAT. DTT treatment and enzyme test showed no change in agglutination strength. Antibody titre was 1024 (IgG) by the (CAT) gel method. Anti-LW was ruled out by testing the patient's serum with DTT-treated RhD-positive cells, which showed no change in reaction strength. Autocontrol, DAT and antibody screen of the maternal blood sample were negative.

Gentle heat elution (at 45°C) of the patient's plasma was performed. DAT after elution was negative. Antibody screen of the eluate confirmed the presence of auto-anti-D. Blood group of the red cells post elution was B RhD positive. Rh and K phenotype of the patient was C+E−c+e+K−. Parents' blood group and Rh K phenotype were also ascertained. The mother's blood sample tested as AB RhD positive, C+E−c+e+K−; the father's sample as B RhD positive, C+E+c+e+K−. The patient had a 10-year-old elder sister whose blood group and Rh K phenotype were AB RhD positive and C+E+c+e+K−.

Previous history revealed that the patient received one transfusion before reporting to our institute around a week earlier. Molecular studies were performed to rule out genetic variants of RhD. Genomic DNA was extracted from peripheral blood with EDTA using the Qiagen Blood Mini kit (QIAGEN, Hilden, Germany). Using polymerase chain reaction-sequence-specific primer (PCR-SSP), the Rh common antigens were genotyped using positive, negative and no template controls. The patient was found to carry the Rh genotype R_1R_1 (*Cde/Cde*) by PCR-SSP (Figure 1).

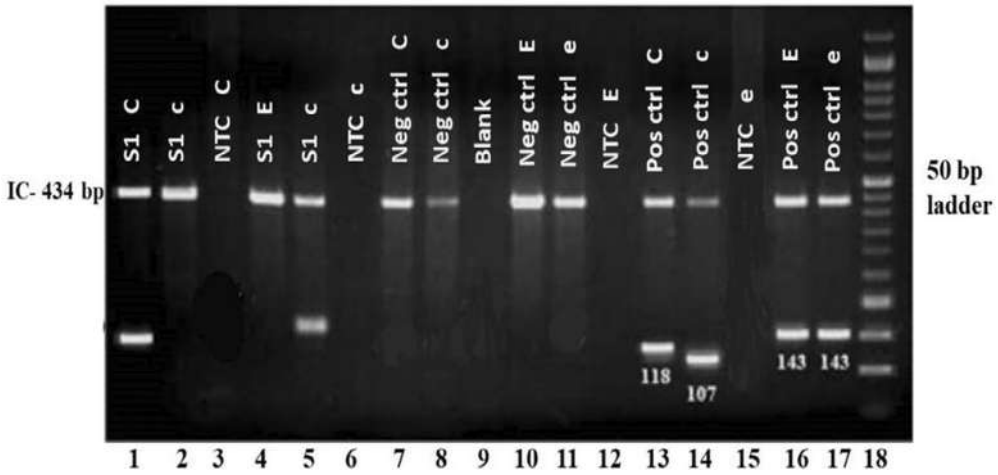

FIGURE 1 Genotyping of C, c, E and e antigens for patient S1 using polymerase chain reaction-sequence-specific primer and appropriate controls.

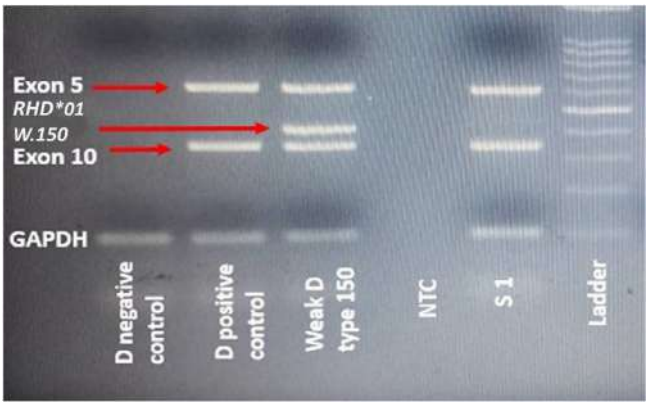

FIGURE 2 Genotyping by Indian-specific RHD genotyping assay. Patient S1 showed the presence of *RHD exon 5*, *exon 10* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*).

On genotyping using the Indian-specific *RHD* genotyping assay, the sample was found to be negative for the *RHD*01W.150* (most common RhD variant in Indians) but positive for *RHD exon 5* and *RHD exon 10* along with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) [4]. The sample was further sequenced for *RHD exons 1–10* by Sanger sequencing and found to be a wild type, thus ruling out the presence of an RhD variant [5] (Figure 2).

One B RhD-negative paediatric PRBC unit, phenotype-matched for Rh and K, was issued by our department. After the transfusion, Hb of the patient rose to 10.2 g/dL. A detailed IH report stating that the patient should receive B RhD-negative blood units for the future transfusions was issued to the patient. The patient was treated with oral prednisolone and responded well. Six months after discharge, the blood group of the patient was re-checked as B RhD positive. Autocontrol and DAT were still positive (2+). Antibody identification confirmed anti-D antibody (titre 64). Hb had risen to 12.6 g/dL. Ten months later, blood group was re-confirmed to be B RhD positive; autocontrol, DAT and antibody screen were negative. Hb was 11.9 g/dL. Prednisolone was stopped and the patient was advised to be maintained on a healthy diet and on regular follow-up.

DISCUSSION

There are a few published case reports of autoimmune anti-D but none from India. Most of these patients were found to have variant RhD phenotypes. Some reports highlight the difficulty in differentiating between allo- and auto-antibodies, especially in patients with a history of recent transfusion. Ouchari et al. reported a case of weak D type 4.0 with auto-anti-D autoantibodies in a 56-year-old patient with myelodysplastic syndrome. Blood group of the patient was O RhD positive, DAT was positive and the eluate contained anti-D [6]. Auto-anti-D has been reported in a partial D woman [7]. The patient was erroneously typed as D positive and became alloimmunized during pregnancy.

In our 3-month-old patient, auto-anti-D antibody titre was high, 1024 (IgG) by the (CAT) gel method. The child presented with pallor and jaundice for 3 days. There was no history of neonatal jaundice at birth. Laboratory reports revealed Hb as 7.1 g/dL. TSB was as high as 19 mg/dL, with an indirect component of 17.8 mg/dL. The high titre of the autoantibody was clinically significant and could explain the clinical features of severe anaemia and severe indirect hyperbilirubinemia (WAIHA). The child was started on prednisolone and responded well. The autoantibody persisted at 9 months of age (titre 64).

Li et al. described auto-anti-D, which interfered with RhD grouping due to blocked D phenomenon in a 19-year-old patient with autoimmune haemolytic anaemia (AIHA) [8]. The patient was initially typed as RhD negative, but the correct blood group (as RhD positive) was determined after elution. DAT was positive with IgG and C_3d specificity. Anti-D was detected in the eluate, suggesting that her red cells were sensitized with auto-anti-D. Another case report described cold AIHA caused by IgM anti-D in a 59-year-old woman (O RhD positive) [9].

Laski et al., in the case series published in 1961, described a case of AIHA in a 2½-year-old due to auto-anti-D [10]. The child presented with jaundice and pallor. Hb was 3 g/dL. Both the patient and the mother were O RhD positive. DAT was weakly positive and anti-D was detected in the patient's serum. The child was transfused RhD-negative blood and was started on prednisolone. The patient was discharged with a Hb of 9 g/dL after 2 months of hospitalization. The patient was again re-admitted after 2 weeks with falling Hb of 4 g/dL. DAT was still positive with anti-D antibody. He required transfusions, and after a month, Hb rose to 7 g/dL and DAT and antibody screen became negative. DTT, enzyme testing, antibody titration and elution studies were not performed. The authors suggested that a viral illness might produce antibody similar to Rh-specificity or might alter the antigen in the RBC or act as a hapten antigenically different to the host. Another theory postulated that an individual loses tolerance to own cells, so the red cells become antigenic and produce an antibody against the patient's own cells.

A similar case report published in 1964 found auto-anti-D in a 9½-month-old infant with anaemia (Hb 3.9 g/dL) [11]. Both the mother and child were O RhD positive, patient's DAT was strongly positive and anti-D was isolated in elution done at 56°C. This patient was transfused with RhD-negative blood every 1½–2 months to maintain the Hb above 6.5 g/dL. In addition, prednisolone therapy was started. DAT positivity persisted till 7 months after discharge. No molecular RhD genotyping was done in both these cases.

Our case is of interest because it involves the rare occurrence of autoimmune anti-D in an RhD-positive patient aged only 3 months. To the best of our knowledge, only two case reports have been published on autoimmune anti-D in infancy (in 1961 and 1964).

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the data, S.M.and D.P. wrote the initial version of the manuscript and S.M., M.R., D.P., M.K., A.J., S.K., D.B. and R.R.S. critically revised it.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SHORT REPORT

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Detection and phenotype analysis of a novel Ael blood group allele

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Abstract

Background and Objectives: The presence of blood subtypes may lead to difficulties in blood group identification; however, third-generation sequencing (TGS) can help in accurately identifying difficult blood groups, and study the serological characteristics and molecular mechanism of Ael subtypes.

Materials and Methods: ABO blood group was identified by the standard serological technique, weak blood group antigen was identified by adsorption-elution experiments, ABH substance in the saliva was determined and glycosyltransferase activity of A and B was detected. The ABO gene full-length sequence and promoter region were amplified by specific primers using single-molecule real-time sequencing, with the amplified products being sequenced directly and analysed in real time.

Results: The patient was serologically identified as Ael subtype, and TGS analysis revealed new intron mutations in Ael patients (c.467C>T; c.29-10T>A).

Conclusion: The discovery of the new allele and the identification of ABO subtypes can be combined with serological characterization and molecular biological methods.

Keywords

ABO blood group, adsorption-elution experiments, Ael subtype, gene sequencing, single-molecule real-time sequencing

Highlights

- The presence of subtypes may lead to difficulties in ABO blood group identification. Serological methods and third-generation molecular sequencing technology can help in identifying them.
- Here we report the discovery of a novel allele of Ael, a group A subtype, using third-generation sequencing.
- The new allele has been submitted to the GenBanK database.

INTRODUCTION

The ABO blood group system, discovered by Karl Landsteiner in 1990, is one of the most important blood group systems in transfusion medicine and organ transplantation [1]. Generally, the ABO blood group system contains four major phenotypes: A, B, AB and O, and the distribution of ABO phenotypes varies among populations and regions [2]. However, because of the polymorphism of the ABO blood group gene, base insertion, deletion, substitution, mutation and splicing errors may affect the activity and specificity of A and B glycosyltransferases, resulting in the weakening or disappearance of A

Cuibi Wang and Yichao Tang contributed to the work equally and should be regarded as co-first authors.

or B antigen expression, which is the reason for the formation of the ABO subtype [3].

The Ael subtype is a relatively rare ABO subtype, in which the red blood cell does not agglutinate with anti-A or anti-AB, and which can be detected only by adsorption-elution experiments [4]. To date, a number of ABO allelic mutations have been identified in individuals, and these mutation sites highlight extensive sequence variations in the coding region of the ABO blood group gene [5]. Here we report a novel mutation c.29-10T>A on the ABO*A1.02 background, which has not been reported in public databases including 1000 Genomes, dbSNP and gnomAD before.

MATERIALS AND METHODS

Study subjects and DNA extraction

The proband is a 30-year-old Chinese man. Informed consent was obtained from the proband for sample collection and related trials, which were approved by the Medical Ethics Committee of Zhongshan Hospital (Xiamen), Fudan University (Approval No: B2022-073). Genomic DNA of the proband was obtained using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The molecular gene detection part was carried out at the Xi'an Haorui Medical Laboratory Co., Ltd.

Serological tests

The blood type of the proband was detected by routine serological methods for A, B and H antigens and anti-A and anti-B antibodies using anti-A, anti-A1, anti-B, anti-AB and anti-H antibody reagents and A1, B and O red blood cells (Shanghai Blood Biotechnology Co., Ltd., Shanghai, China). Absorption-elution experiments were conducted to detect A and B antigens by thermal elution method using anti-A and anti-B antibody reagents and A1, B and O red blood cells (Shanghai Blood Biotechnology Co.) and bovine serum albumin (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China). For saliva experiments, 5 mL of the proband's naturally flowing saliva was boiled according to the standard method, and the supernatant was retained for further use. Anti-A, anti-B and anti-H reagents (Shanghai Blood Biotechnology Co.) were used for multiple dilution, and the '2+' agglutination strength was considered as the optimal dilution. Detailed procedures for the above two tests can be found in the AABB technical manual [6]. Glycosyl transferase activity was also measured by using uridine 5′-diphospho-*N*-acetylgalactosamine disodium salt (UDP-GalNAc), uridine 5′-diphosphogalactose disodium salt (UDP-Gal) (Sigma, USA), imidazole buffer (Beijing Regan Biotechnology Co., Ltd., Beijing, China), bovine serum albumin (Shanghai Yuanye Biotechnology Co.) and manganese chloride (Shanghai McLean Biochemical Technology Co., Ltd., Shanghai, China). The pooled plasma samples of genotype A, B and O were selected as positive and negative controls (the specific steps can be found in the literature [7]).

ABO gene full-length sequencing analysis

We analysed the full-length ABO gene sequence using long-read sequencing. All amplicons were purified by magnetic beads, followed by repeat sequencing and analysis using a PacBio RS sequencer and Sequel System [8]. ABO genotypes were assigned according to the nucleotide sequence of the ABO gene polymorphisms. All nucleotide sequences obtained were compared with known ABO gene polymorphisms ('ABO*A1.01' named by the International Society for Blood Transfusion [ISBT] was used as the reference sequence).

Analysis of the ABO gene haplotype sequence

Amplification with allele-specific primers was carefully designed to generate three sets of primers (Figure 1, Table 1) that efficiently amplify the complete ABO gene sequence, followed by direct sequencing and analysis of the amplicons. At the same time, by rolling circle sequencing of a single DNA molecule, the long-range polymerase chain reaction (LR-PCR) technique was performed by KOD FX Neo (TOYOBO). The PCR cycling conditions were set according to the user manual, and a two-step cycle of 10 min each was used for a total of 30 cycles to achieve high accuracy of sequencing data and determine the haplotype of the sample.

TABLE 1 Primer design for ABO haplotype sequences.

Sequence primers	F[a]	R[b]
Primer 1	5′catccctttcaccttggcattt3′	5′agctacattgaccagagagaga3′
Primer 2	5′gcccaccaaaactccctggaa3′	5′ccagttcctgccagggagagga3′
Primer 3	5′gtgtgaaactcatcaaaacc3′	5′cgcagggattgcagtgagg3′

[a]Forward primer.
[b]Reverse primer.

FIGURE 1 Primer sequence diagram of ABO gene haplotypes. The ABO gene with a length of about 23 kb was broken down into three target gene fragments for amplification, and there was an overlap of more than 1 kb between each amplicon of the same gene, and the single-nucleotide polymorphism (SNP) sites were found in this overlap region to distinguish haplotypes. All amplicons are listed in the figure.

Blood group genotyping using third-generation sequencing

At present, the latest third-generation sequencing (TGS) has two major sequencing platforms. We used Pacific Biosciences' single-molecule real-time sequencing (SMRT), which mainly uses zero-mode waveguide holes to detect the fluorescence generated during DNA polymerization and repeat sequencing through a circular DNA library [9]. In brief, the PCR products were qualified by agarose gel electrophoresis and the library was constructed. A reaction master mixture was prepared before use, which contained 10 μL of the reaction mix containing 4 μL of PCR product, 5 μmol/L barcoded adapter (Integrated DNA Technologies), 1× T4 DNA ligase buffer (Enzymatics), 1 mmol/L ATP (New England Biolabs), 200 μmol/L dNTP (New England Biolabs), 2.5 units of T4 polynucleotide kinase (Enzymatics), 0.75 units of T4 DNA polymerase (Enzymatics) and 180 units of T4 DNA ligase (HC) (Enzymatics). And then, the reaction mix was incubated at 37°C for 20 min, 25°C for 15 min and 65°C for 10 min. After that, exonuclease I and exonuclease III (Enzymatics) were added to remove the failed ligation products, and the library was obtained after purification with 0.6× Ampure PB beads. The final library was bound with sequencing enzymes and primers through a Sequel Binding Kit 2.2 (Pacific Biosciences) and Internal Control Kit 1.0 (Pacific Biosciences). The complexes (150 pM DNA–polymerase) were finally loaded and sequenced with the Sequel II platform (Pacific Biosciences) with 20-h movie time. All procedures were performed in strict accordance with the manufacturer's instructions. SnapGene software was used for sequence alignment and analysis using ISBT Names for ABO (ISBT 001) blood group alleles v1.1 171023 as the reference data source.

RESULTS

Serological characteristics of the ABO phenotype

Table 2 shows the agglutination status of the proband's red cells with anti-A, anti-A1, anti-B, anti-AB, anti-H and serum containing known groups A, B and O red cells. All individual RBCs showed anti-H agglutination of 3+ or 4+ intensity. The results of absorption-elution experiments confirmed the presence of weak A antigen on the patient's RBCs; the proband was secretory type, and substance H was detected in the saliva. A-transferase was not detected in the proband by glycosyl transferase activity assays. According to the above serological characteristics, the proband could be classified as an Ael subtype variant.

ABO gene full-length sequencing analysis

By direct sequencing of the entire ABO coding region, no mutation was found in exons 1–5 and intron 6 of the ABO gene in the proband. There were c.261delG and c.467C>T in exons 6 and 7 and c.29-10T>A heterozygosity in intron 1 of the ABO gene (Figure 2, Table 3).

Analysis of the ABO gene haplotype sequence

Haplotype analysis of the ABO gene showed that the ABO genotype of the proband was ABO*A1.02/ABO*O.01.01 and there was a heterozygous mutation of T>A at position 29–10 in intron 1 (Figure 2, Table 3), which was not included in the ISBT database. This mutation

TABLE 2 The blood group serologic results.

	Forward						Reverse			Adsorption-elution				
Sample	Anti-A	Anti-B	Anti-A1	Anti-AB	Anti-H	Anti-D	Ac	Bc	Oc	Ac	Bc	Oc	Secretor status	Phenotype
Proband	0[a]	0	0	0	3+[b]	4+	0	4+	0	3+	0	0	H	Ael

[a]'0' denotes the serology did not agglutinate.
[b]'+' denotes the agglutination strength of serology.

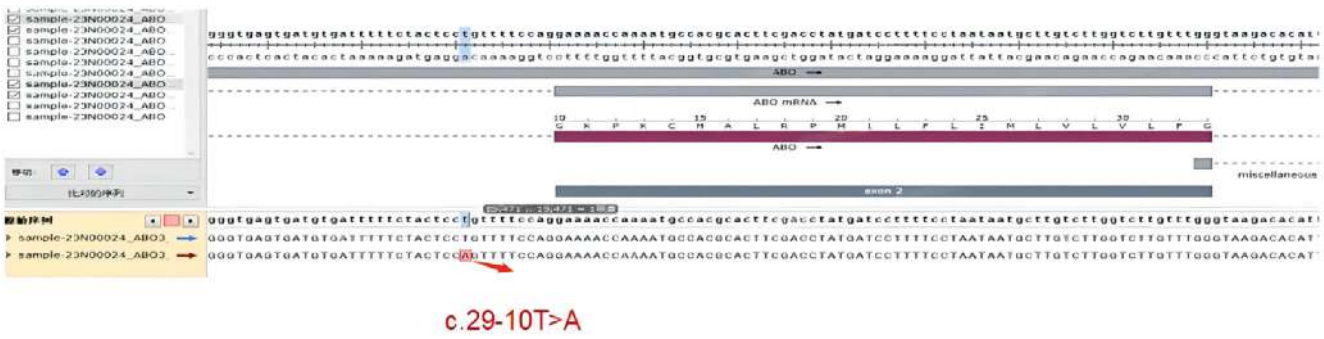

FIGURE 2 ABO gene sequencing results of the proband. *Homo sapiens* ABO, alpha 1-3-*N*-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase (ABO), NG_006669.1 was used as the reference allele sequence.

TABLE 3 ABO genotyping results.

	Haplotype 1			Haplotype 2		
Sample	Phenotype 1	Allele 1	Mutation 1	Phenotype 2	Allele 2	Mutation 2[a]
Proband	O	ABO*O.01.01	c.261delG	A1	ABO*A1.02	c.467C>T c.29-10T>A

[a]The new alleles first identified in our laboratory were not included in International Society for Blood Transfusion but were submitted to the GenBank database.

is located near the splicing region of exon, which may affect the normal splicing of gene products, resulting in the phenotype Ael of the haploid carrying this mutation. Then, we submitted the nucleotide sequence of this new allele of the proband to GenBank database (accession number: BankIt2672115 Seq1 OQ414473).

Blood group genotyping using TGS

According to the sequencing results in Table 3, by using real-time SMRT assay covering the full-length sequence and promoter region of the ABO gene, again we found this novel heterozygous mutation (c.29-10T>A) in intron 1 of the ABO gene. ABO gene-specific primers and PacBio RS sequencing confirmed that the intronic variant was located on the ABO*A1.02 allele. No variant gene was detected in the promoter region and +5.8-kb site of the ABO gene.

DISCUSSION

By December 2022, a total of 44 blood group systems had been recognized by the ISBT. The ABO blood group system is one of the earliest and most clinically significant blood group systems. The majority of subtype A is caused by the inheritance of rare alleles in ABO locus. Clinically, subtype A is divided into A1, A2, A3, Ax, Aend, Am and Ael according to decreasing number of A antigens [10], among which Ael subtype contains the least number of A antigen on the red blood cells. The reactivity of Ael red blood cells can be detected only by absorption-elution experiments, and its serum does not agglutinate or weakly agglutinate with type A red blood cells, that is, A elution type [11–13].

It had been reported that there was no substance A in the secretory saliva of Ael but only substance H. The serum of Ael individuals often contains anti-A1 but can also contain antibodies capable of agglutination with A2 cells [14, 15]. No A-transferase had been detected in the serum or erythrocyte membrane of Ael [16–18]. In this study, the proband was confirmed by absorption-elution experiments because there was weak A antigen on red blood cells and substance H in saliva but no A-transferase was detected on red blood cell membrane, which was consistent with the serological performance of Ael subtype individuals. However, the genotyping of the proband could not be accurately determined, so ABO gene-sequencing analysis was performed. Genotyping results showed that there was C>T mutation at position 467 and a heterozygous T>A mutation at position 29–10 in the intron 1 (which was not included in the ISBT database). The mutation was located near the exon splicing region, which may affect the splicing error during the transcription of gene products, leading to the mutation before and after the variable indirect recognition site and resulting in the weak expression of the antigen and forming the Ael subtype.

The majority of mutations reported so far in ABO subtypes were located in exons 6 and 7. However, ABO subtype pathogenic variants may also occur in regions other than ABO exons 6 and 7 (including exons 1–5 and 6 introns and the regulatory region of the ABO gene), which may have important but rare implications for gene expression [19–22]. In addition, the discovery and genotyping of SNPs in introns are key to the study of intronic gene polymorphisms, and introns are also considered important in assessing genetic diversity [2]. Here, a novel heterozygous mutation in ABO intron 1 (c.29-10T>A) was detected by the third-generation SMRT method for blood group genotyping of the proband. However, it is not certain whether the mutation will cause splicing abnormality, so functional verification is needed. Therefore, it is necessary to use molecular detection techniques to screen all these regions to avoid the phenomenon of weak antigen missed detection, leading to transfusion in the case of blood group discordance.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Towards standardized human platelet lysate production in Europe: An initiative of the European Blood Alliance

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Abstract

Human platelet lysate (hPL) is a supplement for cell culture media that can be derived from platelet concentrates. As not-for-profit blood establishments, we endorse the evolution of maximally exploiting the potential of donated blood and its derived components, including platelets. The decision to use platelet concentrates to supply hPL as a cell culture supplement should align with the principles and values that blood establishments hold towards the use of donated blood components in transfusion. As a consequence, questions on ethics, practical standardization of hPL production and logistics as well as on assuring hPL quality and safety need careful consideration. We therefore propose an opinion on some of these matters based on available literature and on discussions within the proceedings of the Working Group on Innovation and New Products of the European Blood Alliance. In addition, we propose collaboration among European blood establishments to streamline efforts of hPL supply to maximize the potential of hPL and its application in the wider field of medicine.

Keywords

cell therapy, human platelet lysate, platelet lysate, standardization, tissue engineering

Highlights

- Human platelet lysate is a high-quality culture media supplement that supports the development of advanced therapeutic medicinal products, but current human platelet lysates are diverse by nature and need to be standardized across European Union (EU) countries.
- Crucial elements to consider when progressing towards standardization in the EU concern those of a legal and ethical nature as well as manufacturing and quality control.
- This European Blood Alliance position paper is intended to demonstrate the Alliance's central role in bridging the gap between donor and patient across medicine in the EU, beyond classical transfusion.

INTRODUCTION

Platelets contain many bioactive molecules [1] that are stored in α- and dense granules. Even though platelets are small, the relative contribution of platelet molecules to the blood proteome is high

Author list in alphabetical order.

because platelets are abundant, representing 5% of all cells in the human body [2]. The release of platelet granule content is tightly controlled in space and time during haemostasis and subsequent tissue regeneration. The exact role and function of platelet molecules during these complex processes is only partially resolved. Consequently, platelet granule release ex vivo yields a complex mix of bioactive substances that include growth factors, cytokines and coagulation factors. Yet, this blend constitutes a surprisingly efficient growth factor supplement for in vitro cell culture as demonstrated by cell biologists in the 70s and 80s of the previous century [3, 4]. It took an additional 25 years for the platelet's cargo to be re-appreciated [5, 6] when cell therapy was considered the next big revolution in medicine. Because the soluble fraction of the platelet's interior was obtained primarily by cell lysis, the term human platelet lysate (hPL) was coined and stuck. Other methods of retrieving the platelet's bioactive intracellular content are equally effective and hPL now refers to those 'releasates' as well.

hPL LEGISLATION AND NOMENCLATURE

Safety is the primary cornerstone of medical development and this holds for cells and tissues as well. In an ideal supply chain, the final cytotherapeutic product is entirely free of immunogens and pathogens, for example, xeno-free. However, the intrinsic biomolecular complexity of cells and their current production methods are prone to non-ideal conditions. This implies that cell-based advanced therapy medicinal products (ATMPs) often will contain minimal immunogens and that microbial contamination will be reduced to a minimum at best, that is, below limits of detection. These non-ideal production conditions are familiar to blood establishments that are well acquainted with maintaining the quality of an allogeneic tissue ex vivo while minimizing transmission of pathogens.

An important part of ensuring ATMP safety is to avoid supplements [7–9] for cell expansion that are sourced from animals. Animal sera are undeniably immunogenic to humans and increase the risk of zoonotic transmission of adventitious agents [10]. Pioneering work in Europe has revived hPL as a suitable alternative to animal-sourced serum for the expansion of certain cell types [5, 11, 12]. In addition, both the European and US Pharmacopoeia have published general chapters Ph. Eur. 5.2.12 and USP 1043 for guidance [13–15]. Note that these are generic documents not specific to hPL. These are intended to guide the use of materials (nomenclature: *raw materials* [EU]; *ancillary materials* [US]) that come into contact with the ATMP during manufacturing but have no therapeutic activity in itself. A succinct summary of the legislative essentials has recently been published by Oeller et al. [16].

Of note, the prefixes 'clinical grade' and 'current Good Manufacturing Practice (cGMP)' are often used to distinguish a category of hPL that is safe for (pre)clinical use from one that is strictly suitable for in vitro applications. The latter can be called 'research grade' or 'research use only'. However, there is no such thing as a GMP-certified cell culture medium or growth factor supplement. The reason is that hPL does not fall into the category of GMP certifiable products because it is used indirectly as a cell culture medium supplement. This is in contrast to medicines like ATMP that are applied directly to patients. The 'GMP' reference can be confusing to blood establishments, hPL producers and ATMP developers. We suggest that these stakeholders collaborate to rename or at least define the terminology.

The US and EU Pharmacopoeia general chapters stipulate that hPL and ATMP developers must (a) evaluate the impact of the raw/ancillary material on quality, safety and efficacy using a risk-based approach and (b) define responsibilities to qualify the raw/ancillary material, which lies both with the hPL producer as well as the end user of hPL. As GMP certification of hPL is not possible, ISO certification of the quality management systems is highly recommended even when not mandatory. For example, ISO 9001:2015 and ISO 13485:2016 can be obtained, at least assuring rigorous quality management of the production process. Instead of 'GMP', we therefore suggest to define the different categories of hPL as 'Ph. Eur. Compliant' and 'research grade' hPL.

hPL SOURCE MATERIAL

Blood establishments are crucial in transfusion medicine, providing blood components to hospitals and the pharmaceutical industry. In this context, a blood component is a labile substance of human origin that is inherently variable and hence cannot be considered a pharmaceutical by regulatory definition. Consequently, European directives on blood comprehensively define safety and quality criteria that differ from those applying to (pharmaceutical) medicines. Producing hPL as a raw material using platelet concentrates as the starting blood component is a relatively novel activity for blood establishments in the EU and has several consequences.

First, a platelet concentrate is a labile blood component and so its biochemical composition varies by nature. In addition, among (regional) blood establishments, platelet concentrates differ by collection and production methods. A survey among 11 hPL-producing European Blood Alliance (EBA) members showed the following differences: (i) buffy coat derived or apheresis, (ii) storage in (different kinds of) platelet additive solution or not and (iii) implementation of irradiation and/or pathogen inactivation. These were also found in Strunk et al. [17]. Platelet count in both donor and platelet concentrates fluctuates as well, but platelet concentrates contain supraphysiologic cell concentrations of 0.8–1.6 $\times$ 10^9/mL that are optimal for hPL [18, 19]. This implies high growth factor concentrations in the derived hPL, such as platelet-derived growth factor (PDGF)-AB >40 ng/mL and transforming growth factor beta 1 (TGFβ1) >80 ng/mL.

Differences in source material may cause some variation in the biochemical composition of a final hPL [20]. This is inevitably the case for bovine serum [21] as well. It is not entirely clear whether and how such variability impacts cell proliferation at all, because variation in proliferation depends at least as much on the cell clone and the cell type in culture [22]. Pooling of individual hPL preparations will level

content variation [23] and is often performed within, but not among blood establishments. It is not clear how robust a given tissue or cell type is to growth factor variation caused by differences in source product. This requires real-time testing for every cell type or even every clone. We are confident that, in addition to pooling, the high-quality standards on labile blood components in the European Union (EU) and the European Economic Area (EEA) already provide an acceptable level of standardization. Additional research to understand and increase standardization is nonetheless highly recommended [24].

Second, hPL should be produced exclusively from overstock blood components to maintain adequate supply for transfusion purposes. Such overstock could be 'outdated' platelet concentrates or surplus buffy coats. In EU and EEA countries, platelet concentrates are stored at room temperature for 4–5 days, with a maximum of 7 days post donation. This brief storage time is a logistic burden but is maintained because platelet function declines quickly ex vivo [25] and due to the risk on bacterial outgrowth [26]. Note that 'outdated' in this context thus means a legally defined limit for transfusion and not an effective biochemical expiration of the platelet's granule content relating to hPL quality. In fact, hPL quality remains stable during storage of source platelet concentrates up to 7 days post donation [27]. The survey among EBA members indicated that none produce hPL from platelet concentrates that are still suitable for transfusion. Although hPL may become a life-saving product one day, it currently is not. Most ATMPs that (will) rely on hPL are in clinical development and have not yet been approved for routine clinical use. This is different from the source platelet concentrate, which saves many lives worldwide during supportive care in the hospital. In addition, hPL production specifically from outdated platelet concentrates fits with the goals of circular economy and waste minimization. It provides an opportunity for blood establishments to diversify the product portfolio and safeguard their expertise as an intermediary between donor and patient. Finally, it is our obligation towards blood donors to maximally deploy the potential of their donation, and this implies directing platelet donations to transfusion first and redirecting any surplus to hPL or other innovations.

Third, regarding safety, it is important to note that the original platelet concentrate is suitable for transfusion. Blood establishments use multiple layers of safety that are required by national and EU legislation. The donor questionnaire and medical anamnesis interrogate the risk behaviour of the donor and decide on eligibility or deferral. Next, donor screening by laboratory analysis is standard in EU blood establishments. This includes tests for endemic pathogens. Pathogen inactivation is increasingly adopted in EU blood establishments although some risk for breakthrough transmission still exists [28, 29]. Consequently, transfusion-transmitted infection has become increasingly rare, indicating that the high standards for transfusion are an ideal base for developing hPL, especially when produced in a closed system. Data suggest that hPL produced from pathogen-inactivated platelet concentrates is not inferior to hPL produced from platelet concentrates that have not been pathogen-inactivated [27, 30]. Pathogen inactivation of the final hPL is still under investigation. Existing methods of pathogen inactivation used for blood products, for example, photochemical treatment, should be validated and published to move the field forward.

Given the analysis above and the pivotal role of blood establishments as liable mediators between donor and patient, we suggest a close collaboration between European blood establishments to consolidate hPL production based on the generic principles of blood transfusion. This includes the principle of non-remunerated voluntary donation. Ideally, donors and/or donor stakeholder organizations are informed about hPL production. Donors need to be informed about the possible use of their donation for hPL production and give consent. Such principles and cross-border collaborations will increase the chances of standardization of hPL for the many variables discussed here. This should spur research in the fields of tissue engineering, cell therapy and transplantation and increase the quality of the research output. This will require an organizational umbrella that covers legal and ethical facets at minimum, but may go further by centralizing quality control laboratory testing and applying a unique production protocol. The next step is centralized production of hPL and distribution of a standardized product in the EU for the benefit of patients requiring ATMP (Figure 1). This in turn can then advance research into this field to develop hPL supplements that are optimized for specific cell types. Once the organizational umbrella is in place, such developments can then more easily be fitted in and will speed up implementation.

hPL PRODUCTION

Standardization in hPL production

To enable routine clinical use of hPL, it is vital to standardize a production method that is easy to implement in European blood establishments. It is equally important to use existing facilities, build on established protocols and trained staff already operational in blood establishments. While donor screening, pathogen inactivation, storage and component processing are difficult to fully harmonize, the hPL production method can be. The optimal method is still debated. Is hPL prepared in a completely closed system or a semi-closed system? With or without fibrinogen depletion? Such choices influence the infrastructural and operational requirements, but most blood establishments have qualified professionals and equipment that can manage closed-system operations at least for the first steps of hPL production.

Methods for hPL production

There are several possible methods to lyse platelets. These all have down- and upsides and selecting a uniform method is challenging. Such selection is ideally based on scientific data and includes a cost–benefit calculation. Current hPL production either involves platelet lysis or platelet activation. Platelet lysis methods (repeated freeze–thaw cycles, sonication, electroporation) can easily be performed in a closed system. However, these require an additional purification step like filtration that decreases hPL yield and increases cost. The final hPL product also requires addition of heparin to avoid solidifying of culture media. Alternatively, hPL can be prepared by platelet activation and subsequent

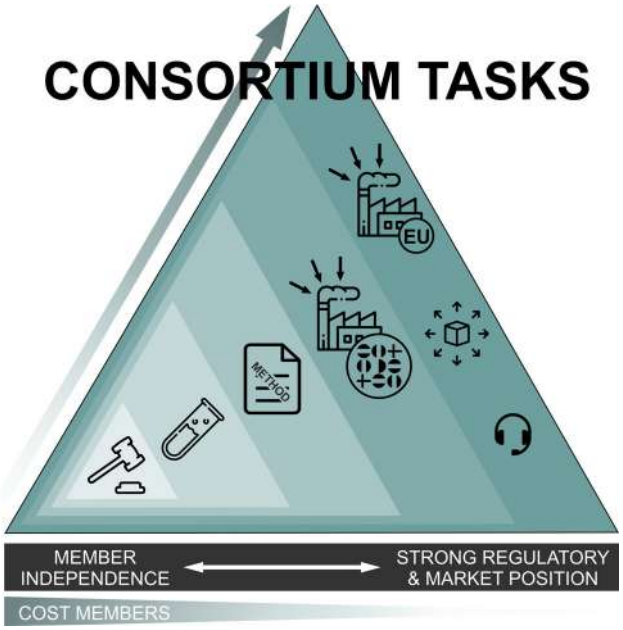

FIGURE 1 Models for cross-border collaboration among EU blood establishment for human platelet lysate (hPL) production. The models have increasing complexity from bottom left to top right and are presented in that order (*model I*, *gavel symbol*). A consortium of blood establishments operates within a common regulatory and legal framework for hPL production and distribution in the EEA and provides regulatory support to blood establishments engaging in hPL production (*model II*, *test tube symbol*). A consortium offers model I as well as validated quality control tests for hPL in a centralized manner (*model III*, *method document symbol*). A consortium is responsible for standardized hPL production protocols and quality guidelines. Blood establishments factually still produce hPL in-house but adhere strictly to the consortium's methods (*model IV*, *industry symbol with separate flags*). This model *decentralized–centralized–decentralized* as the consortium members ship source product to a central facility for hPL production based on standardized methods, but product is never mixed. Country-specific hPL is sent back to consortium members for regional/national distribution (*model V*, *industry symbol with EU*). This model is maximally collaborative, as members ship source product to a central facility for standardized hPL production and pooling. This yields unified batches of European hPL from multiple EU members.

coagulation by adding ionized calcium or thrombin. Platelet fragments are then trapped in the coagulum and therefore do not necessarily require further purification [31]. The resulting hPL is already fibrinogen depleted and therefore avoids the need for heparin addition. The addition of exogenous reagents should be standardized and validated for safety to minimize the risk of contamination. This risk can be kept low as exemplified by current pathogen inactivation methods that use exogenous chemicals like riboflavin [32] or amotosalen [33].

Freeze–thaw cycles

The most commonly used hPL production method is repeated freeze–thawing [34]. Platelet concentrates are frozen at <−20°C and then thawed at 37°C or room temperature and then re-frozen. The amount of freeze–thaw cycles varies between 1 and 5 across protocols [35, 36]. There is no consensus on how long the platelet concentrate should remain frozen during the freeze cycle. There is no consensus at what temperature the platelets should be frozen, and it is not clear whether snap freezing is better than slow freezing. Risks with this method include current storage bags not being compatible or validated for sub-zero temperatures, as well as the use of warm water baths that increase the risk of bacterial contamination. The latter is particularly relevant when scaling up production. It is therefore important to standardize these variables.

Coagulation

In this method, the platelet concentrate is activated using an external coagulation initiator [37]. Coagulation causes the platelets to release granular content by exocytosis. The result is not different from lysis by mechanical rupture such as freeze–thawing; the method yields an equally functional hPL [31, 38]. Using coagulation avoids the need for heparin in the final cell culture medium. The simplest way to initiate coagulation is by increasing the ionized calcium (Ca^{2+}) concentration above chelation. Following an incubation step, the coagulum retracts and the supernatant hPL can be harvested by transfer to a separate container. The coagulum is discarded and the hPL does not necessarily have to be filtered when the procedure is performed in a closed system. The disadvantage is that exogenous addition of a reactant (Ca^{2+}) may increase the risk of contamination.

Sonication

Sonication is based on mechanical disruption of cells by ultrasonic waves. The platelet concentrates are submerged in water that transmits ultrasonic waves at various frequencies (>20 kHz) for various periods of time with a minimum of 30 min, thereby causing thermal and non-thermal effects [39]. One effect is by microbubble formation and cavitation that effectively lyses platelets, thus creating hPL.

Pulse electric field

Pulse electric field (PEF) methods may be used to break up platelet (granule) membranes. This method has been used to generate platelet-rich plasma for wound healing. It is based on electroporation that perforates cell membranes. PEF can be scaled-up to large reactors operating continuously. Different PEF parameters such as pulse width, number and electric field strength have been optimized to selectively lyse platelets and modulate the release of specific growth factors [40]. Further optimizations of PEF parameters are ongoing together with a detailed characterization of the composition and bioactivity of the hPL.

Pooling

Pooling is an important variable (Figure 1). Pooling can be done before or after the hPL production process. The number of blood donors in a

final hPL pool matters. On the upside, pooling cancels out biochemical variability, but on the downside, it increases the risk of pathogen transmission. The latter may be reduced by including pathogen inactivation [41] of the platelet concentrate, of the final hPL or even both [42]. Alternatively, the number of donors whose donations are used for hPL production can be kept to a minimum [43]. A rational choice, supported by guidelines and international consensus, is important to develop a standardized hPL.

Filtration

Filtration is used to remove platelet fragments and to decrease the odds of pathogen transmission. It is especially required with hPL production techniques that cause platelet fragments or when an open system is used [44]. Filters with sequentially smaller pore size can do this, but will inevitably impact hPL yield because of dead volume retention. In addition, large volumes cannot be filtered by simple gravitation, thus requiring pumps. Filtration for sterility reasons suffers from the same technical issues, but is justified and even required when open systems are used during hPL production. Because the end user eventually opens the hPL container, good laboratory practice procedures are crucial in the final stages of hPL use as well. In any case, when filters are used, these should be high performants and have small dead volumes. For restraining bacteria, filters should have pore sizes ≤0.22 μm. All filters should be easy to use, inexpensive and generate reproducible results.

Closed system production

Closed-system hPL production will avoid the need for building, maintaining and continuously accrediting clean room facilities. The technical expertise for working this way already exists in European blood establishments. The connection of various containers like bag sets and filters using sterile welders is often operational and validated. One could even envision a readymade system like a combined bag set where multiple platelet concentrates or hPL units are hooked to a larger satellite bag where the content is mixed [31]. This pooled hPL can be filtered if deemed necessary, and eventually aliquoted in cryocompatible containers for storage and distribution. Appropriate labelling to trace back production up to the individual donor allows for decent quality control. Such a process can be made compatible with existing equipment and methods and in collaboration with one or more industrial partners. This should take into account recent developments in SoHO and medical device regulation as well as plasticizer requirements.

Additional manipulations

Additional manipulations can be performed to make different versions of hPL for different uses. For instance, fibrinogen depletion can be performed on the final hPL. Lyophilization of hPL renders shipping and storage of hPL significantly simpler and less expensive than liquid frozen stocks [45]. Potential loss of yield and quality following reconstitution of lyophilized hPL should be investigated thoroughly, but the technique deserves scientific attention. Pathogen inactivation can be executed on the final hPL as mentioned. A number of ultraviolet (UV) light based methods exist for treating platelets [46] and plasma for transfusion [47]. If hPL is produced in a closed system starting from platelet concentrates that have been treated with a validated pathogen inactivation method, an additional inactivation step may not be cost-effective. However, in open or semi-closed systems, it can still add value. Note that none of the available UV-based methods has been validated for hPL, both for pathogen reduction and impact on quality. Initial work nonetheless indicates limited quality decline in a study that tested amotosalen and UV A light pathogen inactivation on hPL [41]. Besides UV-light-based methods, other pathogen inactivation techniques have been investigated including solvent/detergent treatment and gamma irradiation [30, 48, 49]. These methods also require additional validation.

hPL PRODUCT

Here we provide a rationale and suggestion for the specific minimal release criteria based on an international survey performed by the EBA as well as on the literature. These suggestions are not only useful for guidance in production and quality control testing of hPL but are also essential for the determination of storage conditions and, consequently, the expiration date.

Rationale for general criteria

Minimal release criteria (Table 1) for raw materials include appearance, osmolality and pH. These criteria are considered quality parameters and may vary in hPL prepared from different source platelet concentrates. These parameters are often reported in papers, but generally deal with hPL from a single specific institution where the studies were performed. There is currently no information on hPL that is pooled from multiple sources across institutions, blood establishments and across borders. Such a standardization effort is instrumental for hPL production in a consortium setting and requires international experimental collaboration.

For cell cultures, the initial pH as well as maintaining pH is important because most cellular processes are inefficient at acidic or basic pH. Levels in the range of 7.2–7.4 are regarded as most suitable. Similar to bovine serum, hPL is added at a fraction of total volume. Note that the final pH of complete media is mostly determined by the composition of the basal medium as this contains buffer, often a bicarbonate–CO_2 conjugated pair. Because hPL is generally not added at ratios above 1:5 (hPL:basal medium), the buffer in the medium mainly determines the final pH. As such, a pH between 6.5 and 8.5 in hPL at room temperature is recommended.Besides pH, osmolality is important for cell volume regulation but may also impact growth rate

TABLE 1 Minimal release criteria for platelet concentrate and hPL.

Platelet concentrate	
Red cells and free haemoglobin	$<6 \times 10^9$ red blood cells/L (i.e., visually not red) or 0.018 g/dL haemoglobin
Osmolality	250–320 mOsm/kg
pH	6.5–8.5
Cellular content	$>2 \times 10^{11}$ platelets/unit
	$<1 \times 10^6$ leukocytes/unit
	$>0.8 \times 10^9$ platelets/mL
Bacterial safety	Automated microbial detection and/or pathogen inactivation
hPL	
Osmolality	250–320 mOsm/kg
pH	6.5–8.5
Mycoplasma	Negative
Elemental impurities	Plasticizer concentration during initial validation of production method
Content	PDGF-AB or TGFβ1
	Fibrinogen
	Total protein
	Should be measured and reported
Biological activity	Population cell doublings of a standardized cell (clone) line
Microbiological safety	• Closed system production ○ Pathogen inactivation ○ S/D treatment ○ Gamma irradiation ○ Sterile filtration
Screening for infectious pathogens	• HIV-1/2, HBV, HCV, treponema • HEV, HAV, Parvo B19, HTLV1/2
Bacterial endotoxins	<0.5 EU/mL

Abbreviations: EU, European Union; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV-1/2, human immunodeficiency virus 1 and 2; hPL, human platelet lysate; HTLV, human T-lymphotropic virus; PDGF-AB, platelet-derived growth factor-AB; S/D, solvent/detergent; TGFβ1, transforming growth factor beta 1.

and adhesion properties. Osmolality levels of 280–290 mOsm/kg, which is the average osmolality of serum, are regarded as optimal for most mammalian cell cultures. The same reasoning as for pH can be followed, that is, given that sera and hPL are used at low ratios relative to total basal media volumes, osmolality ranges between 250 and 320 mOsm/kg are considered acceptable.

Rationale for content and biological activity

It is unclear what growth factors in hPL are most important for a specific cell or tissue (culture) and at what concentrations these are optimally functional. These are topics in the field of 'chemically defined media' and are subject to continuous investigation. We therefore define (growth) factors that (a) are reported to support cell growth and could thus serve to benchmark hPL products, (b) are strict platelet granule molecules and could thus serve as a control for efficient platelet lysis and granule release, or (c) are factors that significantly change in concentration or by nature following the hPL production process and thus can serve as a control for efficient hPL production. Finally, total protein content serves as a non-specific measure of consistency across batches.

Growth factors such as PDGF isoform AA, TGFβ1, thrombospondin-1 and chemokine (C-C motif) ligand 5 are abundantly present in platelet granules. These may serve as positive controls for platelet lysis but not necessarily as a measure for hPL potency because not all cell types depend on these molecules for expansion. We argue that markers of sufficient platelet lysis or granule release are good proxies for hPL product potency because it indicates that whatever growth factor is important, it should have been released together with the said sentinel markers.

To confirm the biological activity of hPL and to prevent having to quantify dozens of growth factors, a biological activity assay such as a fibroblast or mesenchymal stem cell proliferation assay can be performed. This test should use a reference hPL or reference serum that is internationally agreed upon. Note that the behaviour of a cell line depends on much more than the culture media, so international agreement on the cell clones and cell lines used to this purpose should be reached as well. For continuous cell lines, this is feasible, but this is much harder for primary cell lines.

When hPL is prepared with fibrinogen depletion, fibrinogen concentration measurements should be performed to ensure complete serum conversion. This is important because even at the low volume fractions of hPL in basal media, fibrinogen-rich hPL will cause medium solidification. This is because all common basal media have Ca^{2+} concentrations that cause coagulation, for example, Ca^{2+} concentrations in Dulbecco's modified Eagle medium are 1.8 mM, and in Iscove's modified Dulbecco's medium they are 1.5 mM.

In summary, we suggest that total protein, fibrinogen concentration and PDGF or TGFβ1 concentration are tested on the final hPL and that a representative cell proliferation assay is included to ensure proper biological activity.

Rationale for microbiological control

Blood establishments have strict donor selection criteria to maximize donor and patient safety. In addition, laboratory screening of donations is performed to minimize the odds for transmission of pathogens. Donations are screened for pathogens according to well-defined national and European legislation (Blood Directive 2002/98/EC) [18]. These tests generally include treponema, human immunodeficiency virus (HIV) 1 and 2, hepatitis B and C virus. Often, selective tests can be performed based on endemic occurrence of pathogens and/or donor risk assessment behaviour. These include but are not restricted to hepatitis E virus, human T-lymphotropic virus and parvovirus B19.

Specific to platelet concentrates, automated microbial detection systems are in place in blood establishments that do not perform pathogen inactivation. Therefore, the products in European blood establishments comply with demands from the cell therapy field [50].

If platelet concentrates are not stored beyond outdating and thus immediately used for hPL production, the risk for microbial outgrowth is not greater than in current transfusion practice. Additional storage beyond 7 days may be applied, but it inherently increases the risk of bacterial outgrowth. We therefore suggest not to store outdated platelet concentrates and move to the first step of hPL production, at least until intermediate cryopreservation. In principle, the commonly used hPL production methods mentioned above allow for this swift transfer following outdating.

If the source platelet concentrate was treated with a validated pathogen inactivation method, we suggest that no secondary microbiological mitigation interventions are required on the condition that hPL production is an entirely closed system and that platelet concentrates are not stored beyond outdating. If the source platelet concentrate was not treated with pathogen inactivation, an additional secondary mitigation intervention on the final hPL is suggested. Treatments such as UV-light-based pathogen inactivation [46, 47], solvent/detergent treatment [49], gamma irradiation [48] or sterile filtration may be considered pending additional validation. The impact of these interventions on the above-mentioned quality criteria of the final hPL then deserves further independent research. Note that Mycoplasma contamination is particularly arduous in cell culture [51], and a negative test for Mycoplasma in hPL will significantly enhance its acceptance by cell culturists. In addition, endotoxins can be determined to indicate the low level of pyrogens.

Rationale for impurity control

Impurities in hPL are currently not defined in certified lists, but free haemoglobin [52] and specific plasticizers [53] are unwanted. Haemoglobin contamination may be assessed visually where 'not red' is equal to roughly $<6 \times 10^9$ red blood cells per litre. Besides haemoglobin, plasticizer contaminants may be present in various amounts. Both apheresis and whole blood collection sets still contain the plasticizer di-ethyl-hexyl pthalate (DEHP). Although DEHP is only present during the collection and processing phase, it will leach into the plasma component and incorporate into cellular membranes to a certain extent. As a result, some DEHP might be present in the final hPL product depending on the amount of plasma. At the time of writing, DEHP is still allowed for manufacturing blood collection sets, but this will change in 2025 as per the EU Medical Device Regulation [54]. From then on, DEHP is only allowed to be used in medical devices below a concentration of 0.1% (wt/wt). Platelet storage bags are currently already often manufactured with butyryl trihexyl citrate or other plasticizer. We propose that plasticizer concentrations are determined as part of the validation of hPL production processes, but it does not require continuous monitoring nor should it be part of a certificate of analysis.

Storage and expiration

Some proteins in hPL may be heat labile, prone to oxidation or denaturation, and this may influence hPL quality following (long term) storage. Longitudinal studies are needed to better understand the impact of storage on hPL quality. Once frozen and maintained at a temperature below −25°C, plasma remains stable for at least 2–3 years. It is conceivable that the (growth) factor content in hPL is stable for at least 2 or 3 years as well when kept in similar conditions as plasma, but experimental evidence is needed. Any intermediate product between platelet concentrate and hPL, once frozen, may be stored at least for 4 months [55]. Based on this, initial expiry may be set to 6 months or 1 year so that validation of longer storage durations may commence as hPL production advances. It is important that the hPL industry reports (long term) stability to assure high quality over storage time.

CONCLUSION AND DISCUSSION

In this article, we have outlined key considerations when striving for an internationally standardized and uniform hPL that adheres to Ph. Eur. requirements. We offer the rationale towards a harmonized hPL production process as well as a set of minimal release criteria on the source material and on the final hPL product. We put forward the possibility of achieving this through international collaboration and harmonization on the level of source material, hPL production method and laboratory tests. A consortium of not-for-profit blood establishments may represent a suitable body to manage these activities. Such collaboration will allow for routine production of a well-defined and internationally recognized European standard hPL. In addition, the interests of our non-remunerated donors can thus be optimally safeguarded by integrating the goals of the blood establishments on the European level to provide the best medical treatments for those in need, also beyond transfusion.

Open questions remain on the scientific level, for example, the impact of pooling either source material or the final hPL product from different blood establishments or the added value of lyophilization. These require output from high-quality research currently ongoing worldwide as the field grows. Profound questions also arise when considering deeply rooted international collaboration for production of a standard hPL, including (but not limited to) legal questions on liability and ethical questions on donor and patient involvement. We feel confident that the expertise both in routine and in experiment-driven operations currently flourishing in European blood establishments provides fertile ground for the development of a standard hPL that is highly valued as a xeno-free supplement for cell culture.

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CONFLICT OF INTEREST STATEMENT

H.B.F. is an author on a pending patent concerning a human platelet lysate manufacturing method. All other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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